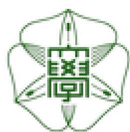
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Expression of guanylyl cyclase genes in medaka hybrids (*Oryzias curvinotus* x *Oryzias latipes*)

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Short title: Expression of guanylyl cyclase genes in medaka fish hybrids

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Abstract

The Hong Kong-originated medaka fish *Oryzias curvinotus* expresses nine genes (*OcGC1~OcGC8* and *OcGC-R2*) for membrane guanylyl cyclases (membrane GCs) and three genes (*OcGCS-*α_1, *OcGCS-*α_2, and *OcGCS-*β_1) for soluble GC subunits. The deduced amino acid sequences of membrane GCs expressed in *O. curvinotus* were quite similar to those expressed in the Japanese medaka fish *O. latipes*, including a novel membrane GC gene, *OlGC8*, which was the first isolated and characterized in *O. latipes*. *O. curvinotus* was able to produce the hybrids with *O. latipes* irrespective of the direction of crossing, and the resulting hybrids expressed both the maternal and paternal soluble GC subunit genes, suggesting the possibility of the formation of a chimeric heterodimer in the hybrids. In the early embryogenesis of the hybrids, however, the maternal soluble GC subunit genes were expressed earlier than the paternal soluble GC subunit genes, suggesting that the maternal soluble GC subunit genes interact more effectively with maternal effector molecules such as transcription factors than with those of paternal origin.

Keywords

gene expression; heterodimer; hybrid; medaka fish; membrane guanylyl cyclase; phylogenetic tree; RACE; RT-PCR; soluble guanylyl cyclase; transcription factor

1. Introduction

Both the soluble and membrane forms of guanylyl cyclases (GCs) catalyze the conversion of GTP to cGMP, which is a ubiquitous second messenger in intracellular signaling cascades and responsible for a wide variety of physiological responses (Drewett and Garbers, 1994; Garbers and Lowe, 1994; Garbers et al., 1994). Soluble GC is a heme-containing heterodimer composed of α and β subunits. In mammals, cDNAs for four soluble GC subunits (α_1, α_2, β_1, and β_2) have been isolated from various tissues (Koesling et al., 1988, 1990; Yuen et al., 1990; Harteneck et al., 1991). Both the α_1/β_1 and α_2/β_1 heterodimers have been shown to be enzymatically active when formed in an *in vitro* expression system (Russwurm et al., 1998). In previous studies, we have isolated the cDNA and genomic DNA clones encoding the soluble GC subunits, *OlGCS-*α_1 and *OlGCS-*β_1, both of which are aligned in tandem on the genome of the medaka fish *Oryzias latipes* (Mikami et al., 1998, 1999) and demonstrated that regulatory parts of both genes are partially overlapped (Yamamoto and Suzuki, 2002). In a recent study, we demonstrated that the NO/cGMP signaling pathway plays critical roles in early embryogenesis of the medaka fish, particularly in the formation of forebrain, eye, and otic vesicles (Yamamoto et al., 2003). We also demonstrated that the medaka fish contains another type of soluble GC subunit gene, *OlGCS-*α_2, which locates on a different linkage group of *OlGCS-*α_1 and *OlGCS-*β_1 (Yao et al., 2003).

To date, mRNAs for nine different membrane GC genes (*OlGC1~OlGC8* and *OlGC-R2*), which is greater than the number found in mammals, have been identified and characterized in various organs of individuals of *O. latipes* (Seimiya et al., 1997; Mantoku et al., 1999; Takeda and Suzuki, 1999; Hisatomi et al., 1999; Yamagami et al., 2003). One of these, *OlGC8*, was a novel membrane GC gene that has not yet been

identified in mammals. Only a few papers have reported on the existence of various GC genes in teleosts and, to our knowledge, there has been no paper describing the existence of *OlGC8* or its homologue even in species of the genus *Oryzias*, with the exception of *O. latipes*. One of the purposes of this study was thus to investigate whether other *Oryzias* species, such as *O. curvinotus*, possess various GC genes like those found in *O. latipes*, including *OlGC8*.

It is known that *O. curvinotus*, which was originally collected in Hong Kong, is able to produce hybrids with *O. latipes* irrespective of the direction of crossing (Hamaguchi and Sakaizumi, 1992). There are several differences in the nucleotide sequence of the gene for soluble GCs between *O. curvinouts* and *O. latipes*. Using specific primers constructed based on the differences, we can detect differential expression of the respective gene derived from *O. curvinotus* genome and *O. latipes* genome. The results will be useful for understanding of the mechanism of regulation of gene expression in the hybrid. Frankel examined solbitol dhydrogenase isozymes that show delay in the appearance of paternally originated isozyme and in case of glucosephophate isomerase B, delays of both paternal and maternal origins are observed in the hybrid fish (Frankel, 1989). Therefore, it is of interest to study whether or not the chimeric soluble GC heterodimer (α_1 or α_2 originated from *O. latipes* and β_1 originated from *O. curvinotus* and *vice versa*) can be formed in the hybrid. Here, we report that *O. curvinotus* contains the all member of GC genes found in *O. latipes*, and their nucleotides and deduced amino acid sequences were quite similar to those of the corresponding GC genes found in *O. latipes*. We also report that in the hybrids formed by crossbreeding between *O. curvinotus* and *O. latipes*, mRNAs for soluble GC α_1, α_2, and β_1 subunits originated from both species were expressed in the adult brain, although the maternal genes were expressed at earlier stages than the paternal genes.

