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A Novel Isoform of Vertebrate Ancient Opsin in a Smelt Fish, *Plecoglossus altivelis*

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Vertebrate ancient (VA) opsin of nonvisual pigment in fishes was reported to exist in two isoforms, i.e., short and long variants with an unusual predicted amino acid sequence length compared to vertebrate visual opsins. Here we cloned an isoform (Pal-VAM) of VA opsin showing the usual opsin length in addition to the long type isoform (Pal-VAL) from a smelt fish, *Plecoglossus altivelis.* Pal-VAM and Pal-VAL were composed of 346 and 387 amino acids, respectively. The deduced amino acid sequences of these variants were identical to each other within the first 342 residues, but they showed divergence in the carboxyl-terminal sequence. Pal-VAL corresponded to the long isoform found in zebrafish and carp, and Pal-VAM was identified as a new type of VA opsin variant. Southern blotting experiments indicated that the VA opsin gene of the smelt is present as a single copy, and RT-PCR analysis revealed that Pal-VAM and Pal-VAL mRNA were expressed in both the eyes and brain. *In situ* hybridization showed that Pal-VAM and Pal-VAL mRNA are expressed in amacrine cells in the retina. Pal-VAM is a new probably functional nonvisual photoreceptive molecule in fish. © 2002 Elsevier Science

Key Words: **vertebrate ancient opsin; nonvisual photoreception; amacrine cell; retina; *Plecoglossus altivelis*.**

Vertebrates can receive light via not only the visual cells but also nonvisual cells of the retina and extraocular organs. These nonvisual photosensitive cells contain opsin-like proteins, and the primary structures of these proteins have been determined in recent years. For example, pinopsin in avian pineal gland, melanopsin in frog melanophores, and exorhodopsin in fish pineal gland and deep brain have been identified (1–3). The contribution of these nonvisual photoreceptors to physiological functions such as, entrainment of circadian rhythms, behavioral orientation, detection of seasonal changes in photoperiod, regulation of body color, and regulation of serum hormone level, were discussed (4).

Vertebrate ancient (VA) opsin was first found in Atlantic salmon as a new member of the nonvisual opsins (5), and other types of VA opsin were also found in zebrafish and carp (6, 7). While the predicted amino acid sequences of these VA opsins retain the conserved features required for opsin function, the lengths of their carboxyl termini vary. Salmon VA opsin has a greatly reduced carboxyl terminal length compared to the vertebrate visual opsins, and that of carp VA opsin is increased (5, 7). Long and short isoforms were found in zebrafish, and these were considered to be splicing variants (6). Salmon VA opsin is a blue-sensitive molecule with maximal absorbance at 451 nm, and is expressed in horizontal and amacrine cells in the retina, cells of the pineal gland, and the subhabenular region of the brain (8, 9). The long isoform of zebrafish VA opsin (zebrafishVAL) was reported to be green-sensitive with maximal absorbance around 500 nm. It was shown to be expressed in horizontal and amacrine cells, and the cells surrounding the diencephalic ventricle of the central thalamus. On the other hand, the short isoform (zebrafish VA) showed no photobleaching signal when bound with 11-*cis*-retinal (6). Carp VA was cloned from the retina and pineal organ (7). These observations suggested that VA opsin is a nonimage-forming photopigment; however, the physiological roles of VA opsin proteins remain unknown (5–9).

Plecoglossus altivelis is a smelt fish belonging to the family Osmeridae, and is distributed in East Asia. There are amphidromous and landlocked forms of this species. This smelt fish shows photoperiodic responses in testicular development and overt circadian rhythms in which extraocular photoreception might be involved (10, 11). This fish seems to be a suitable experimental model for the study of visual and extra-ocular photoreception. In this study, we cloned and characterized the

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cDNA encoding a new type of VA opsin (Pal-VAM) in addition to the long isoform (Pal-VAL) in the smelt fish. Pal-VAM had the usual amino acid sequence length of vertebrate visual opsins, and is a new candidate as a functional nonvisual photoreceptive molecule in fish.

