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

Authors: Masuda, Tomohiro, Iigo, Masayuki, Mizusawa, Kanta, and Aida, Katsumi

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

Tomohiro Masuda[1]*, Masayuki Iigo[2†], Kanta Mizusawa[1], and Katsumi Aida[1]

[1]*Department of Aquatic Bioscience, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo, Tokyo 113-8657, Japan*
[2]*Department of Anatomy, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae, Kawasaki 216-8511, Japan*

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 Hastings and Maywood, 2000; Mangel, 2001).

The photosensitivity of skin was reported earlier in non-mammalian 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 well-developed 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-

* Corresponding author: Tel. +81-3-5841-5289;
FAX. +81-3-5841-5288.
E-mail: masuda@marine.fs.a.u-tokyo.ac.jp
† Present address: Department of Applied Biological Chemistry, Faculty of Agriculture, Utsunomiya University, 350 Mine-Machi, Utsunomiya, Tochigi 321-8505, Japan

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^{TM} 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 primers, 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 μl) consisted of 1xPCR buffer (Takara, Shiga, Japan), dNTPs (200 μM; Takara), Taq DNA polymerase (0.5 U; Takara), Op-Fw (0.5 μM), Op-Rv (0.5 μM) and cDNA template (1 μl). 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 $(A)^+$ RNA was extracted from the brain and retina using Oligotex-dT30 (Takara), and then cDNA ligated adaptor nucleotides including AP1 (5'-CCATCCTAATACGACTCACTATAGGGC-3') and AP2 (5'-ACTCACTATAGGGCTCGAGCGGC-3') sequences was synthesized using Marathon^{TM} cDNA amplification kit (Clontech, Palo Alto, CA, USA). Specific primers were designed for rapid amplification of cDNA ends (RACE): fw1 (5'-TCGTCCTGATGGAGATCTCCTACCTG-3') and fwn1 (5'-AGCGTGGCCTGGTACATCTTCTGC-3') for 3'-RACE; rv1 (5'-AAAGAAGGCCGGGGCTGTCATGAAG-3') and rvn1 (5'-CTTCCCTGATTGCAGAAGATGTACCAG-3') for 5'-RACE. 3'-RACE was executed with the primers AP1 and fw1 (0.5 μM each) in a reaction mixture (total volume of 20 μl) consisted of 1×PCR buffer, dNTPs (200 μM) 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-

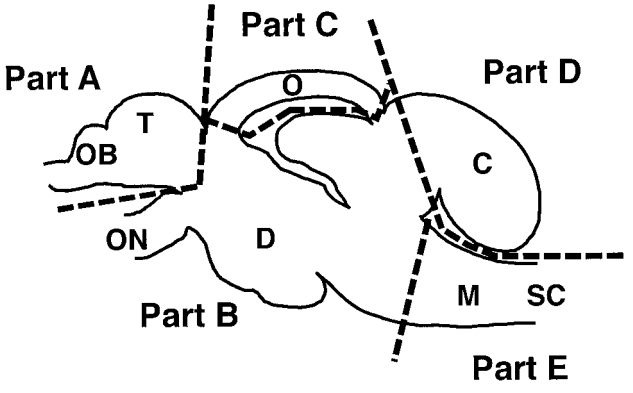

Fig. 1. Schematic representation of the ayu brain (sagittal view) that depicts dissection used for RT-PCR analysis of rhodopsin gene expression in brain areas. The brain was divided into five parts; part A, olfactory bulb and telencephalon; part B, diencephalon; part C, optic tectum; part D, cerebellum; 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 μM) 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-