2. Materials and methods

2.1. Fishes and crossbreeding

Mature adults of the medaka fish *O. curvinotus* were a gift of Dr. Shibata at Sinshu University, and mature adults of the medaka fish *O. latipes* were purchased from a dealer. Both were kept in indoor tanks under artificial reproductive conditions (10 h dark, 14 h light, 27°C). One individual (male or female) of *O. curvinotus* was crossbred with one individual (male or female) of *O. latipes*. The hybrid obtained by crossbreeding in the combination of (*O. curvinotus* ♀ x *O. latipes* ♂) was denoted with a cl and the hybrid obtained with (*O. latipes* ♀ x *O. curvinotus* ♂) was designated as lc. Laid hybrid eggs were collected and allowed to develop in distilled water containing 0.6 ppm methylane blue at 27°C. The developmental stage was expressed in the manner described by Iwamatsu (Iwamatsu, 1994).

2.2. Preparation of RNA and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the hybrid eggs and embryos and various adult organs of *O. curvinotus*, such as the brains, eyes, gills, intestines, and testis, by the acid/guanidinium/thiocyanate/phenol/chloroform extraction method (Chomczynski and Sacchi, 1987). Total RNA (5 μg) was used as the template to synthesize the first strand cDNA using an oligo(dT) primer and Super Script II preamplification system for first-strand cDNA synthesis (Gibco BRL, Tokyo, Japan) according to the manufacturer's protocol.

Three degenerate oligonucleotide primers (P2, 5'-GAYATHGTNGGNTYAC-3'; P6,

5’-GTRTTNACNGTRTCNCC-3’; and P7, 5’-ARRCARTANCKNGGCAT-3’) were synthesized based on the amino acid sequences of three conserved regions (CIVGFT, DTVNTA, and MPRYCL) in

known membrane GCs as described previously (Seimiya et al., 1997). These primers were used for PCR to amplify the *O. curvinotus* cDNA fragments encoding various membrane GCs using cDNA reverse-transcribed from total RNA of brains, eyes, gills, intestines, or testis of *O. curvinotus*. The reaction parameters used for PCR with these primers were as follows: 90 sec at 94°C, followed by 30 cycles of 30 sec at 94°C, 1 min at 41°C, and 1 min at 72°C, and a final cycle of 5 min at 72°C. The DNA polymerase used for PCR was Ex Taq (TaKaRa, Otsu, Japan). The PCR products were separated by electrophoresis on a 0.7% agarose gel, extracted using a MinElute Gel Extraction Kit (QIAGEN K.K, Tokyo, Japan), and re-amplified using primers P7 (a more inward primer than P6) and P2 under the same conditions as described above but for an annealing temperature of 39°C. A 341-bp cDNA fragment amplified in the second PCR was purified and subcloned into the plasmid vector pBluescript II KS(-) (Stratagene, La Jolla, California).

To amplify the *O. curvinotus* cDNA fragments encoding an *OlGC3* homologue (*OcGC3*), an *OlGC5* homologue (*OcGC5*) and an *OlGC-R2* homologue (*OcGC-R2*), PCR was carried out with primers synthesized based on the nucleotide sequence of the *OlGC3*, *OlGC5*, or *OlGC-R2* cDNA (Table 1) and the first-strand cDNA reverse-transcribed from total RNA of the adult eye of an *O. curvinotus* individual. The reaction parameters used for PCR with these primers were as follows: 30 cycles of 30 sec at 94°C, 30 sec at each annealing temperature shown in Table 1, and 1 min at 72°C.

Table 1

A cDNA fragment containing the 3’ non-coding region of an *O. curvinotus OlGC8*

homologue (*OcGC8*), an *O. curvinotus OlGCS-*α_1 homologue (*OcGCS-*α_1), an *O. curvinotus OlGCS-*α_2 homologue (*OcGCS-*α_2) or an *O. curvinotus OlGCS-*β_1 homologue (*OcGCS-*β_1) was amplified by PCR with the first-strand cDNA obtained from brain total RNA of *O. curvinotus*. To obtain the full-length cDNA sequence of *OcGCS-*α_1, *OcGCS-*α_2, or *OcGCS-*β_1, each was amplified as several segments (*OcGC8*: 4 segments; *OcGCS-*α_1: 2 segments; *OcGCS-*α_2: 5 segments; *OcGCS-*β_1: 4 segments) by using primers that were designed based on the nucleotide sequence of cDNA for *OlGC8*, *OlGCS-*α_1, *OlGCS-*α_2, or *OlGCS-*β_1. Primers, segments with nucleotides, and the annealing temperature used at PCR for *OcGC8*, *OlGCS-*α_1, *OlGCS-*α_2, or *OlGCS-*β_1 are shown in Table 1. The reaction parameters used for PCR with these primers were as follows: 30 cycles of 30 sec at 94°C, 30 sec at each annealing temperature shown in Table 1, and 1 min at 72°C, and a final cycle of 5 min at 72°C.

