Promoter activity of the 5'-flanking regions of medaka fish soluble guanylate cyclase α_1 and β_1 subunit genes

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We examined the spatial expression pattern of medaka fish (*Oryzias latipes*) soluble guanylate cyclase α_1 and β_1 subunit genes, *OIGCS- α_1* and *OIGCS- β_1* , and characterized the 5'-flanking region required for expression of both genes by introducing various promoter-luciferase fusion-gene constructs into COS-1 cells and medaka fish embryos. The *OIGCS- α_1* and *OIGCS- β_1* gene transcripts were detected in whole brain and kidney in 7-day and 9-day embryos. Primer-extension analysis demonstrated that there were no differences among various adult organs (brain, eye, kidney, ovary and testis) in the transcription start site of the *OIGCS- α_1* and *OIGCS- β_1* genes. Neither gene contained the functional TATA box within its 5'-flanking region, and the basal promoter activity was found

between nucleotides +33 and +42 in the *OIGCS- α_1* gene and between nucleotides +146 and +155 in the *OIGCS- β_1* gene. In the assay of medaka fish embryos, the 5'-flanking region of the *OIGCS- β_1* gene exhibited lower promoter activity than that of the *OIGCS- α_1* gene. In the experiments on dual-luciferase fusion-gene constructs, the 5'-flanking region of the *OIGCS- α_1* gene connected to the 5'-flanking region of the *OIGCS- β_1* gene was introduced into medaka fish embryos, and the 5'-flanking regions of both subunit genes were shown to mutually influence each other's promoter activity.

Key words: cGMP, co-ordinated transcription, dual-luciferase assay, reporter fusion-gene construct.

INTRODUCTION

Ubiquitous intracellular cGMP is formed from GTP by guanylate cyclase (GC; EC 4.6.1.2), which is present in two forms, a membrane form and a soluble form. The soluble form of GC (soluble GC) is a haem-containing heterodimer composed of α and β subunits [1] and is activated by binding of gaseous ligands, such as NO and CO, to a single haem in the enzyme [2]. The NO-activated soluble GC produces cGMP and subsequently activates cGMP-dependent protein kinases, phosphodiesterases and cation channels. Through the action of these enzymes and channels, the NO/cGMP signalling pathway plays important roles in various physiological phenomena, including vascular smooth-muscle relaxation, synaptic plasticity, signal transduction in various sensory organs, platelet aggregation, neuro-transmission and programmed cell death [3,4].

To date, two isoforms of each subunit of soluble GC have been identified (α_1 and α_2 , β_1 and β_2) and many cDNA clones have been obtained from various species, e.g. humans, cattle, rats, mice, medaka fish (*Oryzias latipes*), sea urchin, *Drosophila* and the moth *Manduca sexta* [5–19]. Moreover, soluble GC has been suggested to be present in *Caenorhabditis elegans* and *Saccharomyces cerevisiae* [20,21]. Each subunit has a catalytic domain in the C-terminal part, but a single subunit itself exhibits no catalytic activity [1]. The active enzyme is formed only by the association of α and β subunits [1], although glutathione S-transferase (GST)-tagged recombinant human α_1 and β_1 subunits have been shown to form catalytically inactive homodimers [22]. Recently, it has been reported that NO-sensitive GC activity can be detected and measured when the rat β_2 subunit is expressed alone in Sf9 cells [23]. However, the most active heterodimeric enzyme is formed from a combination of the α_1 and β_1 subunits [11,24,25]. Despite the rapid accumulation of information concerning the structural nature of soluble GC and the

functional importance of the NO/cGMP signalling pathway, there have been only a few reports related to the regulation of soluble GC gene expression. It has been demonstrated that the level of mRNA for soluble GC subunits is controlled by cAMP-elevating agents, nerve growth factor, and endotoxin or interleukin-1 β [26–28].

The α_1 and β_1 subunit genes are co-localized in human, rat and mouse chromosomes [15,29,30]. Recently, the genomic structure of soluble GC in the *Anopheles* mosquito and mouse has been reported [15,31]. However, with the exception of our report that the α_1 and β_1 subunit genes are aligned in a tandem fashion and separated by a 1 kb spacer sequence in the medaka fish genome [32], there has been no report on the topology of either gene in the genome. Our earlier finding and the fact that the co-expression of both subunit genes is essential for formation of the active enzyme [6,22] suggest that the expression of both genes is co-ordinated. In the present study we characterize the basal promoter region of the *O. latipes* soluble GC (*OIGCS*) α_1 and β_1 subunit genes, *OIGCS- α_1* and *OIGCS- β_1* , and the co-ordinated transcription of both genes.

EXPERIMENTAL

Animals and embryos

Adults and embryos of the orange-red variety of medaka fish *O. latipes* were maintained as described in [33]. The developmental stage was expressed in the manner described by Iwamatsu [34].

Cell culture

Monkey kidney COS-1 cells were cultured in Dulbecco's modified Eagles's medium (Life Technologies, Rockville, MD, U.S.A.) containing 10% fetal bovine serum (HyClone, Logan, UT,

Abbreviations used: AP, activating protein; GC, guanylate cyclase; GST, glutathione S-transferase; *OIGCS*, *Oryzias latipes* soluble GC; RL, *Renilla* luciferase; GL, firefly luciferase; C/EBP, CCAAT-enhancer binding protein; SV40, simian virus 40; GFP, green fluorescent protein; poly(A)⁺, polyadenylated.

