Photoreceptors and olfactory cells express the same retinal guanylyl cyclase isoform in medaka: visualization by promoter transgenics[1]

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Abstract We examined the spatial expression patterns of two orphan receptor guanylyl cyclase genes *OlGC4* and *OlGC5* during embryogenesis of medaka and characterized the 5′ flanking region required for tissue-specific expression of *OlGC4* by introducing promoter–*GFP* fusion constructs into medaka embryos. Expression of *OlGC5* is confined to retinal photoreceptor cells, while *OlGC4* is expressed in the retina, pineal organ, and olfactory pits. The *OlGC4* upstream region between −2374 and +343 is sufficient to drive the sensory organ-specific gene expression. Mutations in the consensus binding sequences for OTX/CRX transcription factors did not impair the reporter gene expression. Our results suggest that the same isoform of guanylyl cyclase is utilized in both photoreceptors and olfactory cells, and that transcription factors other than OTX/CRX primarily activate the *OlGC4* expression.

Key words: Guanylyl cyclase; Retinal photoreceptor cell; Pineal organ; Olfactory neuron; Medaka; Transgenic fish

1. Introduction

In rod and cone photoreceptors of the vertebrate retina, cGMP acts as the excitation transmitter [1]. The pineal organ is also a photo-responsive organ in various vertebrates. Although the signal transduction mechanism in the pineal organ is poorly understood, a cGMP signaling pathway has been shown to be involved in signal transduction in the pineal organ of both mammals [2] and reptiles [3,4]. Recent studies suggested that cGMP also plays an important role in vertebrate olfactory signaling [5–8].

Retinal photoreceptors, pineal cells, and olfactory neurons of vertebrates express specific isoforms of membrane guanylyl cyclase (GC). Rat GC-D is specifically expressed in a subpopulation of olfactory neurons [9]. Two retina-specific GCs (RetGC-1 and RetGC-2 in humans; GC-E and GC-F in rats) have been identified in mammals [10–12]. GC-E is expressed in the eye and pineal organ, whereas the expression of GC-F is confined to the eye [12]. No orphan receptor GC isoform has been reported to be expressed in both photoreceptors and olfactory neurons. Although these GCs are expressed in a highly tissue-restricted manner, mechanisms of their transcriptional regulation are largely unknown.

Medaka *Oryzias latipes* is a small freshwater teleost with various traits that make it useful for developmental and molecular genetic studies [13]. We isolated cDNA clones for three membrane GCs (OlGC3, OlGC4, OlGC5) from a medaka eye cDNA library [14]. In the present study, we examined the spatial expression patterns of *OlGC4* and *OlGC5* during embryogenesis and characterized the 5′ flanking region required for tissue-specific expression of the *OlGC4* gene. Our findings demonstrate that the same isoform of orphan receptor GC is expressed in the retinal photoreceptors, pineal organ, and olfactory cells. Our results also suggest that the photoreceptor-specific expression of *OlGC4* is achieved without direct activation by OTX/CRX transcription factors.

2. Materials and methods

2.1. Animals and embryos

Adults and embryos of the orange–red variety of medaka *O. latipes* were maintained as described [14]. The developmental stage was expressed in the manner described by Iwamatsu [15]. To prevent eye pigmentation, embryos were transferred at stage 20 to distilled water containing 0.2 mM 1-phenyl-2-thiourea and 0.6 ppm methylene blue and were cultured in this solution at 28°C.

2.2. Southern blot hybridization of genomic DNA

Mature males of the *O. latipes* Hd-rR inbred strain [16] fixed in ethanol were provided by Dr. H. Hori (Nagoya University, Japan). The genomic DNA was extracted from one individual. Extraction of genomic DNA, blotting, and hybridization were performed according to standard methods [17]. A cDNA fragment encoding part of the extracellular domain of *OlGC4* (nucleotides 445–884) or *OlGC5* (nucleotides 555–1071) [14] was labeled with [α-^{32}P]dCTP using the Random Primer DNA Labeling kit Version 2 (Takara Shuzo, Japan) and used as the probe. Blots were washed three times with 2×SSC, 0.1% sodium dodecyl sulfate, at 50°C for 15–20 min. Imaging of radioactive signals was performed using a FUJIX Bio-Imaging Analyzer BAS2000 (Fuji Photo Film, Japan).