MATERIALS AND METHODS

cDNA cloning. Landlocked forms of *P. altivelis* were collected from Lake Biwa (Shiga Pref., Japan) and stored at −80°C for molecular analysis. Total retinal RNA was isolated from the smelt by the acid guanidinium isothocyanate phenol–chloroform (AGPC) method using Isogen (Nippon Gene, Toyama, Japan), and single-stranded cDNA was synthesized with a first-strand cDNA synthesis kit (Life Sciences, Inc., St. Petersburg, FL) using T-primer [5′-GCGAATTCGTCGACAAGC(T)17-3′] as a primer. Reverse transcription of total RNA from the retina was performed using 5–25 μg of total RNA and 25 units of avian myeloblastosis virus reverse transcriptase in a 25-μl cDNA reaction mixture, and aliquots of 1 μl of the mixture were used for PCR using two degenerate primers, VA-F1 (CGPDW, 5′-TGYGGNCCNGAYTGG-3′) and VVP-R2′ [YNP(I/V)VY, 5′-CGAAGCTTAYRTANAYNAYNGGRTTRTA-3′] (12), which were designed for amplification of vertebrate opsins. For further amplification, second PCR was carried out using primers VA-F2 (DWYTT, 5′-CCNGAYTGGTAYACNAC-3′) and VVP-R2′. Amplification was performed as follows: denaturation at 94°C for 0.5 min, annealing at 45–50°C for 0.5 min, and extension at 72°C for 0.5 min. The PCR products were electrophoresed on 1% agarose gels, and the 342-bp band was purified. The products were cloned into pGEM easy T vector (Promega, Madison, WI) and sequenced.

RACE (rapid amplification of cDNA ends) was used for sequencing. To sequence the 3′-terminal, PCR was performed using primers VA-F3 (5′-CGGAAACTAAGGAAGGTGTC-3′; 679 to 698) and T-Primer. The products of this PCR were further amplified using a second set of primers, VA-F4 (5′-GCGAATTCAATGGTGGTGGTAATGATCG-3′; 747 to 766) and T-amp (5′-GCGAATTCGTCGACAAGC-3′). In the 3′-RACE procedure, we obtained two kinds of 3′-terminal clones of about 400 and 600 bp, respectively. The 5′-terminal portion of the cDNA was cloned by 5′-RACE (5′-RACE System for Rapid Amplification of cDNA ends, Gibco BRL, Gaithersburg, MD) according to the manufacturer's protocol using the primers VA-R0 (5′-CCGGCTTCCTGGCATTACCC-3′; 711 to 730), VA-R1 (5′-CCCAGCGGCAGTATGAAGCA-3′; 628 to 647) and nested primer VA-R2 (5′-GCGAATTCACGTGAAAAAGAAAGTGATG-3′; 609 to 628). A fragment of a approximately 800 bp was amplified, cloned and sequenced. In the RACE procedure, PCR was carried out as follows: denaturation at 94°C for 0.5 min, annealing at 55°C for 0.5 min and extension at 72°C for 1.5 min.

Finally, to obtain the cDNA including open reading frame, PCR was performed with two primer pairs, VA-ALL-F1 (5′-TGGACCAAAATCTTACAGG-3′; −82 to −64) and VAM-ALL-R1 (5′-AAATACTGGCGTGCATCTA-3′; 1092 to 1110), and VA-ALL-F1 and VAL-ALL-R1 (5′-TAGTGATAGAAGGGTAGAG-3′; 1326 to 1344), which were based on the sequences obtained by RACE. The PCR products were amplified using other sets of nested primers, VA-ALL-F2 (5′-GCGTTAACGGTTTCCTACTACCAACAGG-3′; −65 to −46) and VAM-ALL-R2 (5′-GTATTACATTTAAATTCTGG-3′; 1070 to 1089), and VA-ALL-F2 and VAL-ALL-R2 (5′-GCAAGCTTATAGAAGGGTAGAGCATG-3′; 1322 to 1339).

Sequencing and data analysis. Sequencing reactions were carried out using the dye primer and dye terminator cycle sequencing method according to manufacturer's protocol (Applied Biosystems, Foster, CA). All sequences were determined with independently amplified fragments to avoid PCR errors, and determined in both directions using an ABI-373S automatic DNA sequencer (Applied Biosystems).