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Table 1 Primers used for construction of luciferase fusion genes

Primer	Sequence
Alu-3.8k/ <i>KpnI</i>	5'-CGGGGTACCGCAAGGGTTTATGCAGAG-3'
Alu-2.9k/ <i>KpnI</i>	5'-CGGGGTACCTATGTGGCATCACCACAGG-3'
Alu-2.6k/ <i>KpnI</i>	5'-CGGGGTACCCCAAGAACATGTTAAACACAA-3'
Alu-2.3k/ <i>KpnI</i>	5'-CGGGGTACCGAACATTTGCACAGTCCTCT-3'
Alu-2.2k/ <i>KpnI</i>	5'-CGGGGTACCCTAACATGCCACATTACCAT-3'
Alu-1.9k/ <i>KpnI</i>	5'-CGGGGTACCCATAGAAAGTTCTTTCCG-3'
Alu-1.3k/ <i>KpnI</i>	5'-CGGGGTACCGCTGTGGCTACAACCATA-3'
Alu-1.1k/ <i>KpnI</i>	5'-CGGGGTACCGTGGGACTCAGAGGTTTTTC-3'
Alu-1.0k/ <i>KpnI</i>	5'-CGGGGTACCGTAACAAGAATTTCATGGCA-3'
Alu-0.9k/ <i>KpnI</i>	5'-CGGGGTACCTGTATGGTCACGCTCTTG-3'
Alu-0.3k/ <i>KpnI</i>	5'-CGGGGTACCAAAAGAGGAGAGAAATGTGCA-3'
Alu-0.2k/ <i>KpnI</i>	5'-CGGGGTACCTGCCCTCGCCATAACTG-3'
Alu-0.1k/ <i>KpnI</i>	5'-CGGGGTACCCAGTTTCAGACTTTGTCTGAG-3'
Alu-3/+142/ <i>KpnI</i>	5'-CGGGGTACCTTAGATTACACGCCCC-3'
Alu + 32/+142/ <i>KpnI</i>	5'-CGGGGTACCTACTGCTCTTTTCCCCAGT-3'
Alu + 42/+142/ <i>KpnI</i>	5'-CGGGGTACCTTTTCCCCAGTTTCTATTG-3'
Alu + 48/+142/ <i>KpnI</i>	5'-CGGGGTACCCAGTTTCTTATTGGTGTC-3'
α -1R/ <i>HindIII</i>	5'-GCCCAAGCTTCAATGATCAGCAAGAAGCTG-3'
Blu-1k/ <i>KpnI</i>	5'-CGGGGTACCTGCAATAAATGCTGTCATTGATG-3'
Blu-0.5k/ <i>KpnI</i>	5'-CGGGGTACCCATTCCAGTCCATTGTGTGG-3'
Blu + 9/+189/ <i>KpnI</i>	5'-GGCGGTACCCGACATGTAACACCGC-3'
Blu + 68/+189/ <i>KpnI</i>	5'-GGCGGTACCTTATTCTGATTGTTGAGATTG-3'
Blu + 115/+189/ <i>KpnI</i>	5'-GGCGGTACCACTATTGGACAGCTGAGC-3'
Blu + 124/+189/ <i>KpnI</i>	5'-GGCGGTACCACTGAGCTGTCTGTCAAGT-3'
Blu + 135/+189/ <i>KpnI</i>	5'-GGCGGTACCTCTGTCAAGTCTGCCG-3'
Blu + 145/+189/ <i>KpnI</i>	5'-GGCGGTACCTGCGCACTATACGG-3'
Blu + 155/+189/ <i>KpnI</i>	5'-GGCGGTACCTACTGGGGATGAACAC-3'
Blu + 165/+189/ <i>KpnI</i>	5'-GGCGGTACCGAACACCGACAATCTCAG-3'
β -1R/ <i>HindIII</i>	5'-CCCAAGCTTACAGATGCTGAGATTGTC-3'

U.S.A.), that was heat-inactivated by incubating for 30 min at 56 °C, supplemented with 1 × penicillin/streptomycin/glutamine (Life Technologies), and maintained at 37 °C in an atmosphere of 5% CO₂/95% air. Culture medium was changed every 2–3 days to fresh medium and the cells were subcultured before reaching approx. 70% confluency.

Construction of luciferase fusion genes

DNA fragments with various-sized 5'-flanking regions and non-coding regions of the *OIGCS- α_1* or *OIGCS- β_1* gene were generated by PCR amplification, with various forward primers (upstream primer) with an additional *KpnI* site and receptor sequences around the non-coding exon sequence (downstream primer) with an additional *HindIII* site. Nucleotide sequences of upstream primers are shown in Table 1. The PCR product was digested with *KpnI*/*HindIII* and subcloned into the *KpnI*/*HindIII* site of the vectors pGL-3 enhancer and pGL-3 basic (Promega, Madison, WI, U.S.A.).

Construction of pRLGL dual-luciferase fusion genes

The 5'-flanking region of *OIGCS- β_1* (−1000/+189) was amplified by PCR the following primers: β -up/*SacI*, 5'-GGCG-AGCTCTGCAATAAATGCTGTCATTGATG-3', and β -1R/*HindIII* (see Table 1). The PCR product was subcloned into the *SacI*/*HindIII* site of a pGL-3 enhancer vector in which the *KpnI* site had been replaced with the *XhoI* site. The 5'-flanking region of *OIGCS- α_1* (−3816/+142) was amplified by PCR using the following primers: RLGL-3.8k/*XhoI*, 5'-CCGCTCGAGGCA-AGGGTTTATGCAGAG-3', and α -Rev/*SmaI*, 5'-TTCCCCCGGGTCAAATGATCAGCAAGAAGCTG-3'. The PCR product was subcloned into the *XhoI*/*SmaI* site of the pRL-null

vector (Promega), in which the *BamHI* site had been replaced with a *SacI* site. Both PCR products were digested by *XhoI*/*SacI* and ligated. pRLGL deletion variants were constructed by PCR of the 5'-flanking region of the *OIGCS- α_1* gene using the following primers: RLGL-1.9k/*XhoI*, 5'-CCGCTCGAGCATAGAAGT-TCCTTTCCG-3', RLGL-0.9k/*XhoI*, 5'-CCGCTCGAGTGT-ATGGTCACTCTTTG-3', RLGL-0.3k/*XhoI*, 5'-CCGCTCGAGAAAAGAGGAGAGAAATGTGC-3', RLGL + 48bp/*XhoI*, 5'-CCGCTCGAGCCAGTTTCTTATTGGTGTC-3', and α -Rev/*SmaI*.