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                                                        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  E  Y  P  Q  Y  Y  L  I  N  P  A  A  Y  F  M  L   40
GCCTGCTACATGTTCTTTCTCATCATCACCGGCTTCCCCATCAACTTCCTAACACTGTAC    180
 A  C  Y  M  F  F  L  I  I  T  G  F  P  I  N  F  L  T  L  Y   60
GTCACCATCGAGCACAAGAAGCTGAGGACCGCCCTAAACTACATCCTGCTGAACCTGGCT    240
 V  T  I  E  H  K  K  L  R  T  A  L  N  Y  I  L  L  N  L  A   80
GTGGCTGACCTCTTCATGGTGATCGGTGGCTTCACCACCACATTGTACACATCCATGCAT    300
 V  A  D  L  F  M  V  I  G  G  F  T  T  T  L  Y  T  S  M  H  100
GGCTACTTCGTCTTCGGTAGGACTGGCTGCAACATCGAAGGATTTTGTGCTACCCTCGGT    360
 G  Y  F  V  F  G  R  T  G  (C) N  I  (E) G  F  C  A  T  L  G  120
GGTGAGATTGCCATGTGGTCCCTGGTTGTCCTGGCTATTGAGAGGTGGGTGGTTGTCTGC    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  A  C  A  V  P  P  L  F  G  W  S  R  Y  I  P  180
GAGGGCATGCAGTGCTCATGCGGAATCGACTACTACACCCGTGCCCCCGGCTTTAACAAC    600
 E  G  M  Q  C  S  (C) 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  L  L  C  T  V  K  E  A  A  A  A  Q  Q  E  S  240
GAGACCACCCAGAGGGCCGAGAGGGAAGTTACCCGCATGGTCGTCCTGATGGAGATCTCC    780
 E  T  T  Q  R  A  E  R  E  V  T  R  M  V  V  L  M  E  I  S  260
TACCTGGTGTGCTGGTTGCCCTATGCCAGCGTGGCCTGGTACATCTTCTGCAATCAGGGA    840
 Y  L  V  C  W  L  P  Y  A  S  V  A  W  Y  I  F  C  N  Q  G  280
AGTGAGTTTGGCCCCGTCTTCATGACAGCCCCGGCCTTCTTTGCCAAGAGCTCCGCTCTC    900
 S  E  F  G  P  V  F  M  T  A  P  A  F  F  A  (K) S  S  A  L  300
TACAACCCCCTCATCTACGTGTGCATGAACAAGCAGTTCCGCCACTGCATGATCACCACC    960
 Y  N  P  L  I  Y  V  C  M  N  K  Q  F  R  H  C  M  I  T  T  320
CTGTGCTGCGGAAAGAACCCCTTCGAGGAAGAGGAGGGAGCGTCCACGACAGCCTCCAAG   1020
 L  C  C  G  K  N  P  F  E  E  E  E  G  A  S  T  T  A  S  K  340
ACCGAGGCCTCCTCCGTGTCCTCCAGCTCCGTGGCCCCTGCAtaaatgggctctcatgga   1080
 T  E  A  S  S  V  S  S  S  S  V  A  P  A  *                  354
ccccgcgatccagcgtgcacaaagaagacttctgcacccccgggaaacgactgaaggcta    1140
tcgtatacagaaataacttccttttgtatttttacaaaccagttggttcaacctaatga    1200
cagttgcagaagagggcagcccaagaaaaagttgtttctgtatgtacagagagtccagtg    1260
taacgatgcataagatgttttcttttcctgaaatgaaagcaaaatatcttttatctttta    1320
cagttggagtctatatgttactggcttatttgtgaatgtagaggcatgtaatcaaggcaa    1380
cgtaa[aataaaa]cgcactttgcaaatg                                   1478
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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.