2.3. 3'-Rapid amplification of cDNA ends (RACE)

Total RNA from the brain of *O. curvinotus* was used as the template to synthesize the first strand cDNA using an Oligo dT-3sites Adaptor Primer (TaKaRa, Otsu, Japan) according to the manufacturer's protocol. To amplify the 3'-untranslated region (UTR) of *OcGCS-*α_2 with RT-PCR, GSP1 (5'-CATGCTGTTCTCAGACATTGTGGG -3') and Oligo dT-3sites Adaptor Primer for the first PCR were used. The reaction parameters were as follows: 30 cycles of 30 sec at 94°C, 30 sec at 60°C, and 1 min at 72°C, and a final cycle of 5 min at 72°C. The DNA polymerase used for PCR was Ex Taq (TaKaRa, Otsu, Japan). The first PCR products were reamplified using the primer GSP2 (5'-TTACCAGCAAGTTTGAATCGGGC-3'; a more inward primer than GSP1) and the Oligo dT-3sites Adaptor Primer under the same conditions as described above.

The cDNA fragment amplified in the second PCR was purified and subcloned into the plasmid vector pBluescript II KS(-).

2.4. DNA sequencing, alignment and phylogenetic analysis

The nucleotide sequence of a cDNA fragment was determined by the dideoxy chain termination procedure (Sanger et al., 1977) with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Osaka, Japan) and analyzed with GENETYX-MAC/version 7.2.0 software (Software Development, Tokyo, Japan). The homology search was performed at the NCBI BLAST Web site (http://www.ncbi.nlm.nih.gov/BLAST/). The phylogenetic tree was constructed using the aligned sequences by the neighbor-joining algorithms in the PRTRAS program of PHYLIP ver. 3.572 (Felsenstein, 1994) and the Clustal W program (Thompson et al., 1994). For the neighbor-joining analysis, the evolutionary distance was estimated by Kimura's empirical method (Kimura, 1983) using the amino acid sequences of various membrane GCs and soluble GCs of *O. latipes*, *O. curvinotus*, and *Fugu rubripes*. The GenBank/EMBL/DDBJ accession numbers for the various *O. latipes* GCs used for comparison are as follows: **AB000849** (OlGCS-α_1), **AB109399** (OlGCS-α_2), **AB000850** (OlGCS-β_1), **AB021490** (OlGC1), **AB030274** (OlGC2), **AB000899** (OlGC3), **AB000900** (OlGC4), **AB000901** (OlGC5), **AB016081** (OlGC6), **AB023489** (OlGC7), **AB054814** (OlGC8), and **AB015874** (OlGC-R2). The Ensemble gene IDs of Fugu GCs from the Sanger Institute are as follows: SINFRUG00000140078 and SINFRUG00000140081 (fu1), SINFRUG00000121899 (fu6), SINFRUG00000147481 (fu8), SINFRUG00000133934 (fu9), SINFRUG00000131285 (fu10), SINFRUG00000155431 (fu12), SINFRUG00000155431 (fu13), SINFRUG00000133571 (fu18),

SINFRUG00000140601 (fu19), SINFRUG00000134554 (fu22s), SINFRUG00000140565 (fu23s), and SINFRUG00000140577 (fu24s).

2.5. Expression of mRNAs for soluble GC subunits in hybrids

A specific primer set was used for detection of the respective soluble GC subunit mRNA (*OcGCS-*α_1 or *OlGCS-*α_1, *OcGCS-*α_2 or *OlGCS-*α_2, and *OcGCS-*β_1 or *OlGCS-*β_1) in the hybrids (Table 2). To examine the specificity of each primer set, the first strand cDNA reverse-transcribed from total RNA of the brain of *O. curvinotus* or *O. latipes* was used as the template for control PCR. The reaction parameters for control PCR to amplify *OlGCS-*α_1 and *OcGCS-*α_1 were as follows: 30 cycles of 30 sec at 94°C, 30 sec at 55°C, and 30 sec at 72°C, and those for *OlGCS-*α_2, *OcGCS-*α_2, *OlGC-*β_1, and *OcGCS-*β_1 were the same as described above for *OlGCS-*α_1 but for an annealing temperature of 68°C and 67.8^{o}C. Total RNA extracted from the adult hybrid's brain or whole hybrid embryos at several developmental stages was used as the template to synthesize the first strand cDNA using an oligo(dT) primer as described above. The transcripts of the α_1, α_2, or β_1 subunit genes derived from *O. curvinotus* or *O. latipes* in the hybrids were amplified by PCR using the respective specific primer set. Each PCR product was separated by electrophoresis on a 0.7% agarose gel and visualized by ethidium bromide staining.

Table 2

3. Results

3.1. Isolation and characterization of cDNA clones encoding various O. curvinotus GCs

In the present study, we obtained the full-length cDNA clones for *OcGC8*, *OcGCS-*α_1, *OcGCS-*α_2, and *OcGCS-*β_1 by RT-PCR and 3'-RACE. The cDNA of *OcGC8* was 2,706 bp in length and its ORF predicted a protein of 859 amino acids. The cDNA of *OcGCS-*α_1 was 2,586 bp and its ORF predicted a protein of 678 amino acids. The cDNA of *OcGCS-*α_2 was 2,465 bp and its ORF predicted a protein of 804 amino acids. The full-length cDNA of *OcGCS-*β_1 was 2,488 bp and its ORF predicted a protein of 614 amino acids. The nucleotide and the deduced amino acid sequences of *OcGC8*, *OcGCS-*α_1, *OcGCS-*α_2, and *OcGCS-*β_1 were similar to those of *OlGC8*, *OlGCS-*α_1, *OlGCS-*α_2, and *OlGCS-*β_1, respectively.