CLUSTAL W (13) computer software was used to align the sequences and to make a molecular phylogenetic tree using the neighbor-joining method (14). In constructing the phylogenetic tree, *Drosophila* and octopus rhodopsins were used as outgroups and evolutionary distances were calculated for the region corresponding to P22-Q309 of Pal-VAM and Pal-VAL. Following vertebrate opsins were compared: chicken opsins [rhodopsin (CHK-Rh; GenBank Accession No. D00702), green (CHK-Gr; M92038), blue (CHK-Blue; M92037), violet (CHK-V; M92039), pinopsin (CHK-P; U15762) and red (CHK-Red; X57490)], zebrafish opsins [rhodopsin (ZFH-Rh; AF109370), green1 (ZFH-Gr1; AF109369), green2 (ZFH-Gr2; AF109370), blue (ZFH-Blue; AF109372), UV (ZFH-UV; AF109373) red (ZFH-Red; AF109371), VA (ZFH-VA; AB035277) and VAL (ZFH-VAL; AB035276)], *P. altivelis* rhodopsin (Pal-Rh; AB084484), toad pinopsin (TOD-P; AF200433), chameleon pinopsin (CHA-P; AF134771), lamprey P-opsin (LMP-P; U90671), carp VA (CRP-VA; AF233520), salmon VA (SLM-VA; AF001499), and catfish parapinopsin (CFH-PP; AF028014).

Southern blotting. Southern blotting was carried out using Gene Images Randome-Prime Labeling and Detection System (Amersham–Pharmacia Biotech, Piscataway, NJ). A 221-bp probe was generated using the PCR products amplified with the primers VA-F3 and VA-R3 (5′-GGGTTGTAGCAGGCGGCAGTTT-3′; 1097 to 1118). The probe was labeled with fluorescein using random primers and Klenow fragment of *Escherichia coli* DNA polymerase.

Total genomic DNA was isolated from the muscle of the smelt fish according to the standard methods. DNA was digested with restriction enzymes *Bam*HI and *Hin*dIII, and aliquots of 5 μg were separated on 1% agarose gels, UV cross-linked onto nylon membranes (Hybond-N+, Amersham–Pharmacia Biotech). Membrane-bound digested DNA was hybridized in rapid hybridization buffer at 65°C. After stringency washing in 2× SSC for 20 min, 1× SSC for 15 min and 0.5× SSC for 15 min (each buffer contained 0.1% SDS), membranes were incubated with blocking reagent and then with anti-fluorescein antibodies conjugated to alkaline phosphatase. Positive signals were detected with CDP-*star* detecting reagent (Amersham–Pharmacia Biotech), and photographed using an instant camera (ECL minicamera, Amersham–Pharmacia Biotech).

RT-PCR. Eyes and brains including pineal body were dissected out from the smelt fish and mRNA was isolated from the tissues using an mRNA purification kit (Amersham–Pharmacia Biotech). The polyadenylated mRNA was eluted with 100 μl of 10 mM Tris–HCl (pH 7.5) containing 1 mM EDTA. After DNase treatment of the eluate, mRNA was precipitated by adding 95% ethanol, collected by centrifugation, and finally redissolved in 10 μl of DEPC-treated water.

One-step RT-PCR was performed using a kit purchased from Qiagen (Chatsworth, CA) according to the manufacturer's protocol. RT-PCR analysis was performed with primers VA-RTF (5′-GTCCCCACATCTACATCGAC-3′; 824 to 843) and VAM-RTR (5′-GGCGTGCATCTATTGTATTAC-3′; 1083 to 1103) or VAL-RTR (5′-AAGCTGGGCAAAGGAATTACA-3′; 1111 to 1131). VAM-RTR and VAL-RTR are specific to Pal-VAM and Pal-VAL, respectively. The reaction mixture (50 μl) contained reverse transcriptase, Taq DNA polymerase, dNTP mixture (400 μM of each dNTP), 0.6 μM oligonucleotide primers, 2.5 mM $MgCl_2$, RNase inhibitor (10 units) and 1 μl of mRNA solution obtained by the method described above. As a control, 1 μl of DEPC-treated water was used instead of mRNA. First, reverse transcription reaction was performed at 50°C for 30 min, then the mixture was incubated at 95°C for 15 min to activate *Taq* DNA polymerase, and PCR amplifications were carried out. PCR was performed for 30 cycles of 1 min template denaturation at 95°C, 1 min primer annealing at 45°C, and 1 min primer extension at 72°C. The resultant PCR products were visualized by ethidium bromide staining after 1% agarose gel electrophoresis. The amplified fragments of approximately 300 bp were purified, cloned and sequenced.

