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# Retina-Type Rhodopsin Gene Expressed in the Brain of a Teleost, Ayu (*Plecoglossus altivelis*)

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**ABSTRACT**—Ayu (*Plecoglossus altivelis*) is a teleost whose gonadal development is stimulated by shortened daylength and is a useful model to study the mechanism of photoperiodism. However, localization and characteristics of the photoreceptor that mediates photoperiodism in gonadal development remain to be determined. To identify the photoreceptive molecule that regulates photoperiodic responses, in the present study, we have cloned and characterized the cDNA encoding an opsin gene expressed in the ayu brain, a putative site of the photoreceptor for photoperiodism. The identified opsin was rhodopsin that is identical to the rhodopsin expressed in the retina. Phylogenetic analysis demonstrated that this rhodopsin belongs to the retina-type but not to the pineal-specific rhodopsin group. Genomic polymerase chain reaction (PCR) demonstrated that the ayu rhodopsin gene is intron-less. Southern and Northern blots and reverse-transcription PCR analyses indicate that the same rhodopsin gene is expressed in the retina and the brain but not in the pineal organ of ayu. These results indicate that the rhodospin gene is expressed in the retina and brain and mediates not only visual but also nonvisual functions such as photoperiodism and entrainment of the circadian clock.

Key words: photoreceptor, rhodopsin, brain, retina, ayu Plecoglossus altivelis

# INTRODUCTION

Light regulates physiological, behavioral and biochemical activities in vertebrates through photoreceptors. A typical example is vision. Vertebrates perceive their environment (shapes, colors and irradiance) through rod and cone photoreceptors in the retina. In addition, non-visual functions such as the entrainment of the circadian clock, melatonin secretion, gonadal developments, and body color change are regulated by light (Benoit, 1978; Falcón, 1999; Campbell *et al.*, 2001; Oshima, 2001).

Photoreceptive organs that mediate non-visual functions known so far are the retina, skin, pineal organ, and brain. The retina is involved in the photic entrainment of the circadian clocks in the retina itself in vertebrates and in the suprachiasmatic nucleus in mammals (for review, see Hast-

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ings and Maywood, 2000; Mangel, 2001).

The photosensitivity of skin was reported earlier in nonmammalian vertebrates by direct illumination on the skin (for review, see Oshima, 2001). These results propose that there are photoreceptors in the skin regulating body color change. In fact, a novel opsin named melanopsin has recently cloned in amphibians and is considered as the most probable candidate for the photoreceptive molecule that regulates body color change (Provencio *et al.*, 1998).

Although the photosensitivity of the pineal organ was early inferred by von Frisch (1911), the first direct evidence was obtained by an electrophysiological study (Dodt, 1963). Electronmicroscopic and immunohistochemical studies revealed that there are cone-like photoreceptors with welldeveloped outer segments that are immuno-reactive against opsin antibodies (see Falcón, 1999). Recently, pinopsin in birds and vertebrate ancient (VA) opsin and exo-rhodopsin (pineal-specific rhodopsin) in fish were cloned from the pineal organ (Okano *et al.*, 1994; Soni and Foster, 1997; Mano *et al.*, 1999; Kojima *et al.*, 2000; Philip *et al.*, 2000a).

It has been suggested that the fish brain contains the photoreceptors that regulate body color and locomotor activ-

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ity (von Frisch, 1911; Scharrer, 1928). The existence of photoreceptors regulating gonandal development was subsequently demonstrated in the avian brain (Benoit, 1978). Thus, lines of evidence indicate that the brain of non-mammalian vertebrates is a photoreceptive organ (Tabata, 1992).

Immunohistochemical studies also suggested the existence of photoreceptors in the brain of non-mammalian vertebrates. Opsin-like immunoreactivity was first found in cerebrospinal fluid-contacting neurons in bird (Silver et al., 1988) and later in reptiles and amphibians (for review, see Yoshikawa and Oishi, 1998). In fish, immunocytochemical studies demonstrated the existences of opsin-like molecules in the hypothalamus of lamprey and Atlantic salmon (Garcia-Fernandez et al., 1997; Philip et al., 2000b). In addition, immunoreactivity to the antibody against transducin, G protein involved in photosingal transduction in the retina, was observed in the diencephalon and the telencephalon of lamprey and Atlantic salmon, respectively (Garcia-Fernandez et al., 1997; Philip et al., 2000b). Furthermore, cDNAs encoding rhodopsin and pinopsin have been cloned from the hypothalamus in pigeon and frogs, respectively (Wada et al., 1998; Yoshikawa et al., 1998). In situ hybridization studies revealed the expression of melanopsin gene in the hypothalamus in amphibian and VA opsin gene in the epithalamus in fish (Provencio et al., 1998; Kojima et al., 2000; Philp et al., 2000b). Thus, it is highly probable that these opsins expressed in the brain regulate physiological functions in non-mammalian vertebrates.