By RT-PCR with combinations of three degenerate primers (P2, P6, and P7), we obtained various 341-bp cDNA fragments. The nucleotide sequences of five 341-bp cDNA fragments were quite similar to those of a part of the catalytic domain of the *O. latipes* membrane GC cDNAs—i.e., *OlGC1*, *OlGC2*, *OlGC4*, *OlGC6* and *OlGC7*—and thus were named *OcGC1*, *OcGC2*, *OcGC4*, *OcGC6*, and *OcGC7*, respectively. *OcGC1* was expressed in the eye, *OcGC2* in the eye and gut, *OcGC4* in the eye, *OcGC6* in the eye and gut, and *OcGC7* in the gut and gill. We also obtained the cDNA fragments of *OcGC3*, *OcGC5*, and *OcGC-R2* from the eye total RNA by RT-PCR using the various primers shown in Table 1, indicating that *O. curvinotus* possesses all of the membrane and soluble GCs found in *O. latipes*. The nucleotide and amino acid sequences of these GCs were submitted to GenBank/EMBL/DDBJ and are currently

available under the following accession numbers: *OcGCS-*α_1 (**AB115703**), *OcGCS-*α_2 (**AB115704**), *OcGCS-*β_1 (**AB121231**), *OcGC1* (**AB125138**), *OcGC2* (**AB125139**), *OcGC3* (**AB125140**), *OcGC4* (**AB125141**), *OcGC5* (**AB125142**), *OcGC6* (**AB125143**), *OcGC7* (**AB125144**), *OcGC8* (**AB125145**), and *OcGC-R2* (**AB125146**).

The nucleotide and amino acid sequences of *O. curvinotus* were aligned with those of the corresponding *O. latipes* GCs with the program Clustal W. A phylogenetic tree was constructed by the neighbor-joining method using the amino acid sequences of the catalytic domains of various membrane GCs and soluble GCs. As shown in Fig. 1, *O. curvinotus* possesses all of the isoforms of membrane and soluble GCs found in *O. latipes*. The evolutionary distance between a membrane GC of *O. curvinotus* and the corresponding GC of *O. latipes* is closer than that between the membrane GCs of *O. curvinotus* and *F. rubripes* (Fig. 1B). Similarly, the evolutionary distance of a soluble GC subunit between *O. curvinotus* and *O. latipes* is closer than that between soluble GC subunits of *O. curvinotus* and *F. rubripes* (Fig. 1A).

Fig. 1

It has been reported that soluble GC is composed of an N-terminal domain, a central region, and a catalytic domain, and each domain is more or less similar among the soluble GC subunits bovine GCS-α_1, human GCS-α_2, bovine GCS-β_1, and rat GCS-β_2 (Koesling, 1999). However, the highly similar region (conserved N-terminal region) in the N-terminal domain was restricted to a stretch of just over 100 amino acids which were conserved between the α subunits or the β subunits, respectively, but which were less similar between the α and β subunits. Conceivably, the conserved N-terminal region can be used to define the α subunit or β subunit, respectively. It is expected that the central region is concerned with the dimerization of the α subunit with the β subunit (Koesling, 1999). The comparison of the amino acid sequence of each subunit of *O. curvinotus* soluble GC with the corresponding subunit of *O. latipes* soluble GC

indicated that the *O. curvinotus* GC subunit is also composed of a conserved N-terminal region (OcGCS-α_1, residues 134-240; OcGCS-α_2, residues 174-293; OcGCS-β_1, residues 61-164), a central region (OcGCS-α_1, residues 290-439; OcGCS-α_2, residues 392-539, OcGCS-β_1, residues 213-370) and a catalytic domain (OcGCS-α_1, residues 452-667; OcGCS-α_2, residues 551-766; OcGCS-β_1, residues 383-605) (Table 3). The amino acid sequence of the *O. curvinotus* soluble GC subunit and the corresponding *O. latipes* soluble GC subunit are quite similar to each other; in particular, the similarity in the amino acid sequence of the β_1 subunit between *O. curvinotus* and *O. latipes* was the highest among the three subunits. There were only three amino acid differences (^{335}Arg→Gly, ^{578}Ala→Val and ^{608}Ser→Cys) in the β_1 subunit between *O. curvinotus* and *O. latipes*. The nucleotide sequences of cDNAs for the α_1, α_2, and β_1 subunits were also quite similar between *O. curvinotus* and *O. latipes*, while the similarity in the nucleotide sequence of the 3'-UTR of the α_2 subunit cDNA (93.7%) was slightly lower than that in the amino acid sequence (98.3%).