In situ hybridization. Eyes were enucleated immediately after decapitated, and fixed. cDNA fragments including the full open read-

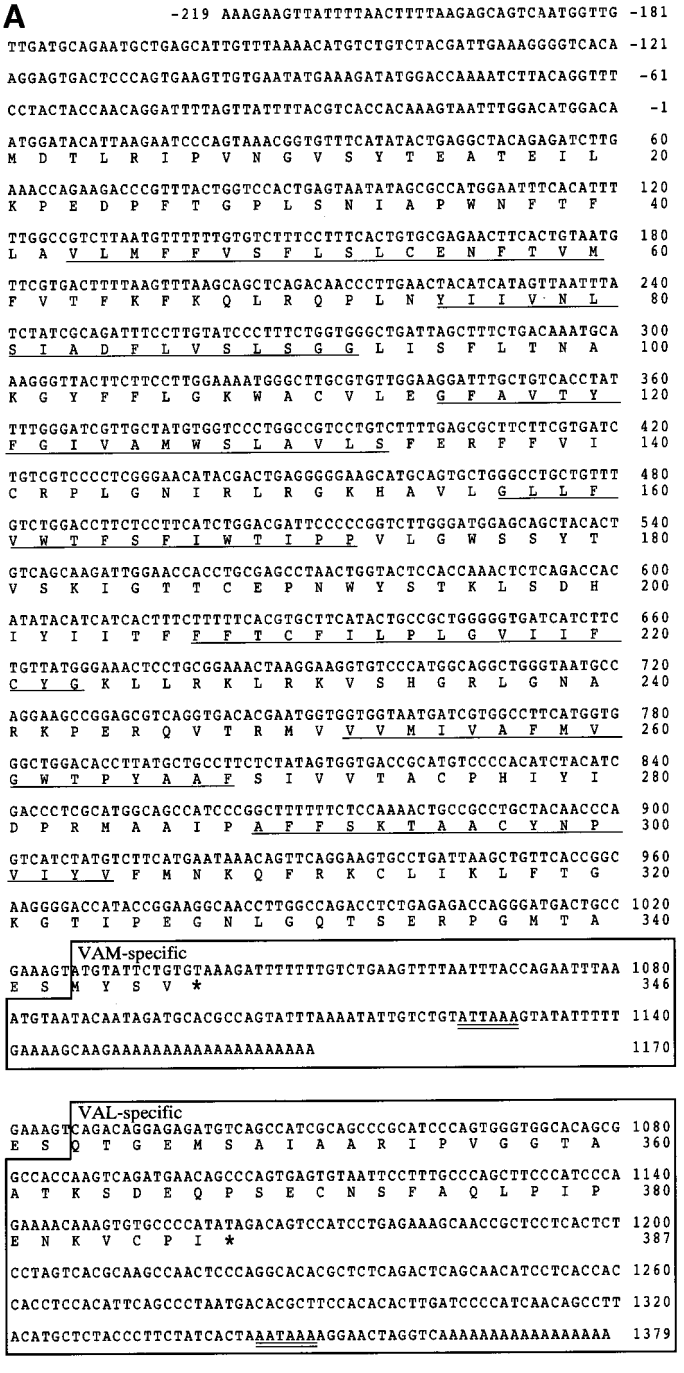

FIG. 1. (A) Nucleotide and deduced amino acid sequences of Pal-VAM and Pal-VAL. The translational stop codon is indicated by the asterisk, and the consensus sequence for the polyadenylation signal is double underlined. The seven predicted transmembrane domains are underlined. The nucleotide sequences of Pal-VAM and Pal-VAL are identical to each other within the first 1026 nucleotides, and the specific sequences of the 3′-termini are boxed. (B) Comparison of the carboxyl termini of Pal-VAM and Pal-VAL with those of other opsins: salmon VA (SLM-VA), zebrafish VA (ZFH-VA), zebrafish rhodopsin (ZFH-Rh), *P. altivelis* rhodopsin (Pal-Rh), zebrafish VAL (ZFH-VAL), and carp VA (CRP-VA). Conserved residues of functional importance are shown in bold. The multiple alignment was conducted with CLUSTAL W 1.6.