2.3. Isolation and analyses of genomic clones and DNA sequencing

An *OlGC4* cDNA fragment (nucleotides 5–1811) was labeled with digoxigenin-dUTP by using a DIG High Prime (Boehringer Mannheim, Germany). A white-strain *O. latipes* genomic DNA library in lambda FIX II vector (Stratagene, La Jolla, CA, USA) was screened with the digoxigenin-labeled DNA probe. DNA fragments containing a 5′ upstream region of the *OlGC4* gene were subcloned into pBluescript II vectors (Stratagene) and sequenced on both strands with an ABI 377 DNA sequencer (PE Biosystems, Foster City, CA, USA).

2.4. In situ hybridization

The pBluescript vectors containing a cDNA fragment encoding the 5′ untranslated region (UTR) and extracellular region of *OlGC4* (nucleotides 5–1811) or *OlGC5* (nucleotides 283–1071) [14] or the entire

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[1] The nucleotide sequence reported in this paper has been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession number AB040099.

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EGFP-coding region (Clontech, Palo Alto, CA, USA) were used as a template to generate digoxigenin-labeled RNA probes using a DIG RNA Labeling kit (Boehringer Mannheim) according to the manufacturer's protocol. Whole-mount in situ hybridization with digoxigenin-labeled RNA probes was carried out according to the protocol described by Westerfield [18] with the following modifications: embryos stored in methanol were rehydrated with PBST and then bleached with 6% hydrogen peroxide in PBST at room temperature for 1 h; the acetylation and RNase treatment steps were omitted; the hybridization and wash were carried out at 55°C for *OlGC* probes and at 60°C for the EGFP probe; after the coloring reaction, the embryos were dehydrated in an ethanol series and incubated in ethanol for 5–10 min; following rehydration with PBST, the embryos were incubated in 25% glycerol in PBST for 5 min and then transferred to 50% glycerol in PBST.

2.5. Primer extension analysis

Total RNA was extracted as described. Primer extension analysis was carried out as described [19]. A ^{32}P-end-labeled oligonucleotide primer (5′-TTCATCGTTTTTGGTGCG-3′) was hybridized with 50 μg of total RNA isolated from adult *O. latipes* eyes.

2.6. Fusion gene constructs

The fusion gene construct GC4(−6350)GFP contains a 5′ upstream region of the *OlGC4* gene (from −6350 to +343) fused with a green fluorescent protein (GFP)-coding sequence. The 5′ upstream region of the *OlGC4* gene was amplified by PCR using a thermostable DNA polymerase with proofreading activity (TaKaRa LA Taq, Takara Shuzo, Japan) and a pair of oligonucleotide primers (5′-TTACG-GATCCTGACTTTTCAATCACACC-3′ and 5′-GACTGAATT-CATCGGTTTTGCTGGTTT-3′). The amplified upstream region was cloned into *Eco*RI/*Bam*HI sites of pEGFP-1 vector (Clontech). The GC4(−6350)GFP construct then served as the template to generate the fusion construct GC4(−2374)GFP. The *OlGC4* upstream region and whole vector DNA was amplified by PCR using LA Taq with a forward primer (5′-ATCGGAATTCGCTTGACAGA-GATGCAGA-3′) and a reverse primer (5′-TTATGGCGACTCCT-GAAG-3′). The amplified DNA fragments were digested with *Eco*RI before ligation. The fusion constructs GC4(−1227)GFP, GC4-(−544)GFP, and GC4(−176)GFP were generated by excision of upstream regions from the GC4(−6350)GFP construct with restriction endonucleases *Hin*dIII, *Xho*I, and *Bgl*II, respectively, followed by self-ligation. The mutant constructs GC4(−2374)GFPmut1, GC4-(−2374)GFPmut2, GC4(−2374)GFPmut3, GC4(−2374)GFPmut2/3 were generated by replacing OTX/CRX consensus elements (TAATCC/T) [20] with the *Nco*I recognition sequence (CCATGG) as described [19]. Nucleotide sequences of upstream regions in the GFP fusion constructs were verified by sequencing with oligonucleotide primers EGFP-F (5′-GCTCACATGTTCTTTCCTGCGT-3′) and EGFP-N (5′-AGAACTTGTGGCCGTTTACGTC-3′), and *OlGC4*-specific primers.