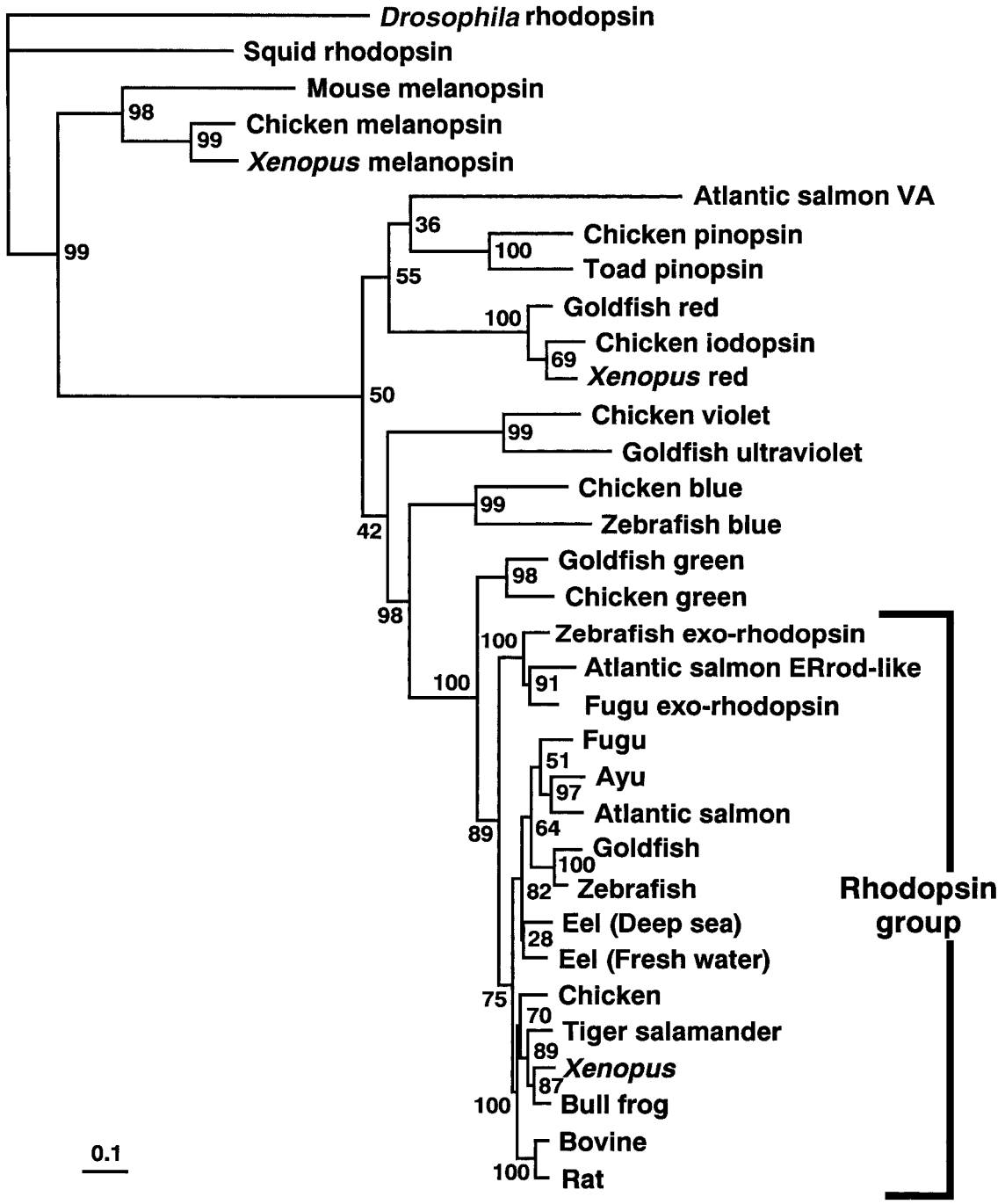
respond to the 5'- and 3'-flanking region, respectively. A reaction mixture (total volume of 20 μl) consisted of 1xPCR buffer, dNTPs (200 μM), Taq DNA polymerase (0.5 U), fw2 (0.5 μM), rv2 (0.5 μM) and cDNA template (1 μl). The PCR was performed as follows: denaturation at 94°C for 3 min, followed by 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 positions 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 μg) 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™ Hybrid-