ing frame (−65 to 1339) of Pal-VAL generated by PCR were cloned into pGEM easy-T vector (Promega), and plasmids were linearized with appropriate endonucleases. Templates for the preparation of sense and antisense cRNA probes were generated. The sense cRNA probe was used as negative control. The cRNA probes were synthesized by run-off transcription from SP6 and T7 promoters with digoxigenin–UTP, as recommended in the manufacturer's protocol (Roche, Nutley, NJ). The cRNA probe was considered to hybridize with both Pal-VAL and Pal-VAM.

Cryosections of *P. altivelis* retina were prepared as described by Barthel and Raymond (15). Briefly, fish were decapitated, and eyes were enucleated, fixed in 4% paraformaldehyde, infiltrated with 20% sucrose in phosphate buffer (100 mM sodium phosphate, pH 7.4), and embedded in 33% OCT compound diluted with 20% sucrose in phosphate buffer. Retinal cryosections 3–5 μm thick were prepared and stored at −80°C until use.

In situ hybridization of retinal cryosections was carried out according to the method of Barthel and Raymond (15), with slight modifications. Briefly, sections stored at −80°C were thawed, dehydrated, incubated in 10 μg/ml proteinase K for 10 min, then treated with 0.25% acetic anhydride in 0.1 M triethanolamine, and hybridized at 65°C overnight with 10–50 ng cRNA probes in 100 μl hybridization solution (16). After washing and blocking, slides were incubated at 4°C for 16 h with anti-digoxigenin antibodies conjugated to alkaline phosphatase, and positive hybridization signals were detected with NCBT–BIP. Sections were rinsed in TE (10 mM Tris–HCl, pH 9.5, and 1 mM EDTA) and coverslipped under 80% glycerol, then photographed using a PM-30 camera attached to a BX50 microscope (Olympus, Tokyo).

RESULTS

Two Isoforms of VA Opsin from P. altivelis

We isolated cDNA clones encoding putative opsin protein, of which the deduced amino acid sequence was similar to that of VA opsins reported in other fish. To obtain a full-length cDNA including the whole coding region, 5′- and 3′-RACE were carried out. As a result, we isolated two kinds of cDNA variants, Pal-VAM and -VAL, respectively.

The nucleotide and deduced amino acid sequence of these two VA opsins are shown in Fig. 1A. The deduced amino acid sequences of these variants were identical to each other within the first 342 residues, and they showed divergence in the carboxyl-terminal sequence. In the nucleotide sequences of Pal-VAM and Pal-VAL, the first ATGs showed good agreement with Kozak's criteria for eukaryotic initiation sites (17), and were followed by an open reading frame of 1038 and 1161 bases predicting proteins of 346 and 387 amino acids, respectively. The nucleotide and predicted amino acid