2.7. Microinjection of promoter–EGFP fusion constructs

The DNA solution at a concentration of 15–25 ng/μl was injected into the cytoplasm of both blastomeres of two-cell embryos as described [19]. The injected embryos were washed with distilled water and incubated at 28°C. GFP fluorescence was examined under a fluorescence microscope (IX70, Olympus, Japan). Embryos were then fixed with 4% formaldehyde in PBS, and *GFP* mRNA was detected by whole-mount in situ hybridization.

3. Results

3.1. Identification of sensory organ-specific GC genes in the medaka genome

Hisatomi et al. [21] independently isolated cDNA clones for three membrane GCs designated as OlGC-R1, OlGC-R2, and OlGC-C from a medaka retina cDNA library. The nucleotide and deduced amino acid sequences of *OlGC-R1* and *OlGC-C* are closely related to but slightly different from those of *OlGC4* and *OlGC5*, respectively. To determine whether OlGC-R1 and OlGC-C correspond to OlGC4 and OlGC5, respectively, Southern blot hybridization of genomic DNA from the Hd-rR inbred strain was performed using a putative exon fragment encoding a part of the extracellular domain of *OlGC4* or *OlGC5* as the probe (Fig. 1). Only a single band was detected in each lane for both *OlGC4* and *OlGC5*. This result strongly suggests that OlGC-R1 and OlGC-C correspond to OlGC4 and OlGC5, respectively. The slight sequence differences could be attributable to the relatively large degree of genetic diversity among different populations of medaka [22]. Thus, medaka has at least four members (OlGC3, OlGC4, OlGC5, OlGC-R2) of the sensory organ subfamily of membrane GC.

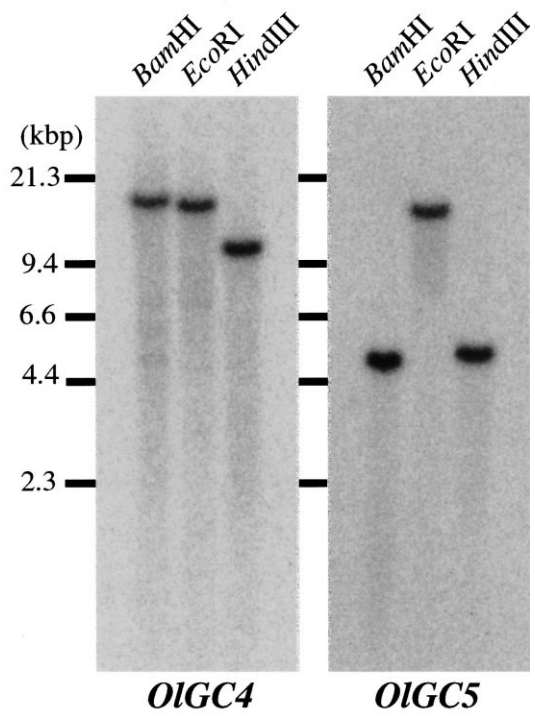

Fig. 1. Southern blot analysis of *O. latipes* genomic DNA digested with *Bam*HI, *Eco*RI, or *Hin*dIII. Each lane was loaded with a digest of 10 μg of DNA from a single individual of Hd-rR inbred strain. The same blot was hybridized with an extracellular-coding region probe of *OlGC4* or *OlGC5*.

3.2. Spatial expression patterns of the OlGC4 and OlGC5 genes in medaka embryos

We examined the spatial expression patterns of *OlGC4* and *OlGC5* in medaka embryos using whole-mount in situ hybridization. At the hatching stage (stages 38–39), both *OlGC4* and *OlGC5* are expressed in the retina (Fig. 2A,B). This is consistent with the previous finding on the *OlGC-C* and *OlGC-R1* expression in the adult retina: *OlGC-C* is expressed in cones, while *OlGC-R1* is expressed in rods [21]. The transcripts of *OlGC5* became detectable around stage 36, while the *OlGC4* expression was first observed at stage 37 (data not shown).