A construct pRL-3.8k/GLΔ + 154 was generated by replacing the 5'-flanking region of the *OIGCS- β_1* gene in pRLGL-3.8k with the 5'-flanking region of the *OIGCS- β_1* gene in Blu + 155/+189 as described above. The boundary of the inserted DNA in all constructs was sequenced using an ABI Prism 377 DNA sequencer to monitor for PCR-associated nucleotide-incorporation error with the following oligonucleotide primers: RV-3, 5'-CTAGCAAAATAGGCTGTCCC-3', GL-3, 5'-CGTATCTCTTCATAGCCTTATG-3', and RL-Rev, 5'-CGACAAGCCAGTTTCT-3'. All plasmid DNAs were purified using a Qiagen maxi kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Transient transfection of a fusion-gene construct into COS-1 cells and luciferase assay

COS-1 cells were transfected by the calcium phosphate precipitation method. One day before transfection, COS-1 cells were plated at 1 × 10⁶ cells on a 100 mm dish. A promoter-luciferase construct (20 µg) and 20 µg of pSV β -gal DNA (Promega) as an internal control were incubated with 1.1 ml of calcium phosphate solution containing 125 mM CaCl₂ and 1 × HBS [140 mM NaCl/25 mM HEPES/1.4 mM Na₂HPO₄ (pH 6.9)] for 10 min at room temperature (approx. 25 °C). After a 24 h incubation in a CO₂ incubator, cells were harvested and shocked in 15% glycerol in 1 × HBS for 3 min. At 48 h after transfection, cells were washed with PBS and then harvested with 900 µl of 1 × Reporter Lysis Buffer (Promega), followed by centrifugation at 7000 g for 2 min at 4 °C to pellet the residual cellular debris. For the luciferase assay, 20 µl of the cell lysate was mixed with 100 µl of the luciferase substrate (Promega) and light units were counted for 100 s using a luminometer (Lumat LB9507; Berthold, Bad Wildbad, Germany). The luciferase activity of various promoter constructs was calculated as the ratio of firefly luciferase activity to β -galactosidase activity for each transfection. The β -galactosidase assay was carried out as described by standard protocols [35].

Transfection of *Renilla* luciferase (RL)/firefly luciferase (GL) constructs and dual-luciferase assay

The dual-luciferase assay was carried out using the Dual-Luciferase Reporter Assay System (Promega). An RLGL construct was transfected into COS-1 cells as described above. Transfected cells were washed in PBS twice and then harvested with 900 µl of 1 × Passive Lysis Buffer (Promega). The cell lysate for the dual-luciferase assay was prepared as described above. The cell lysate (20 µl) was mixed with 100 µl of Luciferase Assay Reagent II for measuring the GL activity and 100 µl of Stop & Glo Reagent (Promega) was added to measure the RL activity. Light units of both luciferase activities were counted for 10 s. For normalization of transfection efficiency, cells were co-transfected routinely with 20 µg of pSV β -gal DNA.

Microinjector of a fusion-gene construct into medaka fish embryo and luciferase assay

For the luciferase assay, various luciferase fusion-gene constructs were injected into two-cell-stage embryos. The fusion-gene construct (50 ng of DNA/ μ l) containing the 5'-flanking region of the *OIGCS- α_1* or *OIGCS- β_1* gene and the internal control vector pRL-TK (50 ng of DNA/ μ l; Promega) were co-injected as described previously [32]. The injected embryos were cultured until the late blastula stage (stage 11) and washed with PBS. The embryos were then homogenized in 150 μ l of 1 $\times$ Passive Lysis Buffer (30 μ l/embryo) and centrifuged at 4000 *g* for 5 min to pellet the residual cellular debris. The resulting supernatant (20 μ l) was used to measure GL or RL activity using a Dual-Luciferase Reporter Assay System kit. RL activity was used to correct for variations in transfection efficiency.

For luciferase assay of embryos injected with dual-luciferase fusion-gene constructs, 50 ng/ μ l dual-luciferase fusion gene and an equal amount of pSV β -gal DNA were microinjected, and the cell lysate was prepared as described above. Luciferase activity of various promoter constructs was calculated as the ratio of activities between RL or GL and β -galactosidase. The same experiments were performed at least four times.

Primer extension

For determination of the transcription-initiation site within both gene promoters, primer-extension experiments were performed using polyadenylated [poly(A)⁺] RNA isolated from the adult medaka fish brain, eye, kidney, ovary or testis as a template. Poly(A)⁺ RNA was isolated from the adult organs using Oligotex-dT30 Super (Roche Diagnostics GmbH, Mannheim, Germany) as described previously [33]. Oligonucleotide primers PE-alpha, 5'-GAAGAAATCCCATCATTCGG-3', and PE-beta, 5'-ACAGATGCTGAGATTGTCCG-3', corresponding to sequence from exon 1 of the *OIGCS- α_1* and *- β_1* genes, respectively, was end-labelled with [α -³²P]ATP (Amersham Pharmacia Biotech, Rainham, Essex, U.K.) using T4 polynucleotide kinase (TaKaRa, Otsu, Japan). Each poly(A)⁺ RNA (2 μ g) was hybridized with isotope-labelled primer (5 $\times$ 10⁴ c.p.m.) 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 90 min. The extension reaction was carried out in 45 mM Tris/HCl (pH 8.3), 3 mM MgCl₂, 10 mM dithiothreitol, 0.5 mM of each dNTP and 200 units of SuperScript II reverse transcriptase (Life Technologies) at 42 °C for 1 h. After RNase treatment (16 μ g/ml, 37 °C for 15 min), the cDNA products were extracted with phenol and chloroform and precipitated with ethanol. The DNA was analysed on a 4.5% polyacrylamide sequencing gel. Dideoxy sequencing products primed by the corresponding primers were run in parallel for size comparison. The radioactive signals were analysed using a FUJIX Bio-Imaging Analyser BAS2000 (Fuji Photo Film, Tokyo, Japan).