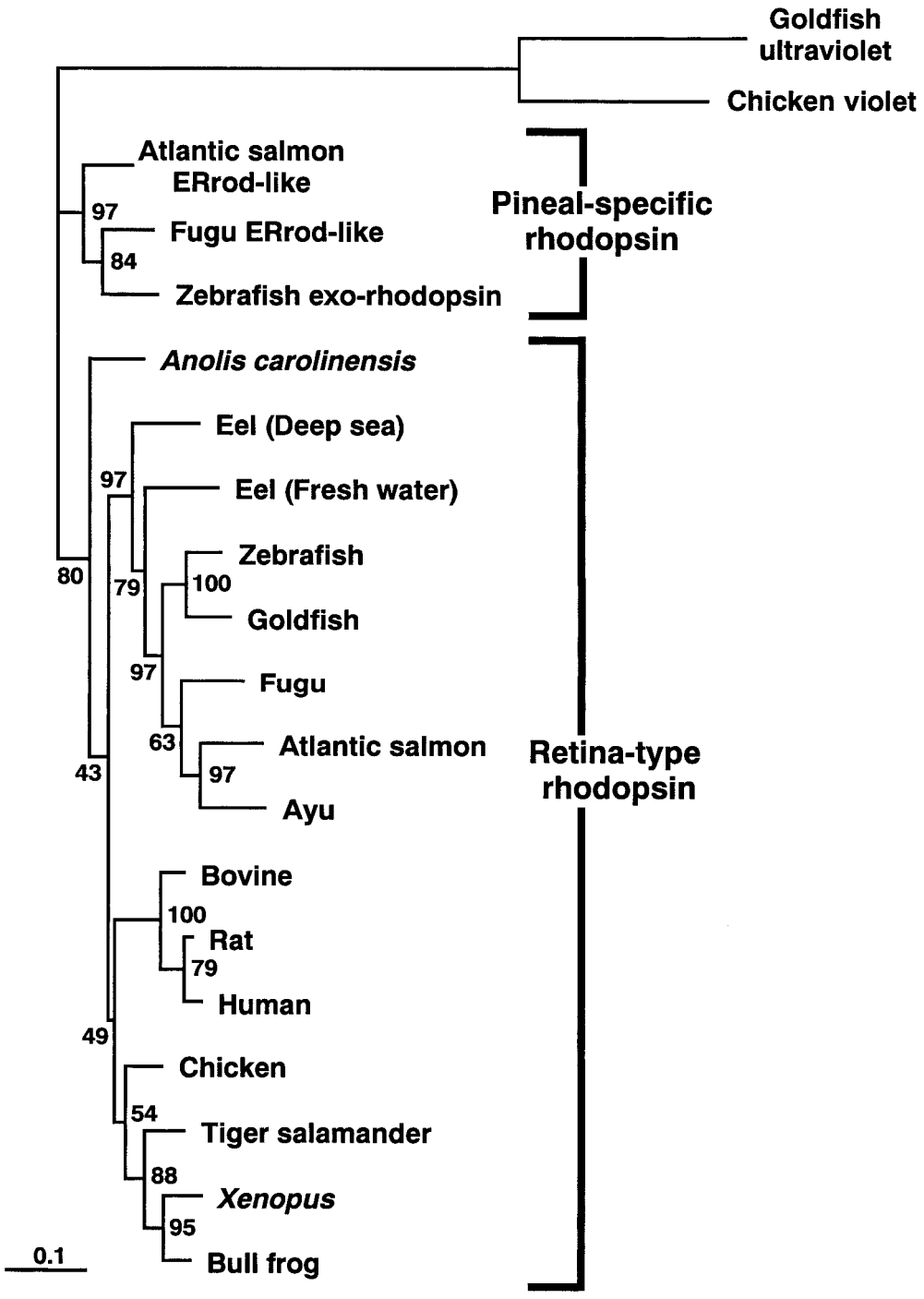


Fig. 3. Phylogenetic analysis of the deduced amino acid sequences of ayu rhodopsin with opsins and rhodopsins of selected species. The deepest root was determined by using the sequences of invertebrate opsins (squid rhodopsin, DDBJ/GenBank/EMBL accession number Z49108; *Drosophila* rhodopsin, XM081147) 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, M920039; goldfish, AF109373); Atlantic salmon ERrod-like opsin, AF201469; fugu ERrod-like opsin, AF201472; 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 ^{32}P -labeled cDNA probes (see below) in PerfectHyb™ 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 [α-^{32}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 μl) consisted of 1×PCR buffer, dNTPs (200 μM), Taq DNA polymerase (0.5 U), fw2 (0.5 μM), rv2 (0.5 μM) and genomic DNA (100 ng). cDNA (3 μg) and total RNA (150 μg) 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)$^+$RNA was purified with Olgotex-dT30 according to manufacturer's instructions. Poly(A)$^+$RNA (10 μg) from the brain and total RNA (10 μg) from the retina were electrophoresed on 0.9% formamide-agarose gel and transferred to a Biodine nylon membrane. The membrane was UV cross-linked (120,000 μJ/cm^2) 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™ 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 μl) consisted of 1xPCR buffer, dNTPs (200 μM), Taq DNA polymerase (0.5 U), fw4 (0.5 μM), rv3 (0.5 μM) and cDNA template (1 μl) 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 pineal-specific 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 non-functional 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

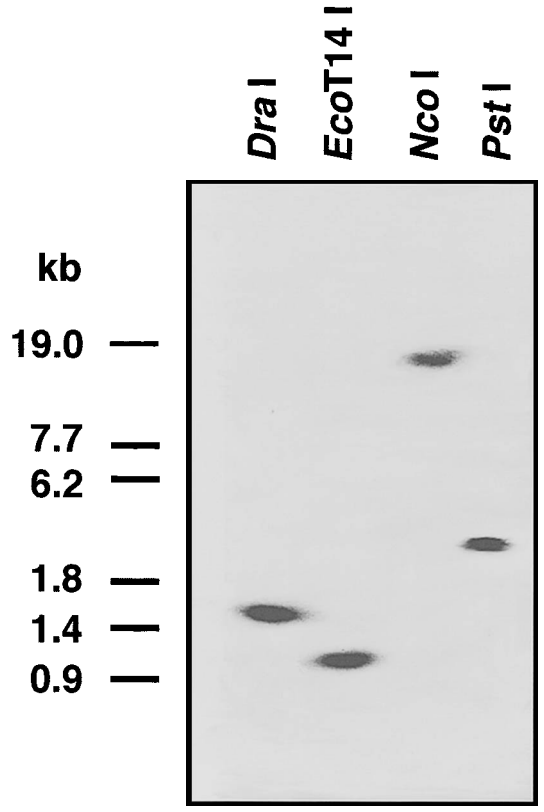


Fig. 4. Genomic Southern blot analysis of the ayu rhodopsin gene. The ayu genomic DNA digested with *Dra*I (lane1), *Eco*T14I (lane2), *Nco*I (lane3) and *Pst*I (lane4) was electrophoresed and hybridized with the [^{32}P]-labelled ayu rhodopsin cDNA specific probe. The DNA size makers are shown on the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