The *OlGC4* gene is also expressed in the embryonic pineal organ (Fig. 2C,D); the expression is evident at stage 32, and it continues to the hatching stage. On the other hand, we did not detect any expression of *OlGC5* in the pineal organ during embryogenesis (Fig. 2E). In the hatching stage embryos, *OlGC4* is also expressed in cells in the olfactory pits (Fig. 2F). The *OlGC4* (*OlGC-C*) expression in olfactory cells has

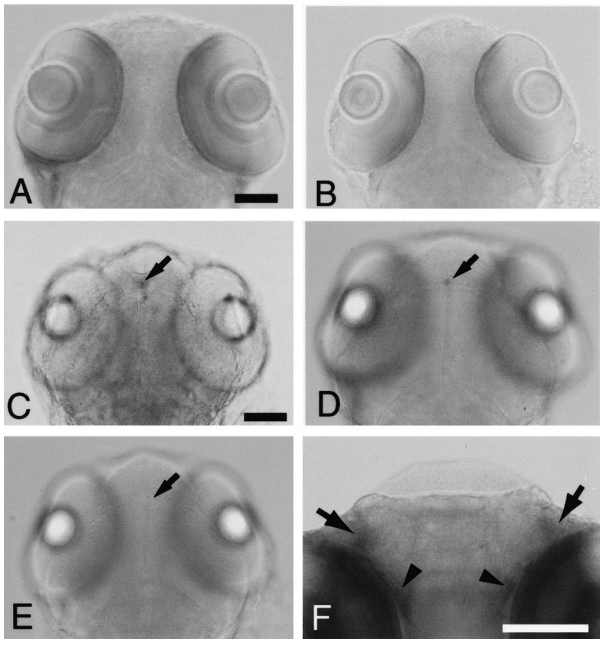

Fig. 2. Expression of *OlGC4* and *OlGC5* in medaka embryos visualized by whole-mount in situ hybridization. A, B: Dorsal view of the head of stage 38 embryos hybridized with an *OlGC4* probe (A) or *OlGC5* probe (B). The *OlGC4* and *OlGC5* transcripts were detected in the retina. C–F: *OlGC4* expression in the pineal organ and olfactory neurons of medaka embryos. Arrows indicate the pineal organ (C–E) or olfactory pits (F). C, D: *OlGC4* is expressed in the pineal organ of medaka embryos. No hybridization signal was detected in the pineal organ of embryos hybridized with an *OlGC5* probe (D). C: Stage 32. D: Stage 38. E: Dorsal view of the head of a stage 39 embryo. Cells in olfactory pits (arrows) and fibrous structures projecting to the olfactory bulb (arrowheads) are stained. Scale bars, 100 μm.

not been reported in the previous study [21]. These cells may be olfactory receptor neurons, because we observed the hybridization signal in fibrous structures projecting to the olfactory bulb (Fig. 2F). These results suggest that the same isoform of orphan receptor GC is expressed in three different sensory receptor cells of medaka, namely, retinal photoreceptors, pinealocytes, and olfactory receptor neurons.

3.3. Characterization of cis-regulatory regions required for the sensory cell-specific expression of OlGC4

The *OlGC4* and *OlGC5* genes, with their highly tissue-restricted and distinct expression patterns, seem to us to be the ideal model system for studying sensory cell-specific gene expression. As the first step towards understanding the mechanisms of cell type-specific transcription of these genes, we analyzed promoter/enhancer activities of the 5′ upstream region of *OlGC4* by microinjecting the GFP fusion constructs into medaka embryos (Figs. 3 and 4).

We isolated the 5′ flanking region of the *OlGC4* gene from a medaka genomic library and analyzed its structure (Fig. 3). The transcription initiation site (+1) determined by the primer extension method was 391 bp upstream from the putative start codon, and it was identical to the 5′ end of the *OlGC4* cDNA [14]. The 5′ UTR of *OlGC4* contains an intron of 99 bp (from +219 to +317). The upstream region contains several consensus binding sequences (TAATCH) for OTX/CRX homeodomain proteins, which are known to be important for photoreceptor cell development [23,24], and Ret1/PCE1 elements (CAATTA) conserved in photoreceptor-specific promoters [25,26] (Fig. 3).