Whole-mount *in situ* hybridization

The pBluescript II KS vector (Stratagene, La Jolla, CA, U.S.A.) containing a cDNA fragment encoding a part of the *OIGCS- α_1* cDNA (nucleotides 195–953) or *OIGCS- β_1* cDNA (nucleotides 252–953) was used for preparation of an antisense or a sense RNA probe. Digoxigenin labelling of the probe was carried out according to the manufacturer's instructions (Roche Diagnostics GmbH).

To avoid eye pigmentation, 1-day embryos (stage 20) were transferred to distilled water containing 0.2 mM 1-phenyl-2-thio urea (Wako Pure Chemicals, Osaka, Japan) and 0.0006 %

Methylene Blue, and cultured in this solution at 27 °C. Embryos were fixed overnight in 4% paraformaldehyde/PBS containing 0.1% Tween-20 (PBST). After fixation, the chorion was removed and the embryo was washed with PBST twice, dehydrated by immersing in 100% methanol and stored at –20 °C until use.

For hybridization, the embryo was rehydrated with 50% and then 30% methanol in PBST. The embryo was washed three times with PBST and treated with 6% H₂O₂ in PBST for 2 h, and then refixed with 4% paraformaldehyde in PBST for 20 min and washed twice with PBST. The embryo was then treated with proteinase K (5 μ g/ml in PBST) for 10 min at room temperature. After two washes in PBST, the embryo was refixed with 4% paraformaldehyde in PBST for 20 min and washed twice with PBST. The treated embryo was incubated in pre-hybridization buffer [50% formamide (Roche Diagnostics GmbH), 5 $\times$ SSC (where 1 $\times$ SSC is 0.15 M NaCl/0.015 M sodium citrate) and 0.1% Tween-20] for 5 min at 55 °C. Then, pre-hybridization buffer was replaced with fresh hybridization buffer containing 5 mg/ml yeast RNA and 50 μ g/ml heparin, incubated for 1 h at 55 °C, and hybridized overnight with digoxigenin-labelled RNA probe (100 ng/ml) in hybridization buffer at 55 °C. The embryo was then washed at the hybridization temperature with 2 $\times$ SSC and 0.1% Tween-20 (SSCT) for 30 min and with 50% formamide in 2 $\times$ SSCT for 15 min, followed by 0.2 $\times$ SSCT for 30 min. Following a wash with PBST for 10 min, the embryo was incubated in 1% blocking reagent (Roche Diagnostics GmbH) and 10% sheep serum (Sigma) in PBST for 1 h at room temperature. After the blocking, the embryo was incubated overnight at 4 °C with 1/2000-diluted anti-digoxigenin Fab fragment (Roche Diagnostics GmbH) containing 10% sheep serum and extracts of medaka fish embryos to reduce the background. Following four washes with PBST for 30 min each, the embryo was washed three times with staining buffer [0.1 M NaCl/50 mM MgCl₂/0.1 M Tris/HCl (pH 9.5)/1 mM levamisole/0.1% Tween-20] for 5 min each time, and then incubated with 20 μ l/ml Nitro Blue Tetrazolium/5-bromo-4-chloroindol-3-yl phosphate stock solution (Roche Diagnostics GmbH) in staining buffer in the dark at 4 °C. After the colour reaction, the embryo was dehydrated in an ethanol series and incubated with 100% ethanol, followed by rehydration with PBST. Then the embryo was mounted in 25% glycerol in PBST and subsequently transferred to 50% glycerol in PBST. The embryo was observed under a light microscope.

RESULTS

Expression of *OIGCS- α_1* and *OIGCS- β_1* in early medaka fish embryos

Whole-mount *in situ* hybridization experiments showed that strong hybridization signals due to both the *OIGCS- α_1* and *OIGCS- β_1* transcripts were detected in whole brain and kidney in the 7-day (stage 37) embryo and 9-day hatched embryo (stage 39; results not shown).

Determination of transcription start sites of the *OIGCS- α_1* and *OIGCS- β_1* genes

To examine whether the tissue-specific transcription start site of the *OIGCS- α_1* or *OIGCS- β_1* genes was present, primer-extension analysis was carried out using various poly(A)⁺ RNAs (2 μ g of each) isolated from various medaka fish tissues, including brain, eye, kidney, ovary and testis. The transcription start sites were determined at nucleotide positions 290 bp (G; *OIGCS- α_1*) and 263 bp (A; *OIGCS- β_1*) upstream of the respective putative initiation codons (Figure 1). There were no differences between

Figure 1 Identification of transcription start sites by primer extension and schematic representation of promoter regions of the *OIGCS-α₁* and *OIGCS-β₁* genes

(A, C) Primer-extension analysis for the identification of the transcription start site of *OIGCS-α₁* (A) and *OIGCS-β₁* (C). A radioisotope-labelled antisense oligonucleotide primer corresponding to the 21 bp (+183 to +203) for *OIGCS-α₁*, or to the 20 bp (+170 to +189) for *OIGCS-β₁*, was hybridized with 2 μ g of poly(A)⁺ RNA isolated from adult medaka fish brain, eye, kidney, ovary and testis, and subjected to primer-extension analysis. For the sequencing ladder, a dideoxy DNA sequencing reaction of *OIGCS-α₁* or *OIGCS-β₁* genomic DNA using the same antisense oligonucleotide primer was performed. The corresponding nucleotide in the sequencing ladder is marked by an asterisk. The transcription start site was determined to be at nucleotide position 290 bp (G) for *OIGCS-α₁*, and 263 bp (A) for *OIGCS-β₁*, upstream of the respective putative initiation codons. Lane 1, brain; lane 2, eye; lane 3, kidney; lane 4, ovary; lane 5, testis. (B, D) Schematic diagrams of the promoter regions of *OIGCS-α₁* (B) and *OIGCS-β₁* (D). The transcription start site is numbered as +1.

tissues for the transcription start site of either gene, and no additional transcription start site was detected in the tissues examined (Figures 1A and 1C).