The ayu, *Plecoglossus altivelis*, is an anadromous teleost fish that belongs to the family Osmeridae. This fish shows clear photoperiodism in gonadal development (Fushiki, 1979). Therefore, ayu is a useful model to study photoperiodism in gonadal development in fish. Interestingly, even after removal of the eyes and pineal organ, photoperiodic responses in gonadal development persisted (Suzuki, 1975), indicating the presence of another photoreceptive organ(s). Considering from the experimental results described above, we hypothesized that the photoreceptive molecule in the brain is a principle regulator of gonadal development in this species. In the first place to elucidate this hypothesis, we have cloned and characterized a cDNA encoding rhodopsin from the ayu brain.

# MATARIALS AND METHODS

#### Molecular cloning of ayu rhodopsin cDNA

The brain and retina were dissected out from the ayu supplied from Chiba-Prefectural Experimental Fresh-Water Industries Station and Chiba-Prefectural Sea-Farming Center, quickly frozen in liquid nitrogen and stored at –80°C until total RNA preparation. Total RNA was extracted from these tissues using RNA extraction solution (Isogen; Nippongene, Toyama, Japan) and used for cDNA synthesis using Ready-To-Go<sup>TM</sup> T-primed first-strand cDNA synthesis kit (Amersham Pharmacia Biotech, Tokyo, Japan) according to manufacturer's instructions.

The cDNAs were subjected to polymerase chain reaction (PCR) amplification with a pair of degenerate oligonucleotide prim-

ers, Op-Fw and Op-Rv, designed from nucleotides corresponding to a region from sixth to seventh transmembrane domain of vertebrate opsins (Yoshikawa *et al.*, 1998). Partial opsin cDNA was amplified in a reaction mixture (total volume of 20  $\mu$ l) consisted of 1xPCR buffer (Takara, Shiga, Japan), dNTPs (200  $\mu$ M; Takara), Taq DNA polymerase (0.5 U; Takara), Op-Fw (0.5  $\mu$ M), Op-Rv (0.5  $\mu$ M) and cDNA template (1  $\mu$ l). The first PCR was performed as follows: denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 42°C for 1 min, and 72°C for 1 min and then 72°C for 10 min. A 10-fold-diluted reaction mixture of the first PCR served as a template for a second PCR with the same primers and conditions. Subcloning and sequencing of the cDNA fragments were carried out as described elsewhere (Okubo *et al.*, 2001).

We obtained two different cDNA fragments from the ayu brain. BLASTN search (Altschul *et al.*, 1997) in October 1999 revealed that one cDNA fragment exhibited the highest homology to the rhodopsin of a teleost, milkfish *Chanos chanos* (DDBJ accession number AF148142) and the other cDNA to vertebrate ancient (VA) opsin of a teleost, Atlantic salmon *Salmo salar* (DDBJ accession number AF001499). In this study, we characterized the former cDNA encoding rhodopsin (see below).

To obtain the full-length rhodopsin cDNA from the brain and retina of ayu, poly (A)<sup>+</sup> RNA was extracted from the brain and retina using Oligotex-dT30 (Takara), and then cDNA ligated adaptor nucleotides including AP1 (5'-CCATCCTAATACGACTCACTAT-AGGGC-3') and AP2 (5'-ACTCACTATAGGGCTCGAGCGGC-3') sequences was synthesized using Marathon<sup>™</sup> cDNA amplification kit (Clontech, Palo Alto, CA, USA). Specific primers were designed for rapid amplification of cDNA ends (RACE): fw1 (5'-TCGTCCT-GATGGAGATCTCCTACCTG-3') and fwn1 (5'-AGCGTGGCCTGG-TACATCTTCTGC-3') for 3'-RACE; rv1 (5'-AAAGAAGGCCGGG-GCTGTCATGAAG-3') and rvn1 (5'-CTTCCCTGATTGCAGAAGAT-GTACCAG-3') for 5'-RACE. 3'-RACE was executed with the primers AP1 and fw1 (0.5  $\mu$ M each) in a reaction mixture (total volume of 20  $\mu$ l) consisted of 1×PCR buffer, dNTPs (200  $\mu$ M) and LA DNA polymerase (1 U; Takara). The PCR was performed as follows: denaturation at 94°C for 1 min, followed by 5 cycles of 94°C for 30 sec and 72°C for 1 min, and 5 cycles of 94°C for 30 sec and 70°C for 1 min, and 30 cycles of 94°C for 30 sec and 68°C for 1 min then 72°C for 10 min. A 100-fold-diluted reaction mixture of the PCR served as a template for a nested PCR with the primers AP2 and fwn1 (0.5 µM each) in a reaction mixture (total volume of 20 µl) con-