We generated a series of *GFP* fusion constructs in which the upstream region was progressively deleted from the 5′ (Fig. 3). GFP fluorescence was not evident in embryos injected with the fusion constructs (data not shown). This is probably due to both weakness of the fluorescence and interference by pigments of the embryo. Therefore, we instead detected the reporter gene expression using whole-mount in situ hybridization. In embryos injected with the construct GC4(−6350)GFP, *GFP* mRNA was detected in the retinal photoreceptors, pineal organ, and olfactory pits (Fig. 4). In addition to photoreceptors, other cells in neural retina and lens cells showed *GFP* expression in a small proportion of injected embryos (two and one out of 24 embryos). Ectopic signals were also observed in a small number of cells of yolk epithelium (data not shown), as previously reported for transgene expression driven by other tissue-specific promoters in medaka embryos [19,27].

When the upstream region was deleted down to −2374, the proportion of embryos with the reporter gene expression in the pineal organ or olfactory pits decreased compared with that of GC4(−6350)GFP-injected embryos, while the retinal expression remained at a high frequency (Fig. 3). Thus, the 5′ flanking region between −2374 and +343 is sufficient for gene expression in the retinal photoreceptors, pineal organ, and olfactory neurons, although additional sequences may be important for pineal and olfactory expression. When the construct GC4(−1227)GFP was injected, fewer percentages of embryos showed the retinal and pineal expression and no olfactory expression was observed (Fig. 3). Therefore, the upstream region between −2374 and −1227 seems to contain regulatory elements that activate transcription in both photoreceptors and olfactory cells. Shorter constructs (GC4(−544)GFP, GC4(−176)GFP) gave no sensory organ-specific expression, whereas ectopic expression in the yolk epithelium remained. Thus, the upstream region between −1227 and −544 contains *cis*-regulatory elements necessary for transcription in photoreceptor cells.

We then tested importance of the OTX/CRX elements in the upstream region −2374/+343 by introducing mutations into GC4(−2374)GFP (Fig. 3). Although the upstream region between −2374 and −1227 contains one OTX/CRX consensus element (TAATCT, −1897/−1902), replacement of this element with *Nco*I sequence did not impair the reporter gene expression in the retina, pineal organ, and olfactory pits. Similarly, the upstream region between −1227 and +343 contains two OTX/CRX consensus motifs (TAATCC) at −339/−344 and −941/−936, but mutations in either or both of the OTX/CRX sequences did not decrease the percentages of positive embryos in retinal photoreceptors, pinealocytes, and olfactory cells. These results clearly indicate that none of the OTX/CRX sequences correspond to regulatory elements shown to be present in the region between −2374 and +343 by the deletion analysis. Although we have not examined expression of the constructs in which all OTX/CRX sequences are mutated, it is unlikely that such mutations severely diminish gene expression in sensory cells. Interestingly, mutations in the OTX/CRX sequences increased the proportion of embryos with pineal and/or olfactory expression (Fig. 3). Some of these sequences might act as negative