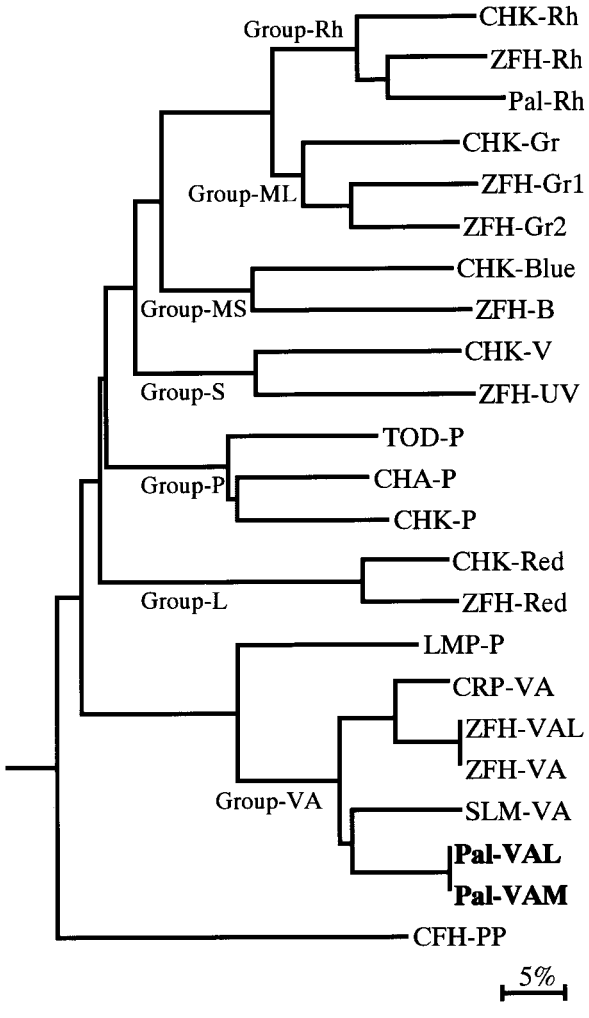

FIG. 2. A molecular phylogenetic tree of vertebrate opsins constructed by neighbor-joining method. *Drosophila* and octopus rhodopsins were used as outgroups. The scale bar indicates 5% replacement of an amino acid per site.

sequences were submitted to GenBank and their Accession Nos. are AB074482 (Pal-VAM) and AB074483 (Pal-VAL).

In the 3′-untranslated region, potential polyadenylation signals were found in both Pal-VAM and Pal-VAL. Consistent with previous reports on VA opsins (5–7), Pal-VAM and Pal-VAL retained the conserved features required for opsin function. Hydropathy plot of the sequence (data not shown) coincided with the typical seven-transmembrane structure as reported for bovine rhodopsin (18) and all other known opsins. The lysine residue (K293) located in putative transmembrane domain VII was a potential site of Schiff base linkage with the chromophore (19), and the glutamic acid (E114) in putative transmembrane domain III seemed to be a counter-ion of protonated Schiff base (20, 21). Cysteine residues (C111:C188) required for conformation of opsin proteins (22) and a probable palmitoylation site (C313) in the carboxyl terminal region were also conserved. Consistent with other non-visual opsins, a doublet of amino acids was lacking in the second extracellular loop (7). In addition, another doublet of amino acids was lacking in the third intracellular loop, similarly to previous observations in salmon VA opsin (5).

The predicted amino acid sequences of Pal-VAM and Pal-VAL showed high degrees of similarity with VA opsins of other fishes. The molecule most similar to these opsins was salmon VA, and both Pal-VAM and Pal-VAL showed the same level of identities with salmon VA, 81% at the amino acid level. A molecular phylogenetic tree among known vertebrate-type opsin proteins was constructed using invertebrate rhodopsins as outgroups (Fig. 2). As expected, Pal-VAM and Pal-VAL were grouped with other VA opsins reported previously. The placement of the vertebrate opsin groups agrees with phylogenetic trees reported previously (5, 23).

As described above, we isolated two isoforms of VA opsin, and found that the first 342 amino acids were identical between the two isoforms. From the long carboxyl terminus and the deduced amino acid sequence of this region, Pal-VAL clearly corresponded to zebrafish VAL and carp VA opsin. In contrast, Pal-VAM was a newly identified variant of the VA opsin group, and has a carboxyl terminus of usual length, that resembles the common vertebrate visual opsin (Fig. 1B). Another shorter variant corresponding to zebrafish VA and salmon VA was not detected.

Southern Blotting

A 221-bp DNA probe recognizing both Pal-VAL and Pal-VAM was used for Southern blotting analysis to

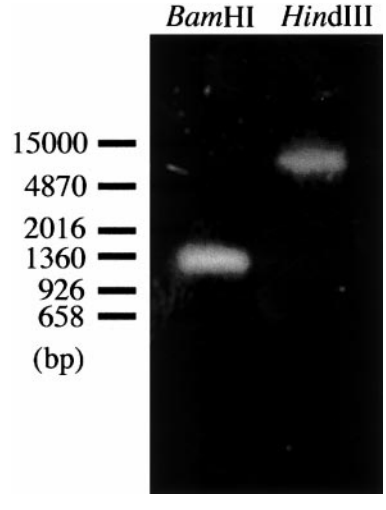

FIG. 3. Southern blotting analysis of the VA opsin gene of *P. altivelis*. Genomic DNA digested with *Bam*HI and *Hind*III was electrophoresed and hybridized with the 221-bp probe. The sizes of markers are indicated on the left.