**Fig. 1.** Schematic representation of the ayu brain (sagittal view) that depicts dissection used for RT-PCR analysis of rhodopsin gene expression in brain areas. The brain was divided into five parts; part A, olfactory bulb and telencephalon; part B, diencephalon; part C, optic tectum; part D, cerebellum; part E, medulla oblongata and spinal cord. C, cerebellum; D, diencephalon; M, medulla oblongata; O, optic tectum; OB, olfactory bulb; ON, optic nerve; SC, spinal cord; T, telencephalon.

sisted of 1xPCR buffer, dNTPs (200  $\mu$ M) and Taq DNA polymerase (0.5 U). The nested PCR was performed as follows: denaturation at 94°C for 1 min, followed by 5 cycles of 94°C for 30 sec, 69°C for 30 sec and 72°C for 30 sec, and 5 cycles of 94°C for 30 sec, 67°C for 30 sec and 70°C for 30 sec, and 30 cycles of 94°C for 30 sec, 65°C for 30 sec and 70°C for 30 sec, then 72°C for 10 min. 5'-

RACE was also executed with the primers AP1 and rv1 and then nested PCR with the primers AP2 and rvn1 under the same conditions used for 3'-RACE. Finally, the cDNA including the entire coding region of the ayu rhodopsin was obtained by PCR amplification with a pair of primers, fw2 (5'-CCATGAACGGCACAGAGGGAC-3') and rv2 (5'-CGCTGGATCGCGGGGTCCATGAGAG-3'), which cor-

ccatctctct	-70	bp
cctgtccagccagacaaaacaacaccaccgaaggctgattgcaaccgcaagaccgcaacc		
ATGAACGGCACAGAGGGACCCTTTTTCTATGTCCCTATGTCAAATGCCTCCGGCATTGTC	60	
M N G T E G P F F Y V P M S (N) A S G I V	20	
AGGAGTCCTTATGAATACCCTCAGTACTACCTTATCAACCCAGCAGCATACTTCATGCTG	120	
R S P Y F Y P Q Y Y I T N P A A Y F M I	40	
GCCTGCTACATGTTCTTCTCATCATCACCGGCTTCCCCATCAACTTCCTAACACTGTAC	180	
	60	
	240	
	00	
	200	
	100	
	100	
GGCTACTICGTCTTCGGTAGGACTGGCTGCAACATCGAAGGATTTTGTGCTACCCTCGGT	360	
GYFVFGRIG(C)NI(E)GFCAILG	120	
GGTGAGATTGCCATGTGGTCCCTGGTTGTCCTGGCTATTGAGAGGTGGGTG	420	
G (E) I A M W S L V V L A I E R W V V V C	140	
AAGCCCATGACCAACTTCCGCTTTGGTGAGAACCATGCCATCATGGGTGTTGCGTTCACC	480	
K P M T N F R F G E N H A I M G V A F T	160	
TGGGTGATGGCCGCTGCCTGTGCTGTGCCCCCACTCTTCGGCTGGTCCCGCTACATCCCA	540	
W V M A A C A V P P L F G W S R Y I P	180	
GAGGGCATGCAGTGCTCATGCGGAATCGACTACTACACCCGTGCCCCCGGCTTTAACAAC	600	
E G M Q C S CO G I D Y Y T R A P G F N N	200	
GAGTCCTTTGTGGTCTACATGTTCATTGTCCACTTCACGCTTCCTCTGACCGTCGTCACC	660	
E S F V V Y M F I V H F T L P L T V V T	220	
TTCTGCTATGGCCGTCTGCTGTGCACCGTCAAGGAGGCAGCTGCTGCCCAGCAGGAGTCC	720	
F C Y G R I I C T V K F A A A A Q Q F S	240	
GAGACCACCCAGAGGGGCCGAGAGGGAAGTTACCCGCATGGTCGTCCTGATGGAGATCTCC	780	
ETTORAFREVTRMVVIMFIS	260	
	8/0	
	280	
	200	
	200	
SEFGPVFMIAPAFFA(K) SSAL	300	
	960	
YNPLIYVCMNKQFRHCMIII	320	
CTGTGCTGCGGAAAGAACCCCTTCGAGGAAGAGGAGGGAG	1020	
L C C G K N P F E E E G A S T T A S K	340	
<u>ACCGAGGCCTCCTCCGTGTCCTCCAGCTCCGTGGCCCCTGCAtaaatgggctctcatgga</u>	1080	
T E A S S V S S S S V A P A *	354	
<u>ccccgcgatccagcg</u> tgcacaaagaagacttctgcacccccgggaaacgactgaaggcta	1140	
tcgtatacagaaataacttcctttttgtatttttacaaaccagttggttcaacctaatga	1200	
cagttgcagaagagggcagcccaagaaaagttgtttctgtatgta	1260	
taacgatgcataagatgttttcttttcctgaaatgaagcaaaatatcttttatcttta	1320	
	1380	
cntaaaataaaatacactttacaaata	1478	
og tadaa tadaapgodo ti tgodda tg	1410	