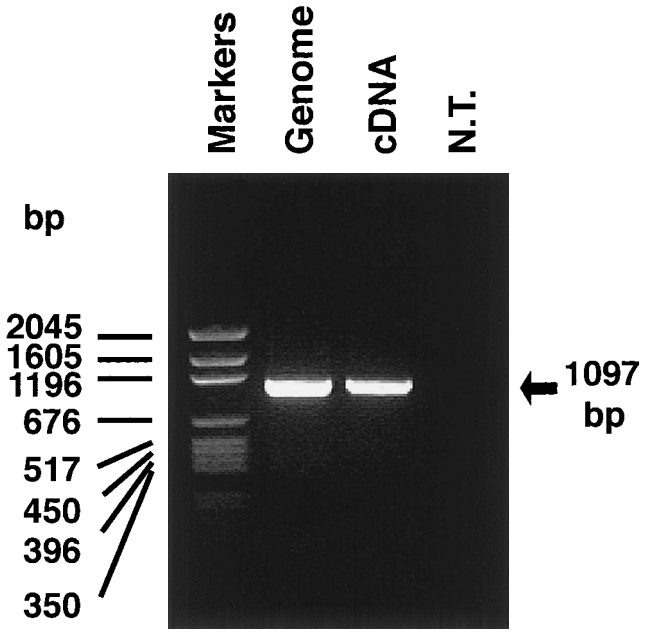


Fig. 5. Genomic PCR analysis of the ayu rhodopsin gene. Ayu genomic DNA (lane1) and cDNA synthesized from ayu brain (lane2) were applied to PCR amplification with primers designed from 5'-(fw2) and 3'-untranslated regions (rv2). The reverse transcriptase treatment for total RNA purified from ayu brain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

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

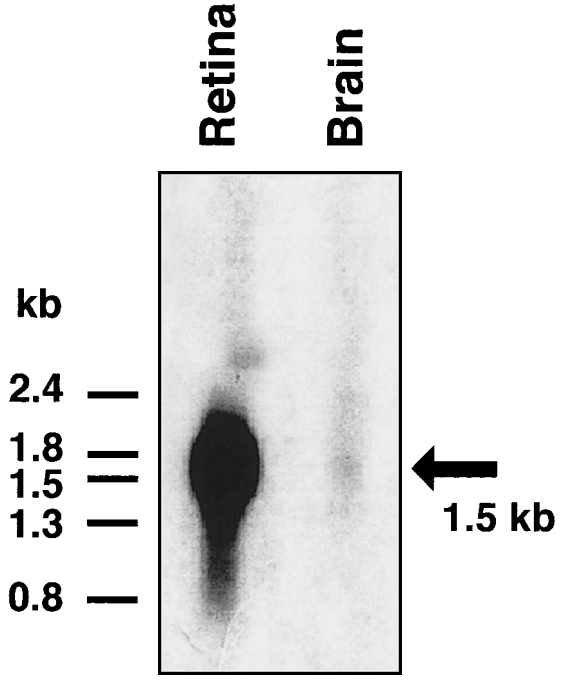


Fig. 6. Northern blot analysis for the ayu rhodopsin mRNA in the retina and brain of ayu. Total RNA (10 μg) from the retina and poly(A)$^+$ RNA (10 μg) from the brain were used. The RNA size makers are shown on the left. An arrow indicates the signal detected with the [^{32}P]-labelled ayu rhodopsin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

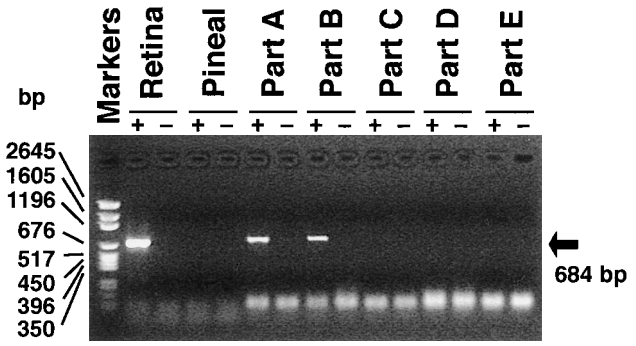


Fig. 7. RT-PCR analysis of the rhodopsin gene expression in the retina, pineal organ and brain areas of ayu. Total RNA obtained from the retina, pineal organ and brain areas (see Fig. 1) was treated (+) or not treated (–) with reverse transcriptase and then amplified by PCR to produce 684 bp band. The 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 α-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 ayu. 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 ayu 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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