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Identification of an olfactory imprinting-related gene in the lacustrine sockeye salmon, Oncorhynchus nerka

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Abstract

The olfactory system of salmonids is essential for imprinting and the subsequent homing migration. Olfactory imprinting-related genes were identified in the olfactory system of 1- and 3-year-old lacustrine sockeye salmon (Oncorhynchus nerka) using a subtractive hybridization technique of representational difference analysis (cDNA-RDA). We have obtained a partial clone from a subtractive cDNA library of 1-year-old fish that contains a 756 bp open reading frame coding for a putative protein with 252 amino acid residues named the Sockeye salmon Olfactory system Imprinting related Gene (SOIG). By northern hybridization, the SOIG mRNA was only expressed in the olfactory epithelium and not in other tissues. In situ hybridization showed that the expression of SOIG mRNA was observed in the olfactory receptor cells and basal cells of the olfactory epithelium. This suggests that SOIG could have specific and important roles in olfactory system.

Key words: Representational difference analysis, Olfactory epithelium, Smolt, Lacustrine sockeye salmon

1. Introduction

Anadromous salmonids are well known for accurate homing migrations guided by an imprinted memory of their natal stream. The olfactory hypothesis proposed by Hasler and Wisby (1951) that smolts memorize specific odors of the natal river during downstream migration is widely accepted. It has been suggested that smoltification of salmonids is controlled by various hormones, including growth hormone and thyroid hormone (Dickhoff et al., 1997). Thyroid hormones in particular are thought to be necessary for imprinting to natal river odors, because they are essential for facilitation of smoltification and down stream migration (Dittman and Quinn, 1996). Olfactory memory has been more thoroughly investigated in mammals, and it is generally thought to be formed by the long-term potentiation (LTP) in synapses (Martin et al., 2000). LTP has also been detected in the olfactory bulb of lacustrine sockeye salmon (Oncorhynchus nerka) at the smolt stage (Satou et al., 1996).

The cDNA representational difference analysis (cDNA-RDA) is a PCR based subtractive enrichment procedure, which has been adapted to enable the isolation of genes with an altered expression between various tissues or cells (Hubank and Schatz, 1994). This technique offers several advantages over other approaches for assessing gene expression, including a low number of false positives, the fact that unwanted

difference products can be competitively eliminated, and genes producing rare transcripts that may not be represented in the currently available database are also detectable.

Behavioral and electrophysiological studies have identified the important functions of the olfactory system (olfactory epithelium, olfactory nerve and olfactory bulb) in salmon (Dittman et al., 1996; Shoji et al., 2000). Recently, an odorant receptor (ASOR1; Atlantic salmon odorant receptor 1) has been first isolated from Atlantic salmon (Wickens et al., 2001). Besides, Dukes et al. (2004) reported the odorant receptor gene (SORB; salmon olfactory receptor B) expression changes during the parr-smolt transformation in salmon. However, the molecular basis of olfactory imprinting of salmon remains poorly understood.

In this study, we have tried to identify imprinting specific genes in the olfactory system of lacustrine sockeye salmon by using cDNA-RDA method.

2. Materials and methods

2.1. Animals

One and three-year-old lacustrine sockeye salmon reared at the Toya Lake Station, Faculty of Fisheries, Hokkaido University, were used. The fish were reared in 1400 L

circular tanks under the natural photoperiod with continuous flow of spring water (pH 6.0-6.7, conductivity 0.139-0.143 mS/cm, turbidity 0.0-0.7 NTV, dissolved oxygen 9.07-11.02 mg/L, and temperature 9.8-10.7˚C). Fish were raised on standard commercial pellets.

We sampled 1-year-old smolts fish (folk length 9.5-16 cm and body weight 8.1-37.6 g) on the 12-May. Three-year-old fish (sub-adults; folk length 15.2-20.4 cm and body weight 37.9-91.8 g) were also sampled on 20-June. In 1-year-old smolt fish, the body color was silver, while their fins were clear with intense black pigment. In salmonids, plasma thyroxine (T_4) increased during smoltification (Dickhoff et al., 1978; Yamauchi et al., 1984). T_4 concentrations of smolt fish used in this experiment were significantly higher than parr fish (data not shown). All the sub-adults sampled did not have smolt characteristics. Twenty fish were used from each age group. Fish were anesthetized with 0.005% eugenol (4-ally-2-methoxyphenol), and then olfactory epithelia, gill, liver, heart, head kidney, spleen, white muscle from dorsal region, the posterior region of the intestine, testis and brain were surgically isolated. Brains were dissected into small regions consisting of olfactory bulb, telencephalon, hypothalamus, optic tectum, cerebellum and medulla oblongata. Total RNA was isolated from each tissue using ISOGEN (Nippongene, Toyama, Japan) according to the manufacturer’s instruction.

2.2. Construction of a forward subtractive cDNA library

Subtractive cDNA libraries were constructed using 2 μg of total RNA extracted from the olfactory bulbs of the 1- (1^+) and 3 (3^+)-year-old fish (for each group, n=20). First and second strand of cDNA synthesis employed a SMART PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA).