**Fig. 2.** The nucleotide sequence of cDNA and deduced amino acid sequence of ayu rhodopsin. Key amino acids (N15, C110, E113, E122, C187, K296) are enclosed in a circle (see text). The translation stop-codon is designated by the asterisk and the polyadenylation signal is enclosed in a square. The sequence of the probe used for genomic Southern and Northern blot analyses is underlined.

respond to the 5'- and 3'-flanking region, respectively. A reaction mixture (total volume of 20  $\mu$ l) consisted of 1xPCR buffer, dNTPs (200  $\mu$ M), Taq DNA polymerase (0.5 U), fw2 (0.5  $\mu$ M), rv2 (0.5  $\mu$ M) and cDNA template (1  $\mu$ l). The PCR was performed as follows: denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 sec and 68°C for 3 min. To determine the sequence of full length ayu rhodopsin cDNA, subcloning and sequencing of these cDNA fragments were carried out as described above.

#### Construction of molecular phylogenetic tree

Amino acid sequences of opsins were obtained from DDBJ (http://srs.ddbj.nig.ac.jp/index-j.html). The sequences were aligned with ayu rhodopsin by Clustal W (Thompson *et al.*, 1994). The posi-

tions containing any gaps were eliminated for construction of molecular phylogenetic trees. A phylogenetic tree was constructed by neighbor-joining methods with bootstrap confidence values based on 1000 replicates by using PHYLIP 3.572 software package (Felsenstein, 1989).

#### Genomic Southern blot analysis

Genomic DNA was extracted from the liver of an individual ayu as described previously (Okubo *et al.*, 2001). Genomic DNA (10  $\mu$ g) was digested with the restriction enzymes, *Dra* I, *Eco*T14 I, *Nco* I, or *Pst* I, electrophoresed on a 0.9% agarose gel and transferred to a Biodine nylon membrane (Pall Gelman Sciences, Ann Arbor, MI, USA). The membrane was prehybridized in PerfectHyb<sup>TM</sup> Hybrid-





**Fig. 3.** Phylogenetic analysis of the deduced amino acid sequences of ayu rhodopsin with opsins and rhodopsins of selected species. The deepest root was determined by using the sequences of invertebrate opsins (squid rhodopsin, DDBJ/GenBank/EMBL accession number Z49108; *Drosophila* rhodopsin, XM081147) as outgroups in opsins and vertebrate opsins (chicken violet opsin, M92039; goldfish ultraviolet opsin, D85863) for rhodopsins. Bootstrap confidence values are based on 1000 replicates. The scale bar is calibrated in substitutions per site. The accession numbers of opsins used for phylogenetic analysis are follows; chicken iodopsin, X57490; Atlantic salmon VA opsin, AF001499; pinopsins (toad, AF200433; chicken; U15762); melanopsins (mouse, AF147789; chicken, AY036061; *Xenopus*; AF014797); red sensitive opsins (*Xenopus*, U90895; goldfish, L11867); green sensitive opsins (chicken, M92038; goldfish, L11865); blue sensitive opsins (chicken, M92037; zebrafish, AF109372); ultraviolet opsins (chicken, M92039; goldfish, AF109373); Atlantic salmon ERrod-like opsin, AF201469; fugu ERrod-like opsin, AF201472; red zebrafish exo-rhodopsin, AB025132; rhodopsins (bovine, M12689; human, U49742; rat, Z46957; chicken, D00702; tiger salamander, U36574; bull frog, S79840; *Xenopus*, U2380; *Anolis carolinensis*, L31503; Atlantic salmon, AF201470; ayu, AB086404; deep sea eel, L78008; fresh water eel, L78007; fugu, AF201471; goldfish, L11863; zebrafish, AF105152).

ization Solution (Toyobo, Tokyo, Japan) at 68°C for 2 hr. Then the solution was replaced and the membrane was hybridized with the <sup>32</sup>P -labeled cDNA probes (see below) in PerfectHyb<sup>TM</sup> Hybridization Solution at 68°C for 18 hr. After hybridization, the membrane was washed in 2×SSC containing 0.1% SDS at 68°C for 5 min. Then the membrane was exposed to X-ray film RX-U (Fuji Film, Tokyo, Japan) at -80°C for 1 week.