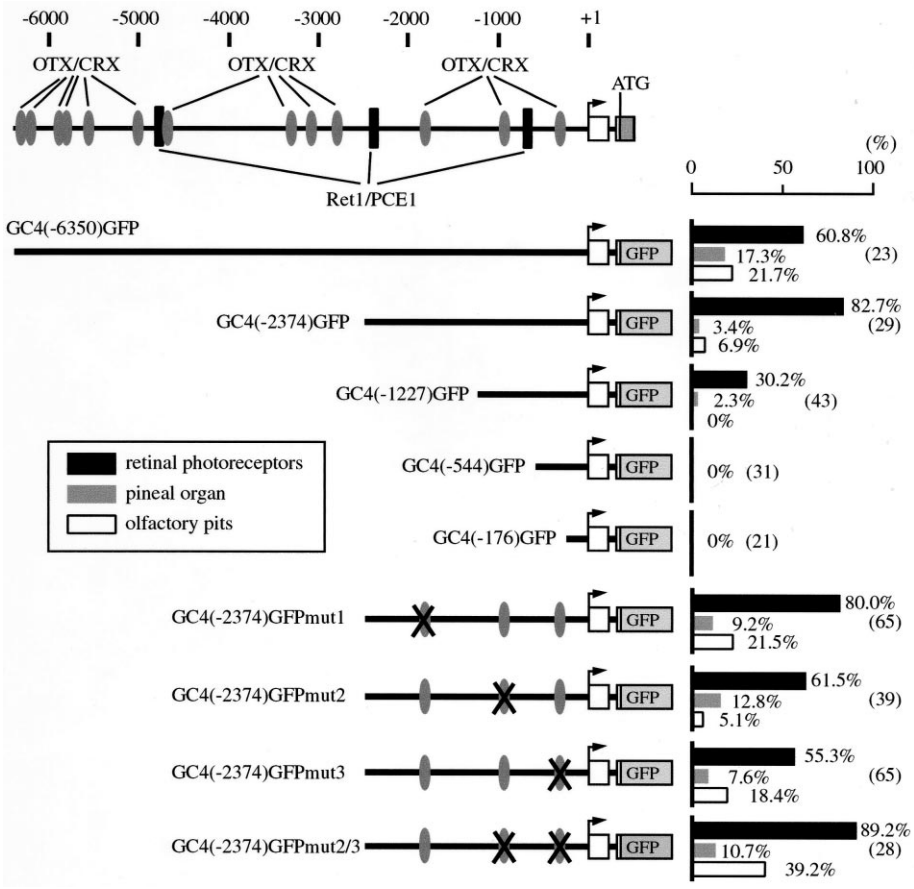

Fig. 3. Summary of the 5′ upstream region analysis using *OlGC4*–GFP fusion constructs. The genomic structure of the endogenous *OlGC4* gene is indicated at the top. White boxes show the 5′ leader sequence, and a shaded box shows the protein-coding region. An intron and untranscribed region are indicated by lines. 'ATG' indicates the start codon of the *OlGC4* open reading frame. Numbers at the top indicate nucleotide positions relative to the transcription initiation site (+1). Consensus sequences for OTX/CRX and Ret1/PCE1 elements are indicated by symbols. In all fusion genes, the *OlGC4*-coding sequence was substituted with the GFP open reading frame. At the right side of the drawing representing each construct, bars indicate the percentage of positive embryos, i.e. individuals with tissue-specific signals out of all embryos scored. Expressions in different tissues are shown by bars of different tones: black, retinal photoreceptors; gray, pineal organ; white, olfactory neurons. Numbers in parentheses indicate the number of embryos scored for each construct.

regulatory elements which spatially or temporally refine the *OlGC4* expression in pineal and olfactory tissues.

4. Discussion

In mammals, GC-E and GC-F are specifically expressed in photoreceptors and GC-D is expressed in olfactory neurons [9,12]. No mammalian GC isoform has been reported to be expressed in both photoreceptors and olfactory neurons. Here we demonstrated that *OlGC4* is expressed in the retina, pineal organ, and olfactory pits of medaka embryos. A phylogenetic tree of the catalytic domain sequences suggests that OlGC4 is most closely related to mammalian GC-D, which is specifically expressed in olfactory neurons [14]. It is unclear, however, whether expression of *OlGC4* is restricted to a subset of olfactory neurons, as seen in rat GC-D [9]. Moon et al. [8] suggested the existence of multiple isoforms of membrane GC in rat olfactory neurons. Therefore, there may be a membrane GC isoform expressed in both retinal photoreceptors and olfactory neurons in mammals, as we demonstrate here in medaka.

The presence of OlGC4 in both retinal photoreceptors and olfactory neurons suggests that the GC activity is regulated by a similar mechanism in these sensory cells. Considering the structure of membrane GC as a cell surface receptor, the same extracellular ligands, if any, may regulate OlGC4 cyclase activity in photoreceptors and olfactory neurons. While no extracellular ligand has been identified for the retinal GCs, two Ca^{2+}-binding proteins, GCAP-1 and GCAP-2, have been shown to be their cytoplasmic activators [11,28,29]. Immunoblot and immunohistochemical analyses suggested that a GCAP-1-like protein, but not GCAP-2, is present in rat olfactory cilia [8]. The expression of a retinal GC isoform presented here provides another piece of evidence for involvement of GCAP-1, GCAP-2, or related molecules in olfactory signaling.

Another intriguing feature of *OlGC4* is its expression in pinealocytes in earlier stages of embryogenesis. Expression of the *OlGC4* gene in the pineal organ begins as early as stage 32, about 3 days before the onset of the retinal expression of *OlGC4* and about 4–5 days before hatching. Thus, the pineal organ may be a major photosensory organ in the embryo, and OlGC4 is probably required for its function. Because the hatching of medaka embryos is regulated by light conditions [30], the cGMP signaling pathway mediated by OlGC4 might be involved in controlling when hatching occurs.