Promoter analysis of the 5'-flanking region of the *OIGCS-α₁* gene

The proximal promoter region of the *OIGCS-α₁* gene contains several known *cis* elements, such as activating protein (AP-2), GATA, Oct-1, CCAAT box, CCAAT-enhancer binding protein (C/EBP) and a TATA-like sequence [32]. To determine whether the 5'-flanking region of the *OIGCS-α₁* gene has the promoter activity, a 3958 bp genomic DNA fragment from -3816 to +142 (where +1 is the transcription start site) was connected to the upstream region of a GL reporter-gene pGL-3 enhancer vector or a GL reporter luciferase gene pGL-3 basic vector, and injected into medaka fish two-cell-stage embryos. As shown in Table 2, the 5'-flanking region of the *OIGCS-α₁* gene directed to express the luciferase gene, and a degree of considerable luciferase activity, was detected. Removal of the nucleotides -3816 to +31 (Alu +32/+142; Table 1) resulted in a decrease in luciferase activity to a level slightly higher than that of the promoterless pGL-3 enhancer vector. By further deletions, the level of luciferase activity decreased to slightly less than that of the promoterless vector (Alu +42/+142). The promoter

assay of medaka fish embryos thus produced valuable results, but was time-consuming and inadequate for the assay of many of the fusion-gene constructs. In addition, the results sometimes varied, particularly in the case of the dual-luciferase assay (see below). We therefore employed a promoter assay system using COS-1 cells for most of the following experiments.

As shown in Figure 2, among the 17 reporter fusion-gene constructs examined, Alu-1.3k showed the highest luciferase activity. Deletion of the nucleotides from -3816 to -2890 (Alu-2.9k) resulted in an approx. 40% decrease in luciferase activity compared with that of Alu-3.8k, whereas deletion of the nucleotides from -3816 to -2605 (Alu-2.6k) restored the activity. Removal of the nucleotides from -3816 to -2304 (Alu-2.3k) resulted in a 2-fold increase in luciferase activity compared with that of Alu-3.8k, and removal of the nucleotides from -3816 to -991 (Alu-1.0k) led to an approx. 50% decrease in luciferase activity. When the nucleotides from -3816 to +41 were deleted (Alu +42/+142), the luciferase activity due to the reporter gene fell to the level of the promoterless pGL-3 enhancer vector. Furthermore, a construct (Alu-3/+142) without a TATA-like sequence (TATAATA, -45/-39) showed the same level of luciferase activity as the construct with the TATA-like sequence (Alu-0.2k). The simian virus 40 (SV40) enhancer in the pGL-3 enhancer vector did not affect the nature of the *OIGCS-α₁*

Table 2 Luciferase activities in medaka embryos injected with various fusion-gene constructs

Data significantly differing from control values (promoter-less vectors) are indicated with an asterisk ($P < 0.05$, Student's t test). The results are expressed as means $\pm$ S.E.M. of raw luciferase units (RLU) normalized against β -galactosidase activity, which was measured at an absorbance of 420 nm.

Construct	Luciferase activity	<i>n</i>
5'-Flanking region of α_1 subunit gene		
Alu-3.8k (−3816/+142) (+SV40)	59.3 $\pm$ 6.1*	8
Alu-3.8k (−3816/+142) (−SV40)	36.1 $\pm$ 3.4*	5
Alu +32/+142 (+SV40)	35.8 $\pm$ 2.7*	5
Alu +42/+142 (+SV40)	7.04 $\pm$ 1.0	5
5'-Flanking region of β_1 subunit gene		
Blu-1k (−1000/+189) (+SV40)	29.5 $\pm$ 5.0	6
Blu-1k (−1000/+189) (−SV40)	14.7 $\pm$ 2.8	5
Blu +155/+189 (+SV40)	35.9 $\pm$ 8.4	5
Blu +165/+189 (+SV40)	14.9 $\pm$ 2.5	7
Promoterless control vector		
pGL-3 enhancer vector (+SV40)	22.8 $\pm$ 4.9	5
pGL-3 basic vector (−SV40)	23.0 $\pm$ 6.9	4

Figure 2 Promoter activity of serial deletion constructs of the *OIGCS- α_1* subunit gene in COS-1 cells

COS-1 cells were transfected with sequentially deleted luciferase reporter fusion-gene constructs. The promoter activity of each construct is expressed as a percentage of that of Alu-1.3k. The pGL-3 enhancer vector is used as a promoterless control vector. Transient transfection and luciferase assay were performed six times independently, and the data are expressed as means $\pm$ S.D. of raw luciferase units (RLU) normalized against β -galactosidase activity, which was measured at an absorbance of 420 nm.

promoter, with the exception that it induced higher promoter activity. Luciferase activity with the pGL-3 enhancer vector was 40-fold higher than with the pGL-3 basic vector when assayed in

Figure 3 Promoter activity of serial deletion constructs of the *OIGCS- β_1* subunit gene in COS-1 cells

The promoter activity of each construct is expressed as a percentage of that of Blu +9/+189. Blu Δ -213/+167 lacked the nucleotides from −212 to +166 of the 5'-flanking region of the *OIGCS- β_1* gene. The pGL-3 enhancer vector was used as a promoterless control vector. Transient transfection and luciferase assay were performed six times independently, and the data were normalized to β -galactosidase activity and expressed as means $\pm$ S.D.