The cDNA probe for ayu rhodopsin (396 bp: from nucleotide 701 in the open reading frame to 1095 in the 3'-untranslated region) was generated and labeled with  $[\alpha$ -<sup>32</sup>P]dCTP (Amersham Pharmacia Biotech) by PCR amplification. The cDNA probe did not overlap any of the four restriction sites used to digest the genomic DNA.

## **Genomic PCR**

Genomic DNA was applied for PCR amplification with a pair of ayu rhodopsin specific primers, fw2 and rv2. A reaction mixture (total volume of 20  $\mu$ l) consisted of 1×PCR buffer, dNTPs (200  $\mu$ M), Taq DNA polymerase (0.5 U), fw2 (0.5  $\mu$ M), rv2 (0.5  $\mu$ M) and genomic DNA (100 ng). cDNA (3  $\mu$ g) and total RNA (150  $\mu$ g) obtained from the ayu brain were also used as templates as the control. The PCR was performed as follows: denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 sec and 68°C for 3 min. Each reaction mixture was electorophoresed on 2% agarose gels and stained with ethidium bromide.

#### Northern blot analysis

Total RNA was isolated from the brain and retina as above. From the brain total RNA, poly(A)<sup>+</sup>RNA was purified with OlgotexdT30 according to manufacturer's instructions. Poly(A)<sup>+</sup>RNA (10  $\mu$ g) from the brain and total RNA (10  $\mu$ g) from the retina were elecrotophoresed on 0.9% formamide-agarose gel and transferred to a Biodine nylon membrane. The membrane was UV cross-linked (120,000  $\mu$ J/cm<sup>2</sup>) using Spectrolinker XL-1500 (Westburg, NY, USA) and air-dried in a dry oven at 80°C for 20 min. Following procedures for hybridization and the labeled probe were the same for the Southern blot analysis.

#### **Reverse-transcription (RT)-PCR**

The brain was dissected out from the ayu and quickly divided into five parts; part A, olfactory bulb and telencephalon; part B, diencephalon; part C, optic tectum; part D, cerebellum; part E, medulla oblongata and spinal cord (Fig. 1). Total RNA was isolated from the retina, pineal organ and each brain area as described above. Total RNA from each tissue (3 µg) was treated with DNase (Promega Biosciences, Inc., CA, USA) and then used for cDNA synthesis using Ready-To-Go<sup>TM</sup> T-primed first-strand cDNA synthesis kit according to manufacturer's instructions. These cDNAs were subjected to PCR amplification with a pair of ayu rhodopsin specific primers, fw3 (5'-CCATGAACGGCACAGAGGGAC-3') and rv2 in a reaction mixture (total volume of 20 ul) consisted of 1xPCR buffer. dNTPs (200 µM), Tag DNA polymerase (0.5 U), fw4 (0.5 µM), rv3 (0.5  $\mu$ M) and cDNA template (1  $\mu$ I) or total RNA (negative control; 1 µl). The PCR was performed as follows: denaturation at 94°C for 3 min, followed by 40 cycles of 94°C for 30 sec and 68°C for 3 min. Then the reaction mixture was electorophoresed on 2% agarose gels and stained with ethidium bromide.

# **RESULTS AND DISCUSSION**

### Characterization of opsin expressed in the ayu brain

Fig. 2 shows the nucleotide sequence of cDNA and deduced amino acid sequence of ayu rhodopsin. We identified 1478 bp of full-length ayu rhodopsin cDNA except  $poly(A)^+$  tail. The rhodopsin cDNA sequence obtained from the brain was identical with that from the retina. This cDNA

encodes a protein with 354 amino acid residues.

The deduced amino acid sequence shows typical features of vertebrate rhodopsin: a Schiff base linkage with the chromophore (K296), a counter-ion of the protonated Schiff base (E113), glycosylated site (N15), a disulfide bond (C110, C187), and glutamate (E122) found commonly in vertebrate rhodopsins (Karnik *et al.*, 1988; Ohguro *et al.*, 1994; Sakmar *et al.*, 1989; Shichida *et al.*, 1994; Wang *et al.*, 1980; Zhukovsky *et al.*, 1989) are conserved.

A molecular phylogenetic relationship of vertebrate opsins is shown in Fig. 3. In this tree, ayu rhodopsin cDNA was classified into the rhodopsin group but not to the other opsin groups. Fig. 2 also shows a molecular phylogenetic tree of vertebrate rhodopsins. Ayu rhodopsin was classified into the retina-type rhodopsin subgroup. The ayu rhodopsin expressed in the brain was clearly different from the pinealspecific rhodopsin (exo-rhodopsin or ERrod-like opsin) in teleosts. Thus, the retina-type and pineal-specific rhodopsins had diverged at the very early stage in the course of molecular evolution of teleost rhodopsins.