Table 3

Membrane GC is composed of an extracellular domain, a single transmembrane domain, a protein kinase-like domain, and a cyclase catalytic domain. The similarities in the respective domain of each membrane GC between *O. curvinotus* and *O. latipes* are shown in Table 3. The mature OcGC8 protein is composed of a small extracellular domain (residues 1-235), a single transmembrane domain (residues 236-254), a protein kinase-like domain (residues 287-570), and a cyclase catalytic domain (residues 591-817), each of which is highly similar to that of OlGC8. The similarity in the amino acid sequence of the catalytic domain between OcGC8 and OlGC8 is the highest (99.6%) among all of the domains. Although several differences in the nucleotide sequences of various membrane GCs between *O. curvinotus* and *O. latipes* are seen, no difference is found in the amino acid sequences between OcGC2 and OlGC2; OcGC3

and OlGC3; OcGC4 and OlGC4; OcGC6 and OlGC6; or OcGC-R2 and OlGC-R2.

3.2. Soluble GC expression in the hybrid embryos at several developmental stages and adult hybrids

Using a specific primer set, we detected the soluble GC subunit gene transcripts originated from the *O. curvinotus* or *O. latipes* in the hybrid. We checked the specificity of the specific primer sets used for RT-PCR. As shown in Fig. 2, when RT-PCR was carried out with the brain total RNA of *O. latipes* as the template and a primer set specific to *OlGCS-*α_1, *OlGCS-*α_2 or *OlGCS-*β_1, no transcript of *OcGCS-*α_1, *OcGCS-*α_2 or *OcGCS-*β_1 was detected, and conversely, RT-PCR with the brain total RNA of *O. curvinotus* as the template and another primer specific to *OcGCS-*α_1, *OcGCS-*α_2 or *OcGCS-*β_1 resulted in successful detection of *OcGCS-*α_1, *OcGCS-*α_2 or *OcGCS-*β_1 but not *OlGCS-*α_1, *OlGCS-*α_2 or *OlGCS-*β_1. RT-PCR with these primer sets and cDNA reverse-transcribed from total RNA extracted from the hybrid adult brain demonstrated that mRNA for the α_1, α_2 and β_1 subunits originated from both species were expressed in the adult individual brain of the hybrids irrespective of the crossing direction.

Fig. 2

In order to determine the temporal expression pattern of each subunit mRNA of soluble GC, we carried out RT-PCR with total RNA obtained from the hybrid embryos at several developmental stages (stages 1, 16, 18, 22, and 24) and an appropriate specific primer set. Thirty cycles of PCR resulted in no detectable amplification of the *OlGCS-*α_2 and *OcGCS-*α_2 cDNA fragments, but 35 cycles of PCR resulted in successful amplification of the *OlGCS-*α_2 and *OcGCS-*α_2 cDNA fragments. Therefore, in later experiments we used 35-cycle PCR with *OlGCS-*α_2 and *OcGCS-*α_2. As shown in Fig. 3, in hybrid lc (*O. latipes* ♀ × *O. curvinotu*s ♂), the *OlGCS-*α_1 transcript was

Fig. 3

clearly detected in the sample obtained from the hybrid embryos at stage16, but the *OcGCS*-α_1 transcript was only faintly detected. Conversely, in hybrid cl (*O. latipes* ♀ × *O. curvinotus* ♂), the *OlGCS*-α_1 transcript was not detected at stage 16, but the *OcGCS*-α_1 transcript was clearly detected at stage 16. In hybrid lc, the *OlGCS*-α_2 transcript was detected in the sample obtained from the hybrid embryos at stage 22, but the *OcGCS*-α_2 transcript was not detected. Conversely, in hybrid cl, the *OlGCS*-α_2 transcript was not detected at stage 22, but the *OcGCS*-α_2 transcript was detected at stage 22. At stage 24, all subunit mRNAs originated from *O. latipes* and *O. curvinotus* were detected in hybrid embryos (lc and cl). On the other hand, the *OlGCS*-β_1 transcript was detected in hybrid lc (the maternal parent is *O. latipes*) but not detected in hybrid cl (the maternal parent is *O. curvinotus*) at stage 1, and the *OcGCS*-β_1 transcript was clearly detected in hybrid cl and faintly detected in hybrid lc at stage 1.

4. Discussion

In this study, we demonstrated that *O. curvinotus* expresses all membrane and soluble GCs identified in *O. latipes*, and the numbers of GCs expressed in both species were greater than the numbers expressed in mammals. This may be explained by the observation that fishes contain more members of the same gene family than mammals do (Wittbrodt et al., 1998). Moreover, we showed that the deduced amino acid sequences of membrane GCs expressed in *O. curvinotus* are quite similar to those expressed in *O. latipes*. In particular, the identification of *OcGC8* in *O. curvinotus*, which is homologous to a novel membrane GC, *OlGC8*, was the first finding in a species other than *O. latipes*. OlGC8 expressed transiently in COS-7 cells exhibited only the basal GC activity, and none of the known ligands and various medaka fish

tissue extracts stimulated the basal activity (Yamagami et al., 2003). In this study, we found that GC8 was expressed not only in *O. latipes,* but also in *O. curvinotus*, suggesting that the membrane GC would be functional in both species.