Subtraction was performed in two directions. In the forward subtraction experiment the olfactory bulb of 1^+ fish was the tester, and in reverse subtraction, the tester consisted of 3^+ fish. The cDNA-RDA technique was performed according to the protocol of Niwa et al. (1997). Briefly, double stranded cDNA was digested with MboI and ligated to R-Bgl-12 and R-Bgl-24 at 16˚C for 16 h (For primer sequences see Table 1). Amplicons for both tester and driver were generated with PCR reactions using R-Bgl-24. MboI digestion was used to remove the R-adaptors from both tester and driver amplicons before tester was ligated to the J-Bgl-12/24. Subtractive hybridizations were performed at 67˚C for 20 h. To generate a first difference product (DP1), 0.4 μg of J-ligated tester was mixed with 40 μg of driver at a ratio of 1:100. DP1 was digested with MboI to remove J-adaptors before ligation of N-Bgl-12/24. To generate DP2, 50 ng of N-ligated tester was mixed with 40 μg of driver at a ratio of

1:800 (DP2). To generate a third difference product (DP3), 100 pg J-ligated DP2 was mixed with 40 µg at a ratio of 1:400,000. Finally, DP3 was digested with MboI and the forward subtraction product subcloned into the Bam HI site of the pBluescript II SK (+) vector (Stratagene, La Jolla, CA, USA). The libraries were plated onto agar plates (50 µg/ml ampicillin) and incubated at 37˚C for 14 h and then briefly at 4˚C to allow the blue/white staining to be clearly distinguishable.

2.3. Differential screening

Differential screening was carried out to identify the false positive clones in forward subtractive cDNA library. The protocol of developed by Robert et al. (2000) was followed in the screening process. Nine hundred sixty white colonies from the forward subtraction library were randomly picked. The insert of each colony was amplified by PCR using T7 and T3 primers. The amplification profile comprised of an initial cycle of 4 min at 94˚C followed by 30 cycles as follows: 94˚C for 30 sec (dissociation), 55˚C for 30 sec (annealing), 72˚C for 1 min (extension). A 2 µl aliquot of each amplification was dot-blotted on to a Hybond-N^+ membrane (Amersham Biosciences, Buckinghamshire, UK). Two identical membranes were used for all the PCR amplifications. DIG High Prime (Roche, Mannheim, Germany) was used for

generation of hybridization probes and for hybridization of membranes.

After the membranes were treated with denaturing solution (0.5 M NaOH, 1.5 M NaCl), neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl pH7.2, 0.001 M EDTA) and fixation solution (0.4 M NaOH), membranes were prehybridized for 4 h in a solution of 50% formamide, 5× standard saline citrate (SSC; 16.65 mM NaCl, 16.65 mM sodium citrate, pH7.0), 0.1% N-Lauroylsarcocine, 0.02% sodium dodecyl sulfate (SDS) and 1.0% blocking reagent (Roche) at 42˚C. Membranes were then hybridized in the same buffer at 42C for 16 h with forward and reverse subtraction products probes. Membranes were washed twice in 2× SSC, 0.1% SDS for 5 min followed by 0.1× SSC, 0.1% SDS for 15 min at 68˚C solutions. Membranes were incubated for 30 min with alkaline phosphatase-conjugated sheep anti-digoxigenin Fab fragments (Roche) at 37˚C. The dots were detected with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) reaction.

The clones that hybridized to the forward subtraction probe only were collected and purified. Sequence determination was performed by ABI PRISM ™ 377 DNA Sequencer (PerkinElmer Life Sciences, Tokyo, Japan) using the Big dye terminator RR mix (Cycle Sequencing Ready Reaction Kit, Applied Biosystems, Warrington, UK), and their sequences were submitted for a DNA Data Bank of Japan (DDBJ:

http://www.ddbj.nig.ac.jp).

2.4. Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Single-stranded cDNAs were synthesized from 2.5 μg of olfactory bulb total RNA from 1^+ and 3^+ fish using a Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. PCR amplification was performed with 0.5 μl of the first strand cDNA solution using primers for clone 1, 2, 3 and 4 (Table 2). The amplification profile comprised an initial cycle of 2 min at 94˚C followed by 35 cycles as follows: 94˚C for 1 min, 58˚C for 1 min, and 72˚C for 1 min. Amplification of ß-actin was also performed with the same first strand cDNA using primers ß-actin designed by reference in Atlantic salmon (accession number **AF012125**, Table 2). The amplification profile comprised an initiating cycle of 2 min at 94˚C followed by 35 cycles as follows: 94˚C for 30 sec, 57˚C for 30 sec, and 72˚C for 30 sec. The amplified products were electrophoresed on a 3% agarose gel (Nippongene).

2.5. Northern blot analysis

Total RNA (10 μg) was electrophoresed on a 1% formaldehyde agarose gel and

transferred to Hybond-N^+ membrane. The blot was air dried and baked at 60°C for 30 min. A RNA probe was synthesized employing a non-radioactive method using a DIG RNA Labeling Kit (Roche). A partial cDNA was subcloned into pBluescript II SK (+) vector, and then RNA probes were transcribed in vitro using T3 or T7 RNA polymerase.