COS-1 cells (results not shown) and 2-fold higher than with the pGL-3 basic vector in medaka fish embryos (Table 2).

Promoter analysis of the *OIGCS- β_1* 5'-flanking region

The proximal promoter region of the *OIGCS- β_1* gene contains a consensus TATA box, a CCAAT box and other putative regulatory elements [AP-3, GATA-1, C/EBP and hepatocyte nuclear factor (HNF)-5 site]. In our previous investigation of several *OIGCS- α_1* -GFP and *OIGCS- β_1* -GFP fusion-gene constructs (where GFP corresponds to green fluorescent protein), we suggested that although the upstream region of the *OIGCS- α_1* gene alone was sufficient for the expression of GFP-derived fluorescence, the upstream region of the *OIGCS- β_1* gene alone was not sufficient for such expression. However, GFP-derived fluorescence is not sensitive enough for detection and not adequate for quantitative determination of the promoter activity of serially deleted fusion-gene constructs. Therefore, in the present study, we generated 11 *OIGCS- β_1* fusion-gene constructs using a luciferase gene as a reporter and examined whether the 5'-flanking region of the *OIGCS- β_1* gene had promoter activity. Reporter activity in the embryo injected with the construct Blu-1k (Table 1), containing a 1 kb 5'-flanking region of the *OIGCS- β_1* gene, was much lower than that of the *OIGCS- α_1* gene and was at the same level as the reporter activity induced by the promoterless vector (Table 2). In contrast, when assayed in the COS-1 cell system, the Blu-1k construct exhibited considerable luciferase activity due to the reporter gene compared with that of the promoterless pGL-3 enhancer vector.

Although deletions from −1000 to +67 had no significant effects on the expression of luciferase activity, deletion from +68 to +114 resulted in a 50 % reduction of the activity of Blu +9/+189. Further deletions (Blu +155/+189) reduced the promoter activity to the level of that with the pGL-3 enhancer vector alone. Removal of the TATA box (TATAGAA, −16/−11) did not

Figure 4 Analysis of the co-ordinated transcriptional activity of both subunit genes in the COS-1 cell system

(A) Schematic drawing of pRLGL dual-luciferase fusion-gene constructs. Eight fusion-gene variants were constructed. The pRL-3.8k/GL Δ +154 construct lacked the nucleotides from -1000 to +154 of the 5'-flanking region of the *OIGCS- β_1* gene. (B) Luciferase activities of dual-luciferase fusion-gene constructs in COS-1 cells. COS-1 cells were transfected transiently. RL (left) and GL (right) activities were normalized by β -galactosidase activity. The results are the means $\pm$ S.D. from six different experiments.

affect the expression of luciferase activity (construct Blu+9/+189 in Figure 3). The SV40 enhancer in the fusion-gene construct did not change the character of the *OIGCS- β_1* promoter, as seen in the case of the *OIGCS- α_1* gene.

Luciferase assay with dual-luciferase fusion-gene constructs

To examine quantitatively whether the 5'-flanking region of the *OIGCS- α_1* and/or *OIGCS- β_1* genes affects the expression of either or both of the subunit genes, we used two reporter genes (RL and GL) to generate several fusion-gene constructs that mimicked the genomic organization of two medaka fish soluble

GC subunit genes and introduced the constructs into COS-1 cells or medaka fish embryos. This unique fusion-gene construct differentially and simultaneously allowed us to measure two luciferase activities in the same preparation. As shown in Figure 4, in the COS-1 cell system the promoter activity of the β_1 subunit gene (GL activity) decreased 40% when the 5'-flanking region of the *OIGCS- α_1* gene containing the RL gene was connected to Blu-1k (pRLGL-3.8k). Deletion of the nucleotides from -3816 to -1873 in the 5'-flanking region of the *OIGCS- α_1* gene (pRLGL-1.9k) increased the RL activity, whereas the GL activity stayed at the original level. The removal of the nucleotides from -3816 to -884 in the 5'-flanking region of

Table 3 Dual-luciferase assay in medaka embryos injected with pRLGL constructs

Data significantly differing from Blu-1k GL activity values are indicated with an asterisk ($P < 0.05$, Student's t test). The results are expressed as means $\pm$ S.E.M. ($n = 6$). Activities are given as raw RL units or raw GL units normalized against β -galactosidase activity (measured at an absorbance of 420 nm).

Construct	RL activity	GL activity
Blu-1k (OIGCS- β -1000/+189) + GL		245.5 $\pm$ 35.1
pRLGL-3.8k (OIGCS- α -3816/+142) + RL + (OIGCS- β -1000/+189) + GL	1128.5 $\pm$ 189.6	492.9 $\pm$ 97.0*
pRLGL + 48bp (OIGCS- α +48/+142) + RL + (OIGCS- β -1000/+189) + GL	9972.2 $\pm$ 1264.7	944.0 $\pm$ 142.9*

OIGCS- α_1 (pRLGL-0.9k) resulted in a 1.5-fold increase in RL activity compared with pRLGL-1.9k, but GL activity was not changed significantly. Further deletion of the 5'-flanking region of OIGCS- α_1 (pRLGL +48bp) increased the RL activity 2-fold and the GL activity 3-fold compared with the activity of pRLGL-0.3k. A construct lacking the 5'-flanking region necessary for the basal promoter activity of the OIGCS- β_1 gene (-1000/+154; pRL-3.8k/GL Δ +154) linked to the 4 kb of the upstream region of the OIGCS- α_1 -RL fusion-gene construct exhibited dramatically decreased GL activity, whereas RL activity was at the same level as for pRLGL-3.8k.

In contrast to the results obtained with the COS-1 cell system, GL activity in medaka embryos injected with the pRLGL-3.8k construct was higher than that in embryos injected with the Blu-1k construct (Table 3), whereas deletions of the nucleotides from -3816 to +48 (pRLGL+48bp) increased not only the RL activity but also the GL activity, as in the COS-1 cell system. Removal of the SV40 enhancer from these constructs resulted in a dramatic decrease in both luciferase activities, but the results were essentially identical to those obtained in the above experiments (results not shown).