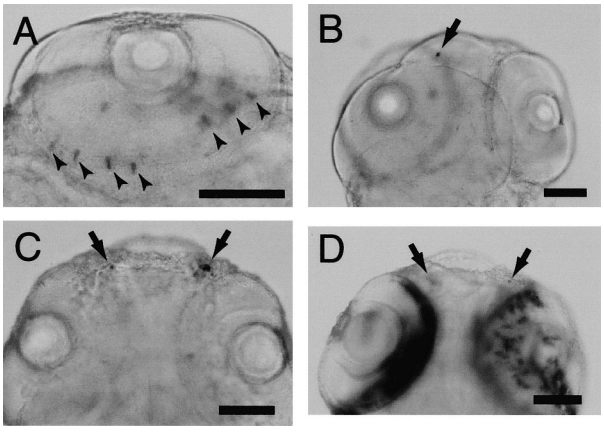

Fig. 4. Expression of *GFP* mRNA in transgenic medaka embryos bearing *OlGC4* promoter–*GFP* fusion constructs. *GFP* transcripts were detected at stage 39 by whole-mount in situ hybridization. A–C: Embryos injected with GC4(−6350)GFP. D: Embryos injected with GC4(−2374)GFPmut3. The *GFP* reporter gene was expressed in the retinal photoreceptors (A, D), pineal organ (an arrow in B), and cells in the olfactory pits (arrows in C and D). Scale bar, 100 μm.

We analyzed the *cis*-regulatory region required for tissue-specific expression of *OlGC4* by microinjecting the *GFP* fusion constructs into embryos and monitoring the reporter gene expression in the injected embryos. This method has been successfully used to investigate transcriptional regulation of several tissue-specific genes in medaka embryos [19,27]. In these analyses, although the reporter gene expression was highly mosaic, the embryonic regions in which given *cis*-regulatory sequences would activate transcription were properly determined by observing many embryos. The previous analysis also showed that the frequency of embryos with reporter gene expression roughly correlated with expression levels [19]. This procedure enabled us to test many fusion gene constructs containing deletions or mutations in sites of interest in a much shorter time compared to what would be needed for the establishment of transgenic lines of animals [19]. Thus, the gene transfer into medaka embryos can serve as a unique in vivo system for studying transcriptional regulation of cell type-specific genes.

The spatial expression pattern of *OlGC4* was reproduced by the reporter gene expression driven by the *OlGC4* upstream regions. Deletion analysis of the upstream region suggested that multiple *cis*-regulatory sequences are important in different ways for the *OlGC4* gene expression in different sensory receptor cells. The homeodomain protein CRX is a photoreceptor-specific transcriptional activator that binds to the OTX elements in upstream regions of several photoreceptor-specific genes and can stimulate transcriptional activity of the opsin and IRBP promoters in non-retinal cells [23,24]. CRX may also play a crucial role in regulating pineal gene expression [31]. Here we demonstrated that the OTX/CRX-binding consensus sequences are dispensable for transcription of the reporter gene from the *OlGC4* promoter in retinal photoreceptor cells, pinealocytes, and olfactory cells. Thus, some photoreceptor-specific genes may be transcribed without direct activation by OTX/CRX transcription factors.

The homeodomain protein RX binds to the Ret1/PCE1 elements in upstream regions of many retina-specific genes and activates transcription of these genes [32]. Two Ret1/PCE1 elements are present in the 5′ flanking region up to −2374 of *OlGC4*. Both sequences are within the region shown to be important for the photoreceptor-specific expression of *OlGC4* in the present study: the distal Ret1/PCE1 element locates at −2190/−2185 and the proximal element at −682/−677. Therefore, RX protein may regulate the sensory cell-specific expression of *OlGC4* via the Ret1/PCE1 elements.

The mechanism by which overlapping but distinct sets of genes are activated in different types of sensory receptor cells is largely unknown. Four GC genes, *OlGC3*, *OlGC4*, *OlGC5*, and *OlGC-R2*, are differentially expressed in rods, cones, pinealocytes, and olfactory cells of medaka. Future comparative studies on the transcriptional regulation of these genes will shed light on the molecular mechanisms regulating the differentiation of different types of sensory receptor cells.

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