The deduced amino acid sequences of the soluble GC subunits expressed in *O. curvinotus* were quite similar to those in *O. latipes*, and there were only three amino acid replacements in the β_1 subunit between *O. curvinotus* and *O. latipes*. As shown in Table 3, the identity of amino acid sequences of the soluble GC α_1 and α_2 subunits between *O. curvinotus* and *O. latipes* are 98.2% and 98.5%, respectively. The dimerization region of human soluble GC-β_1 extends over 205 residues of its regulatory and central domains, and two discontinuous sites of 41 and 30 residues, respectively facilitate binding of β_1 to the α_1 subunit of human soluble GC (Zhou et al., 2004). There is only one difference in the dimerization region (residues 204-408), moreover, there is no difference in the two discontinuous sites of 41 and 30 residues (residues 204-244 and 379-408, respectively) between *O. curvinotus* and *O. latipes*. Therefore, we presume that an active chimeric soluble GC heterodimer can be formed in the hybrids between *O. curvinotus* and *O. latipes*. Actually, mRNAs for the α_1, α_2, and β_1 subunits originated from both species were detected in the adult individual brain of the hybrids irrespective of the crossing direction, suggesting that a chimeric heterodimer may form in the hybrid. In this regard, it should be mentioned that soluble GC has been shown to play critical roles in medaka fish embryogenesis, and the knock-down of soluble GCs using morpholino resulted in severe abnormality in formation of the organs (Yamamoto et al., 2003). In the present study, we demonstrated that *O. curvinotus* is able to produce the hybrid with *O. latipes* irrespective of the direction of crossing, and the hybrids develop to adulthood without apparent abnormality despite their inability to reproduce. This also supports the above idea that an active chimeric soluble GC

heterodimer is formed in the hybrids.

However, the temporal expression patterns of the maternal genes were not completely identical with those of the paternal genes in the hybrids. In the early embryogenesis of the hybrids, the maternal soluble GC subunit genes were expressed earlier than the paternal soluble GC subunit genes, suggesting that the soluble GC subunit genes of maternal origin interact more effectively with the maternal effector molecules, such as transcription factors, while the soluble GC subunit genes of paternal origin interact less effectively with these molecules in the hybrids. However, we presume that the differences in such interaction may be too small to affect the organogenesis in the hybrids.

On the other hand, in a previous study we demonstrated that the *OlGCS-*β_1 transcript was detected in *O. latipes* embryos at stage 1 and reported that *OlGCS-*β_1 was expressed maternally (Yamamoto et al., 2003). In the present study, we demonstrated that the *OcGCS-*β_1 transcript was also detected in the hybrid embryos of lc at stage 1, although it was detected only faintly, indicating that the soluble GC β_1 subunit gene can be expressed not only maternally but also zygotically at stage 1. To make clear the differences between maternal and paternal soluble GCs in the hybrid, it is important to analyze the proximal promoter regions in the 5'-flanking region of soluble GC subunit genes expressed in *O. curvinotus*.

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Captions to figures

Fig.1. Molecular phylogenetic analysis of various teleost GCs. The amino acid sequences of a part of the catalytic domain of soluble GCs (130 residues) **(A)**, and membrane GCs (102 residues) **(B)** were subjected to phylogenetic analysis by the maximum parsimony method. The sea urchin sperm membrane GC (HpGC) was used as an outgroup. The numbers on each node represent the bootstrap pseudoreplication values as a percentage. Sources, references, and accession numbers for the respective GC sequences are described in Materials and methods.

Fig.2. RT-PCR analysis of various transcripts of the soluble GC subunit gene transcripts in the adult brain of *O. latipes*, *O. curvinotus*, and the hybrids between *O.latipes* and *O. curvinotus*. The cDNAs reverse-transcribed from adult brain total RNA (5 μg) were used as the template for the PCR. The PCR products containing the cDNA fragments of various GCs (*OlGCS-*α_1, *OcGCS-*α_1, *OlGCS-*α_2, *OcGCS-*α_2, *OlGCS-*β_1, and/or *OcGCS-*β_1) were separated on a 0.7% agarose gel and the gel was stained with ethidium bromide. Similar results were obtained in two independent experiments.

Fig.3 RT-PCR analysis of the soluble GC subunit gene transcripts expressed during embryogenesis of the hybrids. Reverse transcription of total RNA obtained from approximately 20 embryos at various developmental stages (stage 1, stage 16, stage 18, stage 22, and stage 24) was carried out using Superscript II and oligo(dT)$_{12\text{-}18}$ primers. The resultant cDNA was used as a template for the PCR. The PCR products containing the cDNA fragments of various GCs (*OlGCS-*α_1, *OcGCS-*α_1, *OlGCS-*α_2,

OcGCS-α_2, *OlGCS*-β_1, and/or *OcGCS*-β_1) were electrophoretically separated on a 0.7% agarose gel and the gel was stained with ethidium bromide. Similar results were obtained in two independent experiments.

Tables

Table1. Primers and the annealing temperature used for amplification of the cDNA fragment of *OcGC3*, *OcGC5*, *OcGC-R2*, *OcGC8*, *OcGCS*-α_1, *OcGCS*-α_2, or *OcGCS*-β_1 by PCR

Table 2. Specific primer sets used for amplification of the cDNA fragments of the soluble GC subunit transcripts

Table 3. Sequence similarity of the soluble GC subunits and the catalytic domain of various membrane GCs between *O. latipes* and *O. curvinotus*.