DISCUSSION

Soluble GC has been reported to be expressed in a wide variety of adult vertebrate tissues [36]. In mammals, soluble GC is expressed abundantly in the lung, brain and central nervous system [6,37]. Similarly, in medaka fish, high-level expression of mRNAs for both the α_1 and β_1 subunits of soluble GC have been detected in the adult brain by Northern-blot and reverse transcriptase PCR analysis, and significant activity of sodium nitroprusside-activatable GC has been measured in adult brain homogenate [16]. In the present study, using whole-mount *in situ* hybridization experiments, we demonstrated that both subunit genes (OIGCS- α_1 and OIGCS- β_1) of medaka fish soluble GC are expressed abundantly in the embryonic brain. This specific expression of the soluble GC subunit genes in the embryonic and adult brain implies that the NO/cGMP signalling pathway plays important roles in the formation of the nervous system during embryogenesis and in the function of the nervous system of adult fish. In the present study we also demonstrated that both subunit genes of medaka fish soluble GC are expressed in the embryonic kidney. This is in good agreement with previous studies reporting that NO synthase and soluble GC are co-localized in macula densa in rat kidneys [38] and that NO-derived cGMP mediates the inhibition of renin secretion [39] and tubulo-glomerular feedback in the rat [40], and suggests that the pathway involving soluble GC plays an important role in the fish embryonic kidney.

It has been reported that neuronal NO synthase and erythroid transcription factor GATA-1 are expressed in multiple tissues [41,42]. These genes possess tissue-specific transcriptional regulation units and the tissue-specific transcription start site. The expression of medaka fish soluble GC subunit genes is known to

be widespread in various tissues [16]. However, no differences in the transcription start site in the medaka fish soluble GC subunit genes was found among tissues in the present study (Figures 1A and 1C). This suggests that each of the subunit genes is regulated by the same core promoter, irrespective of tissue type.

It has been well established that co-expression of the α and β subunit genes is essential for the formation of functionally active soluble GC [6,11,24]. In addition, there have been a few studies (e.g. [22]) demonstrating that a single soluble GC subunit (α_1 or β_1) expressed *in vitro* forms a functionally active holoenzyme, although a recent study suggests that the β_2 subunit exhibits NO-sensitive GC activity despite lacking a heterodimeric structure [23]. These results imply that simultaneous or co-ordinated gene expression is critical for the formation of functional soluble GC. Nevertheless, there is very little published information about the promoter of the soluble GC subunit genes and the mechanism of transcriptional regulation of these genes. In a previous study [32], we demonstrated that the OIGCS- α_1 and OIGCS- β_1 genes are organized in tandem in the medaka fish genome and that the injection of fusion-gene constructs consisting of the 5'-flanking region of OIGCS- α_1 and the GFP reporter gene into medaka fish two-cell-stage embryos resulted in detection of GFP fluorescence in the embryonic brain. In the present study, we carried out experiments focused on identifying the proximal region of the OIGCS- α_1 and OIGCS- β_1 genes using various fusion-gene constructs containing a luciferase gene, instead of a GFP gene, as a reporter. Deletion analyses of the various 5'-flanking regions of the OIGCS- α_1 gene in COS-1 cells showed that the basal promoter activity was in the +32/+142 promoter region of the sequence, which did not share similarity with any known *cis* elements. Further analysis indicated the presence of a silencer region at nucleotides -2889 to -2303 and the enhancer regions -3816 to -2890 and -1323 to -991 (Figure 1B). Considering the origin of COS-1 cells, we presume that several *cis* elements in these regions are important for kidney-specific transcription of the OIGCS- α_1 gene. Removal of a TATA-like sequence (TATAATA, -45/-39) in the proximal promoter region of the OIGCS- α_1 gene did not affect the expression of luciferase activity (Figure 2), suggesting that there is no functional TATA box in the 5'-flanking region of the OIGCS- α_1 gene. However, the 5'-flanking region of the OIGCS- α_1 gene has been shown to lack the GC-rich region and multiple transcription start sites characteristic of a TATA-less promoter [43].

When the medaka fish embryo system using fusion-gene constructs containing a luciferase reporter gene was used for measuring the promoter activity of the upstream region of both subunit genes, the promoter activity of the OIGCS- β_1 gene was at almost the same level as that of the promoterless control vector, whereas the same experiments with the OIGCS- α_1 gene exhibited positive promoter activity (Table 2). This may explain our previous results [32] that the upstream region of the OIGCS- β_1 gene alone was insufficient for the expression of the reporter gene (GFP), as distinct from the case of the OIGCS- α_1 gene.

Surprisingly, however, when the COS-1 cell system was used for assaying promoter activity of the *OIGCS- β_1* gene, the 5'-flanking region of the *OIGCS- β_1* gene exhibited a level of basal promoter activity sufficient for the expression of the luciferase gene, and this level was somewhat higher than that for the *OIGCS- α_1* gene. In this regard, our result is in agreement with the recent finding that the β_1 subunit gene of mouse soluble GC exhibits a significant basal promoter activity when assayed in N1E-115 mouse neuroblastoma cells [15]. Serial deletion of the *OIGCS- β_1* upstream region in the fusion-gene construct demonstrated that the minimal nucleotides necessary for exhibiting the maximum promoter activity are +145 to +189 in the 5'-flanking region and suggested the presence of a weak enhancer region at nucleotides +68 to +115 (Figure 1D). Nucleotides +146 to +155, necessary for basal promoter activity of the *OIGCS- β_1* gene, share similarity with no known *cis* element. In addition, removal of the TATA box (TATAGAA, -16/-11) did not affect the promoter activity, suggesting that transcription of the *OIGCS- β_1* gene is independent of the TATA box. These deletion analyses also demonstrated that the promoters of both subunit genes of medaka fish soluble GC are regulated by the nucleotide in the first exon, much as in the case of genes such as collagen $\alpha 1$ (type XII), phosphodiesterase 5A and the tenascin-R gene [44-46]. This is a unique gene-regulation mechanism because most of the genes are regulated by *cis* element(s) in the upstream region sequences of the transcription start site. The results mentioned above and from the experiments using dual-luciferase fusion-gene constructs, which showed that the level of promoter activity of the *OIGCS- β_1* gene was significant when the COS-1 cell system was applied, but not significant in the medaka fish embryo system, indicate that the 5'-flanking region of the *OIGCS- β_1* gene has its own very weak promoter activity that is enhanced by the 5'-flanking region of the *OIGCS- α_1* gene.