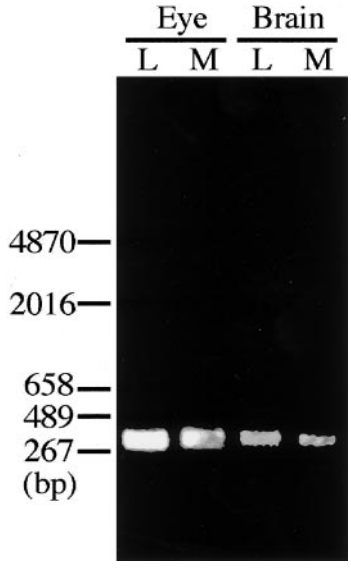

FIG. 4. RT-PCR of Pal-VAL (lane L) and Pal-VAM (lane M) mRNA in the eye and brain of *P. altivelis.* PCR products were separated on 1% agarose gel. The gel was stained with ethidium bromide. The sizes of markers are indicated on the left.

determine the copy number of the gene. Genomic DNA was digested with recognition enzymes, *Bam*HI and *Hind*III, and hybridized with the VA opsin probe. Southern blotting analysis revealed a single hybridization band in each lane (Fig. 3). These results indicated that the VA opsin gene of *P. altivelis* is present as a single copy.

RT-PCR

To investigate transcription of Pal-VAM and Pal-VAL in the eye and brain, RT-PCR analysis was performed using mRNA isolated from these tissues of the smelt. Amplification of fragments of about 300 bp was detected in both tissues (Fig. 4). The fragments were found to correspond to partial sequences of the Pal-VAM and Pal-VAL cDNA.

The detected bands were derived from cDNA and not from contaminating genomic DNA because we performed DNase treatment of the mRNA solution and no bands were detected when reverse transcription (RT) was omitted (data not shown). These observations indicated that Pal-VAM and Pal-VAL mRNA were expressed in the eye and brain.

In Situ Localization of VA Opsins in the Retina

We investigated the localization of Pal-VAM and Pal-VAL mRNA in the retina of the smelt fish. The hybridization signal was detected in a small fraction of amacrine cells in the inner nuclear layer (Fig. 5). No hybridization signals were found when sense probe was used as negative control. Salmon and zebrafish VA opsin were reported to be expressed in horizontal cells in addition to amacrine cells. However, no positive hybridization signals were detected in horizontal cells of the smelt fish.

DISCUSSION

We isolated two isoforms, Pal-VAM and Pal-VAL, of VA opsin from the retina of a smelt fish, *P. altivelis*, and found that the first 342 amino acids were identical between these two isoforms (Fig. 1). Pal-VAM was a newly identified variant of the VA opsin family, and Pal-VAL was a variant corresponding to the long isoform of VA opsin found in zebrafish and carp (6, 7). In this study, another variant corresponding to the short isoform found in salmon and zebrafish was not found (5, 6). Pal-VAM and Pal-VAL retained the conserved features required for opsin function, suggesting that these two isoforms encode functional opsin proteins.

Pal-VAM and Pal-VAL lacked of two doublets of amino acids in the second extracellular loop and the third intracellular loop region. All vertebrate type non-visual opsins except exo-rhodopsin lack this doublet in the second extracellular loop region (7), whereas they are present in all image-forming visual opsins of vertebrates (24). Further, invertebrate-type opsin proteins including melanopsin lack these two residues. Although the molecular phylogenetic tree (Fig. 2) showed that long wavelength-sensitive opsins (Group-L) separated prior to pinopsins (Group-P), this insertion or deletion of doublet suggested that visual opsin groups separated to each group after separation of Group-P. Among all vertebrate-type opsins characterized to date, only salmon VA (5), Pal-VAM and Pal-VAL show deletion of amino acids in the third intracellular loop region, which is considered to interact with the G protein transducin (25, 26). These deletions in salmon and smelt fish suggest that VA opsin lacked this doublet in the Salmoniformes lineage, and that these opsins use different phototransduction cascades from other known opsins. Pal-VAM had seven serine and threonine residues in its carboxyl terminal, whereas Pal-