Table 1

GCs	position of primers		Annealing
	forward	reverse	temp. (^{o}C)
OcGC3	2549-2567 bp	2963-2980bp	55
OcGC5	3216-3233 bp	3716-3733 bp	50
OcGC-R2	2808-2825 bp	3222-3239 bp	54
OcGC8	1331-1353 bp	2099-2117 bp	62
	2049-2069 bp	2816-2935 bp	62
	2593-2610 bp	3271-3288 bp	59
	3220-3222 bp	4126-4046 bp	59
OcGCS-β_1	20-37 bp	935-953 bp	52
	576-593 bp	1478-1495 bp	51
	1195-1212 bp	1988-2008 bp	54
	1844-1861 bp	2614-2631 bp	48
OcGCS-α_1	1-20 bp	936-953 bp	52
	731-750 bp	2525-2542 bp	47
OcGCS-α_2	146-165 bp	744-765 bp	60
	532-551 bp	1019-1038 bp	58
	901-920 bp	1636-1656 bp	60
	1543-1562 bp	2167-2186 bp	58
	2110-2129 bp	2813-2832 bp	60

Table 2

type of subunit	position of primers	
	forward	reverse
OlGCS-α_1	1880-1999 bp	2324-2343 bp
OcGCS-α_1	1840-1859 bp	2284-2303 bp
OlGCS-α_2	353-272 bp	817-836 bp
OcGCS-α_2	306-325 bp	770-789 bp
OlGCS-β_1	1342-1361 bp	1625-1644 bp
OcGCS-β_1	1302-1342 bp	1588-1607 bp

Table 3

Type of GC	Identity (%) (Oc/Ol)	
	Nucleotides	Amino acids
GCS-α_1	98.0	98.2
GCS-α_2	98.3	98.5
GCS-β_1	98.5	99.5
GC1	97.4	98.0
GC2	98.4	100
GC3	96.7	100
GC4	99.3	100
GC5	97.3	96.9
GC6	97.7	100
GC7	97.4	97.1
GC8	97.4	98.0
GC-R2	97.0	100