A comparison of the genomic structure and the 5'-flanking region of both subunit genes of soluble GC between human (the Celera Human Genome Database) and *Fugu* fish (C. Morinaga, T. Yamamoto, Y. Moriya and N. Suzuki, unpublished work; accession numbers AB062169, AB062170, AB062171 and AB062172) indicated that the two subunit genes of soluble GC in both species are aligned in tandem on their respective genomes, as seen in the medaka fish, but that the size of the spacer sequence in humans (about 28 kp) is much larger than that in *Fugu* fish (162 bp), which is less than one-sixth of the size of that in medaka fish. This small spacer sequence suggests that there may be co-ordinated transcriptional regulation between both subunit genes in *Fugu*, as has been suggested in the medaka fish. On the other hand, there are several *cis* elements, such as GATA, a CCAAT box, C/EBP and myocyte enhancer factor-2 (MEF-2), in the 5'-flanking region of both subunit genes in both species, as seen in the corresponding regions of both subunit genes of the medaka fish. These elements may be important for the tissue-specific expression of the respective soluble GC. The 5'-flanking region of the *Fugu* soluble GC α_1 subunit gene contains the sequence 5'-CTGCTCTTTTC-3', corresponding to nucleotides +35 to +46 in the *OIGCS- α_1* gene, which is necessary for the basal promoter activity of the *OIGCS- α_1* gene.

As shown in Figure 4, in COS-1 cells removal of the nucleotides from -3816 to -1873 in the 5'-flanking region of the *OIGCS- α_1* gene in the dual-gene fusion construct resulted in a 3-fold increase in RL activity, which was consistent with the results obtained in the promoter analysis of the *OIGCS- α_1* upstream region alone. However, deletion of the nucleotides from -3816 to -334 in the 5'-flanking region of the *OIGCS- α_1* gene in the dual-gene fusion construct resulted in an unexpected increase in RL activity, suggesting that this region interacts with the 5'-

upstream region of the *OIGCS- β_1* gene and enhances the transcription of the *OIGCS- β_1* gene. Furthermore, deletion of the nucleotides from -3816 to +47 in the 5'-flanking region of the *OIGCS- α_1* gene in the dual-gene fusion construct resulted in an increase in both reporter activities, implying that there is mutual interaction between the 5'-upstream regions of both subunit genes. These tendencies were also seen when the medaka fish embryo system was used and were irrespective of the presence or absence of the SV40 enhancer within the reporter vector (results not shown).

It has been reported that, at the protein level, both α_1 and β_1 subunits of human soluble GC are expressed only in brain and lung [6]. Moreover, it has been demonstrated that upon co-expression of the respective GST-tagged human soluble GC subunit gene in Sf9 cells, GST- α_1/β_1 or GST- β_1/α_1 heterodimers are formed preferentially [22]. In addition to these, all of the results obtained in the present study show that transcriptional regulation of the two subunit genes, *OIGCS- α_1* and *OIGCS- β_1* , was regulated through interactions between their 5'-flanking regions. These results suggest that co-expression of the α_1 and β_1 subunit genes is essential for *in vivo* formation of NO-sensitive heterodimeric soluble GC and that the regulation of expression of both subunit genes is co-ordinated. In this regard, it should be mentioned that a recent paper [15] demonstrated that the 5'-flanking region of the α_1 and β_1 subunit genes of mouse soluble GC have the independent ability to drive transcription of the respective subunit gene, based on the separation of both genes by a large spacer (2% of the total chromosomal length) and the independent promoter activities when assayed in neuroblastoma cells. The former is the case found in the corresponding human genes and the latter was followed experimentally in the present study. However, considering that, in our study, (i) the promoter activity of *OIGCS- β_1* was much less than that of *OIGCS- α_1* in the medaka embryo and (ii) the 5'-flanking region of both of the subunit genes influenced each other's promoter activities in dual reporter-gene constructs, we presume that the evidence presented for mouse genes does not necessarily exclude the possibility of co-ordinated transcriptional regulation between the α_1 and β_1 subunit genes. To clarify the mechanisms of the transcriptional regulation of both genes, identification of the *trans* elements bridging these two genes will be critically important. In addition, considering that the soluble GC transcribed from the α_1 and β_1 subunit genes is detected in almost all animal tissues, the mechanisms of their transcriptional regulation may differ among tissues. The regulatory mechanisms of the tissue-specific expression of soluble GC are also an important problem to be solved. To this end, the medaka fish is the most suitable experimental animal, not only for understanding the basic and/or co-ordinated transcriptional mechanisms of the soluble GC gene, but also for clarifying the mechanisms of the tissue-specific transcriptional regulation of soluble GC subunit genes. This model is preferable to the mammalian soluble GC subunit genes due to the ease of handling the medaka embryos and the compact genomic structure of the medaka soluble GC.

We thank the staff of the Research Center for Molecular Genetics and the Center for Experimental Plants and Animals, Hokkaido University, Sapporo, Japan, for facilitating the use of molecular biology equipment and culturing of medaka fish. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan (grant no. 11236202).

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