These results indicate that the cDNA cloned from the brain of ayu encodes functional rhodopsin, although functional expression and reconstitution experiments should be required to confirm its photosensitivity.

#### Genomic Southern blot and genomic PCR

The probe specific to the ayu rhodopsin cDNA sequences was used for genomic Southern blot analysis in order to examine the copy number of the ayu rhodopsin gene. Hybridization with the ayu rhodopsin cDNA specific probe gave a single band of approximately 1.8, 1.1, 16.4, and 3.1 kb for *Dra* I, *Eco*T14 I, *Nco* I, and *Pst* I digestions, respectively (Fig. 4). These results indicate that the ayu rhodopsin is coded by a single copy gene.

Genomic PCR was performed to test whether the ayu rhodopsin gene has intron or intron-less. The size of the genomic DNA fragments amplified was 1097 bp that was identical with those amplified from the ayu brain cDNA (Fig. 5). Thus, we concluded that the ayu rhodopsin gene is intron-less. Such a copy is commonly thought to be a nonfunctional processed pseudogene. However, it is reported in teleosts that rhodopsin genes lack introns (Fitzgibbon *et al.*, 1995), suggesting that the ayu rhodopsin is intron-less but functional.

#### Expression sites of the ayu rhodopsin gene

Northern blot analysis was first carried out to detect the ayu rhodopsin mRNA in the brain and retina. A single band of approximately 1.5 kb, corresponding to the full-length ayu rhodopsin cDNA sequence, was observed in the brain as well as the retina (Fig. 6). This size was the same size expected from the full-length ayu rhodopsin cDNA sequence.

The RT-PCR analysis was also carried out in order to analyze the tissue distribution of the ayu rhodopsin mRNA in the retina, pineal organ and brain areas. A representative



**Fig. 4.** Genomic Southern blot analysis of the ayu rhodopsin gene. The ayu genomic DNA digested with *Dral* (lane1), *Eco*T14I (lane2), *Ncol* (lane3) and *Pst*I (lane4) was electrophoresed and hybridized with the [<sup>32</sup>P]-labelled ayu rhodopsin cDNA specific probe. The DNA size makers are shown on the left.

result is shown in Fig. 7. The PCR amplification yielded a band of predicted size (684 bp) in the retina and brain part A and B but not in the pineal organ and brain part C, D and E. No product was detected from the negative control.

These results indicate the brain and retina are the expression sites of the rhodopsin gene in ayu. In the genomic Southern blot analysis, we suggested that the ayu rhodopsin is coded by a single copy gene. Taken together, we concluded that the same rhodopsin gene is expressed in both the brain and retina.

During the revision of this manuscript, Minamoto and Shimizu (2003) reported the cDNA sequence encoding the ayu rhodopsin and examined its expression site in the retina. The sequence of cDNA is almost identical with our sequence and the deduced amino acid sequences are identical. By *in situ* hybridization histochemistry, the rhodopsin mRNA was detected in the rod photoreceptor cells in the retina. However, they did not examine the expression of the rhodopsin gene in the brain.

In the present study, we confirmed that the rhodopsin gene is expressed in the ayu brain by Northen blot and RT-PCR analyses. However, the exact sites of expression remain to be determined. In some fish species, the existence of putative photoreceptors in the brain has been



**Fig. 5.** Genomic PCR analysis of the ayu rhodopsin gene. Ayu genomic DNA (lane1) and cDNA synthesized from ayu brain (lane2) were applied to PCR amplification with primers designed from 5'-(fw2) and 3'-untranslated regions (rv2). The reverse transcriptase treatment for total RNA purified from ayu brain was omitted in lane 3 (N.T.). The DNA size makers are shown on the left.



**Fig. 6.** Northern blot analysis for the ayu rhodopsin mRNA in the retina and brain of ayu. Total RNA (10 µg) from the retina and poly(A)<sup>+</sup> RNA (10 µg) from the brain were used. The RNA size makers are shown on the left. An arrow indicates the signal detected with the [<sup>32</sup>P]-labelled ayu rhodopsin cDNA specific probe.

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**Fig. 7.** RT-PCR analysis of the rhodopsin gene expression in the retina, pineal organ and brain areas of ayu. Total RNA obtained from the retina, pineal organ and brain areas (see Fig. 1) was treated (+) or not treated (-) with reverse transcriptase and then amplified by PCR to produce 684 bp band. The reaction mixture was fractionated on an agarose gel, and stained with ethidium bromide. The DNA size makers are shown on the left.

suggested in by immunohistochemical studies: opsin-immunoreactive cells in the diencephalon and  $\alpha$ -transducin-immunoreactive cells in the telencephalon were observed in lamprey and Atlantic salmon (Garcia-Fernandez *et al.*, 1997; Philip *et al.*, 2000b). Philp *et al.* (2000b) also reported the presence of cone-opsin-immunopositive cells in the preoptic area (POA). This area is located in the boundary of brain part A and B of this study. Therefore, the telencephalon, diencephalons and POA may be possible sites of rhodopsin gene expression in the ayu brain. Further *in situ* hybridization and immunohistochemical studies will be required to localize rhodopsin in the brain.