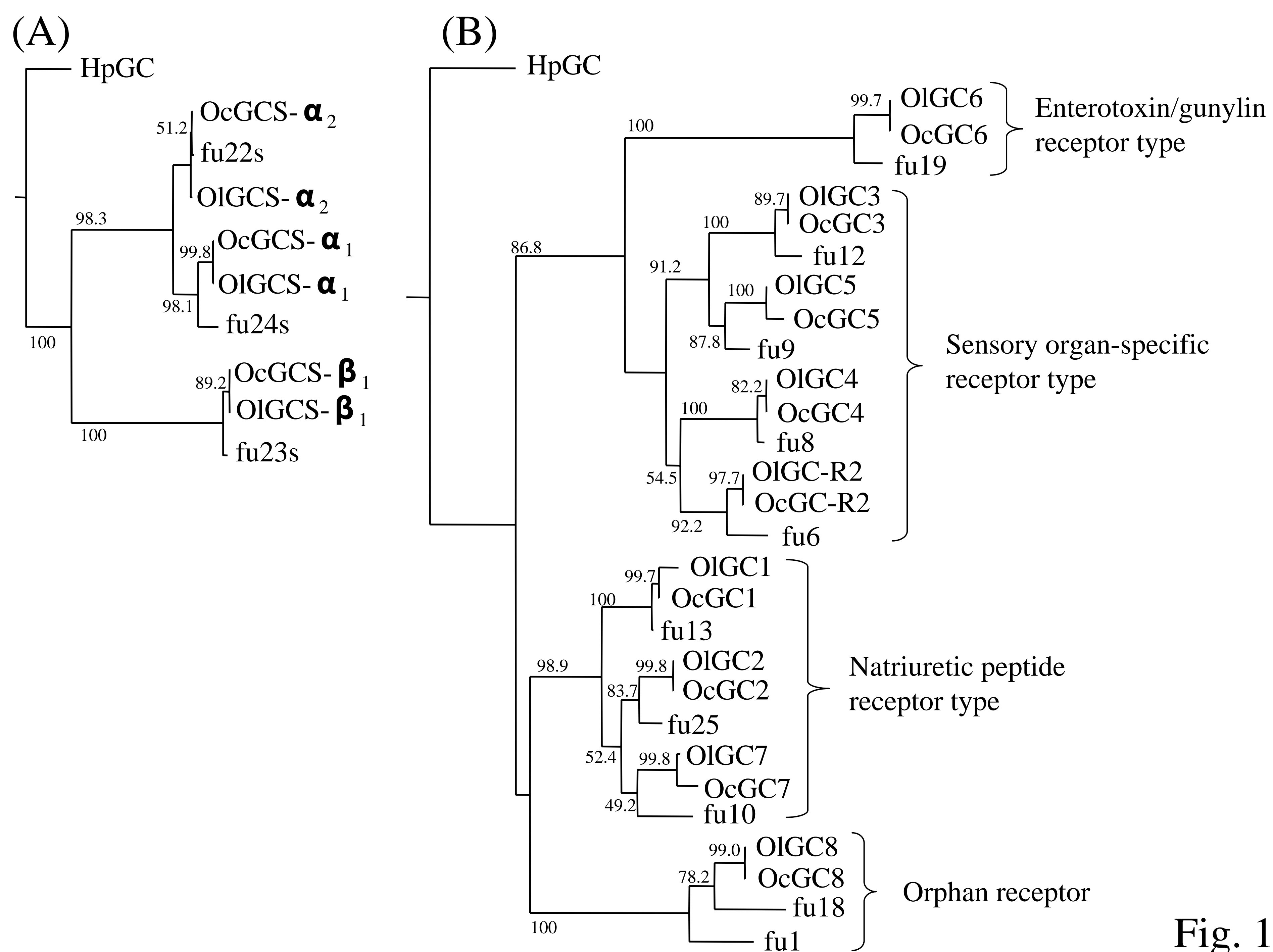

Fig. 1

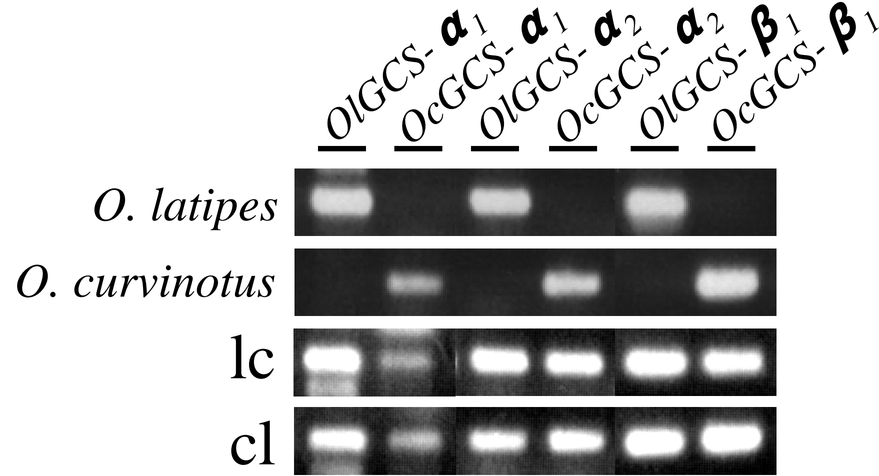

Fig. 2

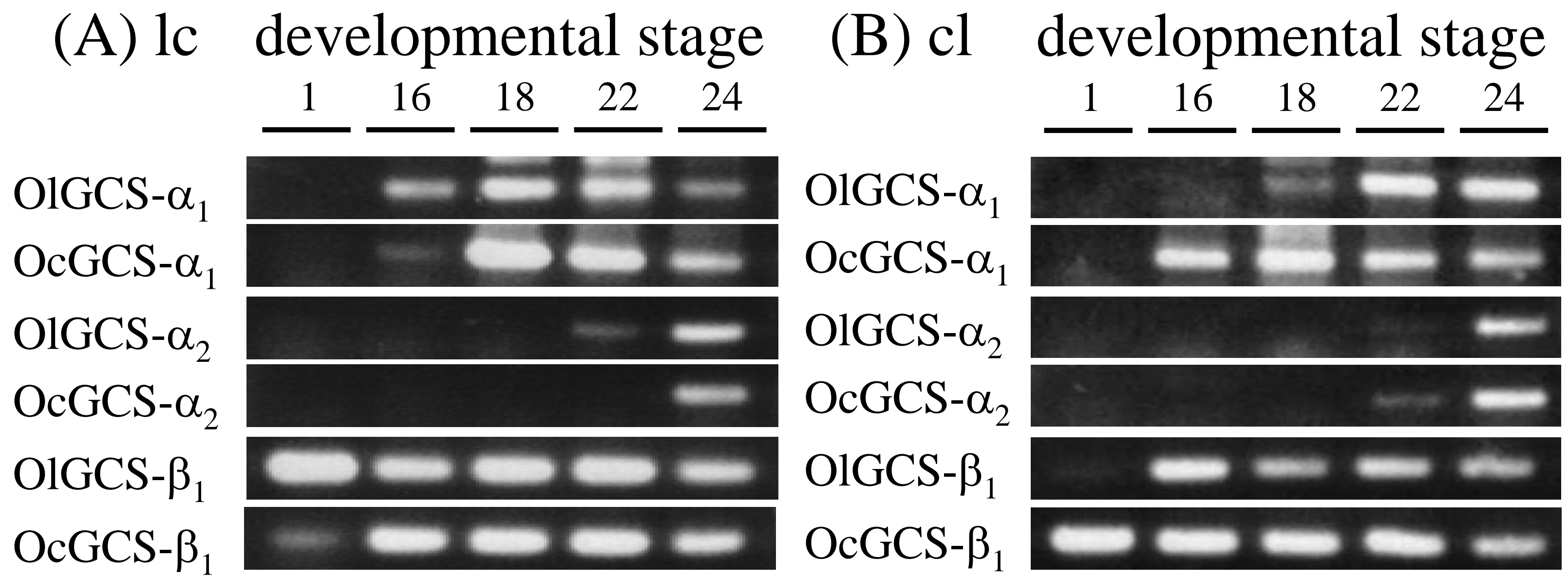

Fig. 3