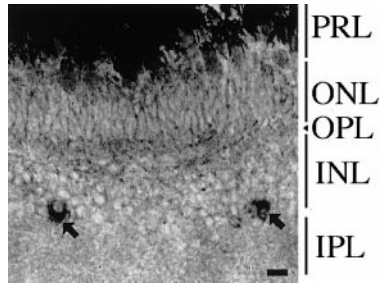

FIG. 5. *In situ* localization of Pal-VAM and Pal-VAL mRNA in *P. altivelis* retina. The hybridization signals (arrows) were located in a subset of amacrine cells in the inner nuclear layer (INL). PRL, photoreceptor layer; ONL, outer nuclear layer; OPL, outer plexiform layer; IPL, inner plexiform layer. Scale bar indicates 10 μm.

VAL had 13 residues. Phosphorylation of serines and threonines in the carboxyl terminal of rhodopsin is required for the termination of phototransduction (27, 28). Thus, Pal-VAL may allow rapid deactivation of the G protein-mediated cascade, whereas Pal-VAM may allow extended activation of the cascade.

Soni and Foster (5) first isolated salmon VA opsin, and this new opsin had a very short carboxyl terminus. Kojima *et al.* (6) reported that two VA opsin isoforms exist in the zebrafish retina. The shorter of the two, zebrafish VA opsin, is considered to be produced by intron retention at the splice site conserved among vertebrate opsin genes, whereas the longer variant, zebrafish VAL opsin, is spliced at this site. Based on the sequence comparison between the fourth intron of the carp VA opsin gene and the 3′-untranslated region of salmon VA opsin, Moutsaki (7) suggested that carp potentially have a short isoform of VA opsin. Southern blotting analysis was carried out using a 221-bp probe specific to the identical region of Pal-VAM and Pal-VAL. The results showed that only a single copy of the VA opsin gene exists in the genome of this smelt fish (Fig. 3), and indicated that these isoforms are derived from this single gene. However, the boundary between the common and isoform-specific region is not the conserved splice site. This suggested that the generation mechanism of the two isoforms in the smelt is different from that in zebrafish.

von Frisch (29) first showed the existence of a nonvisual photoreceptor, which regulates the body color of fish (European minnow), in the brain. Deep brain photoreceptors are known to regulate nonvisual photoresponses such as circadian rhythms and photoperiodic responses, and these responses have been characterized in many nonmammalian vertebrate species (30, 31). However, the photoreceptive molecules responsible for these phenomena have not to be identified. Salmon VA opsin and zebrafish VA and VAL opsin were reported to be expressed in both the eyes and brain (6, 8). As expected, RT-PCR indicated that Pal-VAM and Pal-VAL opsins were expressed in both the eyes and brain of the smelt (Fig. 4). Pal-VAM and Pal-VAL opsin are candidates for the molecule responsible for such nonvisual photoreception.

The results of *in situ* hybridization indicated that VA opsins were expressed in a subset of amacrine cells in the smelt retina (Fig. 5). In contrast to previous studies indicating expression of VA opsins in the horizontal cells (6, 8), the hybridization signals were not detected in the horizontal cells of the smelt. The reasons for this discrepancy remain unknown. The expression pattern might change with the time of the day, the growth stage of the fish or environmental photic conditions.

The physiological functions of nonvisual photoreceptors are as follows: entrainment of circadian rhythms, behavioral orientation, detection of seasonal changes in the photoperiod, regulation of body color, and regulation of serum hormone levels (4). As each role requires a distinct wavelength and distinct time of response, vertebrates would need to obtain photopigments with different characteristics and/or different phototransduction cascades. Pal-VAM and Pal-VAL might play different roles in some of these important functions, although they are derived from only a single-copy genes. Further studies of the photoreception in amacrine and other nonvisual cells and of the expression pattern of VA opsins are required.

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