# **Functional considerations**

The brain photoreceptors are known to regulate physiology and behavior of fish such as the change in body color, locomotor activity and gonadal development. von Frisch (1911) examined the effect of direct illumination into the brain on the body color and proposed that cells of the ependymal layer were photosensitive. Concerning locomotor activity, the presence of photoneuroendocrine cells within the hypothalamic nucleus magnocellularis preopticus (NMPO) that regulates swimming and feeding reflexes was proposed (Scharrer, 1928, 1964). Since then, lines of evidence indicate the presence of brain photoreceptors in fishes (see Tabata, 1992). However, the photoreceptive molecules in the brain have not been identified until a few years ago.

Recently, a novel opsin named VA opsin was cloned from Atlantic salmon (Soni and Foster, 1997) and then its variant VA-long (VAL) opsin in zebrafish was identified (Kojima *et al.*, 2000). The expression sites of VA and VAL opsins were subependymal cells in epithalamus (Kojima *et al.*, 2000; Philp *et al.*, 2000b), suggesting that these opsins regulate body color change. Immnohistochemical studies also demonstrated the presence of opsin-like immunoreactivity in the NMPO in Atlantic salmon (Philp *et al.*, 2000b). This site nicely fits the localization of photoneuroendocrine cells by Scharrer (1928). Further studies will be required to elucidate whether the NMPO contains photoreceptors that regulate locomotor activity.

There is little information on the roles of brain photoreceptor that regulates gonadal development in fish. However, removal of both lateral eyes and pineal organ did not affect photoperiodism in gonadal development in ayu (Suzuki, 1975). Furthermore, opsin-immunopositive fibers passing through basal hypothalamus to hypophysis were observed in Atlantic salmon (Philp *et al.*, 2000b). Thus, gonadal development may be directly regulated by the photoreceptors in the brain.

# CONCLUSIONS

Now we have cloned a cDNA encoding rhodopsin from the ayu brain. Expression of the rhodopsin gene in the brain was demonstrated by Northern blot and RT-PCR analyses. The results of RT-PCR indicated that the rhodopsin gene is expressed in the telencephalon and/or diencephalon of avu. In addition, during preparation of this manuscript, VAL opsin and another VA opsin variant named VAM opsin were also cloned from ayu and expression of these genes in the brain was demonstrated in by RT-PCR (Minamoto and Shimizu, 2002). Therefore, further studies on these brain opsins in avu may reveal the roles of brain photoreceptors in the regulation of physiology and behavior in fishes. Localization of the expression sites of opsin genes in the brain by in situ hybridization and immunohistochemistry will be an important step toward elucidation of the functions of brain photoreceptors in this species.

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#### REFERENCES

- Altschul SF, Thomas LM, Alejandro AS, Jinghui Z, Zheng Z, Webb M, David JL (1997) BLASTN 2.0.11, distributed by the author, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389–3402
- Benoit JM (1978) Chronobiologic study in the domestic duck. II. Physiological mechanism of the chronobiologic action of visible light on the gonads of the male duck. Chronobiologia 5: 158– 168
- Campbell SS, Murphy PJ, Suhner AG (2001) Extraocular phototransduction and circadian timing systems in vertebrates. Chronobiol Int 18: 137–172
- Dodt E (1963) Photosensitivity of the pineal organ in the teleost, *Salmo irideus* (Gibbons). Experientia 19: 642–643
- Falcón J (1999) Cellular circadian clocks in the pineal. Prog Neurobiol 58: 121–162

- Felsenstein J (1989) PHYLIP (Phylogeny inference package) version 3.5c, distributed by the author, Department of Genetics, University of Washington, Seatlle, WA
- Fitzgibbon J, Hope A, Slobodyanyuk SJ, Bellingham J, Bowmaker JK, Hunt DM (1995) The rhodopsin-encoding gene of bony fish lacks introns. Gene 164: 273–277
- Fushiki S (1979) Studies on the effect of long photoperiod in spring season on gonadal maturation of Ayu-fish, *Plecoglossus altivelis* T. et S. Doctral dissertation, University of Tokyo, Tokyo
- Garcia-Fernandez JM, Jimenez AJ, Gonzalez B, Pombal MA, Foster RG (1997) An immunocytochemical study of encephalic photoreceptors in three species of lamprey. Cell Tissue Res 288: 267–278
- Hastings M, Maywood ES (2000) Circadian clocks in the mammalian brain. BioEssays 22: 23–31
- Karnik SS, Sakmar TP, Chen HB, Khorana HG (1988) Cysteine residues 110 and 187 are essential for the formation of correct structure in bovine rhodopsin. Proc Natl Acad Sci USA 85: 8459–8463
- Kojima D, Mano H, Fukada Y (2000) Vertebrate ancient-long opsin: a green-sensitive photoreceptive molecule present in zebrafish deep brain and retinal horizontal cells. J Neurosci 20: 2845– 2851
- Mangel SC (2001) Circadian clock regulation of neuronal light responses in the vertebrate retina. Prog Brain Res 131: 505– 518
- Mano H, Kojima D, Fukada Y (1999) Exo-rhodopsin: a novel rhodopsin expressed in the zebrafish pineal gland. Brain Res Mol Brain Res 73: 110–118
- Minamoto T, Shimizu I (2002) A novel isoform of vertebrate ancient opsin in a smelt fish, *Plecoglossus altivelis*. Biochem Biophys Res Commun 290: 280–286
- Minamoto T, Shimizu I (2003) Molecular cloning and characterization of rhodopsin in a teleost (*Plecoglossus altivelis*, Osmeridae). Comp Biochem Physiol B134: 559–570
- Okano T, Yoshizawa T, Fukada Y (1994) Pinopsin is a chicken pineal photoreceptive molecule. Nature 372: 94–97
- Okubo K, Nagata S, Ko R, Kataoka H, Yoshiura Y, Mitani H, Kondo M, Naruse K, Shima A, Aida K (2001) Identification and characterization of two distinct GnRH receptor subtypes in a teleost, the medaka *Oryzias latipes*. Endocrinology 142: 4729–4739
- Oshima N (2001) Direct reception of light by chromatophores of lower vertebrates. Pigment Cell Res 14: 312–319
- Philp AR, Bellingham J, Garcia-Fernandez J, Foster RG (2000a) A novel rod-like opsin isolated from the extra-retinal photoreceptors of teleost fish. FEBS Lett 468: 181–188

- Philp AR, Garcia-Fernandez JM, Soni BG, Lucas RJ, Bellingham J, Foster RG (2000b) Vertebrate ancient (VA) opsin and extraretinal photoreception in the Atlantic salmon (*Salmo salar*). J Exp Biol 203: 1925–1936
- Provencio I, Jiang G, De Grip WJ, Hayes WP, Rollag MD (1998) Melanopsin: An opsin in melanophores, brain, and eye. Proc Natl Acad Sci USA 95: 340–345
- Sakmar TP, Franke RR, Khorana HG (1989) Glutamic acid-113 serves as the retinylidene Schiff base counterion in bovine rhodopsin. Proc Natl Acad Sci USA 86: 8309–8313
- Scharrer E (1928) Die Lichtempfindlichkeit blinder Elritzen I. Untersuchungen uber das Zwischenhirn der Fische. Z Vergl Physiol 7: 1–38
- Scharrer E (1964) Photoneuroendocrine systems: general concepts. Ann NY Acad Sci 117: 13–22
- Silver R, Witkovsky P, Horvath P, Alones V, Barnstable CJ, Lehman MN (1988) Coexpression of opsin- and VIP-like-immunoreactivity in CSF-contacting neurons of the avian brain. Cell Tissue Res 253: 189–198
- Soni BG, Foster RG (1997) A novel and ancient vertebrate opsin. FEBS Lett 406: 279–283
- Suzuki K (1975) The significance of photoperiod on the gonadal development of ayu *Plecoglossus altivelis*. Doctral dissertation, University of Tokyo, Tokyo
- Tabata M (1992) Photoreceptor organs and circadian locomotor activity in fishes. In "Rhythms in Fishes", Ed by MA Ali Plenum Press, NY, pp 223–234
- Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673–4680
- von Frisch K (1911) Beitraage zur Physiologie der pigmentzellen in der fischhaut. Pflugers Arch Gesamte Physiol Menschen Tiere 138: 319–387
- Wada Y, Okano T, Adachi A, Ebihara S, Fukada Y (1998) Identification of rhodopsin in the pigeon deep brain. FEBS Lett 424: 53– 56
- Wang JK, McDowell JH, Hargrave PA (1980) Site of attachment of 11-cis-retinal in bovine rhodopsin. Biochemistry 19: 5111–5117
- Yoshikawa T, Oishi T (1998) Extraretinal photoreception and circadian system in nonmammalian vertebrates. Comp Biochem Physiol B 119: 65–72
- Yoshikawa T, Okano T, Oishi T, Fukada Y (1998) A deep brain photoreceptive molecule in the toad hypothalamus. FEBS Lett 424: 69–72

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