Membranes were prehybridized for 2 h in a buffer of 50% formamide, 5× SSC, 0.1% N-Lauroylsarcocine, 0.02% SDS and 2% blocking reagent (Roche) at 50˚C. Membranes were then hybridized in the same buffer at 50˚C for 16 h with RNA probes (100 ng/ml). Membranes were washed under the same conditions as used for differential screening. The signals were detected with a CSPD (Roche) reaction.

2.6. Tissue preparation for in situ hybridization

Olfactory epithelia and olfactory bulbs of lacustrine sockeye salmon (1^+) were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2; PB), rinsed with 0.1 M PB, dehydrated in a graded series of ethanol, and embedded in paraffin (Histosec; Merck, Darmstadt, Germany). Serial sections were cut at 5 μm using a microtome and stained with Carazzi's hematoxylin and eosin counter-stain (Carazzi, 1911).

2.7. In situ hybridization

For in situ hybridization, DIG labeled single stranded sense and anti-sense RNA probes were synthesized from the same template clones as for generation of Northern blot probes using T3 or T7 RNA polymerase. Serial sections were cut at 5 μm using a microtome, and mounted on MAS-coated glass slides (Matsunami, Osaka, Japan). Serial section were deparaffinized with xylene. Deparaffinized sections were treated with proteinase K (10 μg/ml) at 37˚C for 15 min ; then hybridization was carried out at 55˚C for 16 h with hybridization buffer containing 500 ng/ml DIG-labelled RNA probe. The composition of buffer was 50% formamide, 17 mM Tris-HCl (pH 8.0), 2 mM EDTA, 1× Denhard's solution (0.02% BSA, 0.02% polyvinyl pyrrolidone, 0.02% Ficoll 400), 250 mM NaCl, 80 μg/ml yeast tRNA and 8% Dextran Sulfate. The sections were washed as follows: (1) 2× SSC/50% formamide at 50˚C for 30 min; (2) TNE (10 mM Tris-HCl pH7.5, 500 mM NaCl, 1 mM EDTA) at 37˚C for 10 min ; (3) RNase A treatment (20 μg/ml) at 37˚C for 30 min; (4) TNE at 37˚C for 10 min; (5) 2× SSC at 50˚C for 30 min; (6) two washes in 0.2× SSC at 50˚C for 20 min. For signal detection, samples were incubated with anti-DIG antibody coupled to alkaline phosphatase (Roche) at a 1:1,000 dilution at 4˚C for 16 h. The signals were detected with NBT and BCIP reaction.

2.8. Construction of cDNA library

The mRNA was extracted from olfactory epithelium total RNA of 1^+ fish using an Oligotex™-dT30 (Super) mRNA Purification kit (Takara, Shiga, Japan) according to the manufacturer's instruction. Five μg poly(A)$^+$ RNA was used to synthesize cDNA primed with an oligo(dT) adaptor primer using a cDNA synthesis kit (Stratagene, La Jolla, CA) according to the instructions supplied by the manufacture. The cDNA insert containing the EcoRI and XhoI linkers were then ligated into EcoRI/ XhoI sites of Uni-ZAP XR vector (Stratagene). Approximately 1.2×10^6 primaly clones were recovered with an average insert size of 2.0 kb.

2.9. Screening and cloning

Screening of the library was performed with DIG (Roche) labeled probes. After plaques were transferred onto Hybond-N$^+$ membranes, the membranes were treated with denaturing solution, neutralizing solution and fixation solution. The membrane were prehybridized for 2 h in a buffer of 50% formamide, 5× SSC, 0.1% N-Lauroylsarcocine, 0.02% SDS and 1.0% Blocking reagent (Roche) at 42˚C. Membranes were then hybridized in the same buffer at 42˚C for 16 h with DIG labeled probes (25 ng/ml). Membranes were washed under the same conditions as used for differential screening.

Positive plaques were piked up to SM buffer and in vivo excision was performed using ZAP-cDNA Gigapack III Gold Cloning Kit (stratagene) according to the manufacturer's instructions. The obtained sequences were searched against 'Fasta Sequence Similarity search' (DDBJ) and motif database (PROSITE: http://br.expasy.org/prosite/).

3. Result

3.1. 1^+ specific cDNAs

We obtained four clones that hybridized to the forward subtraction probe from differential screening. These sequences were compared with those in the DDBJ (Table 3). Of the four sequences, the insert in clone 1 is 98% similar to the rainbow trout (O. mykiss) TAT-binding protein-1, and 3 shows 67% similarity to the gene for the frog (Xenopus laevis) glutamate carboxypeptidase-like protein. For the other two sequences, the insert in clone 2 is 60% similar to the mouse (Mus musculus) gene, and 4 shows 82% similarity to the gene for the zebra fish (Danio rerio), but the function of either gene is unclear.

3.2. Semi-quantitative RT-PCR analysis

Semi-quantitative RT-PCR was performed with the aim of finding a difference in the

expression levels of mRNA between olfactory bulb of 1^+ and 3^+ fish. No differences in the expression levels of clones 1, 3 and 4 were detected in the olfactory bulbs of the two age-classes of fish, but clone 2 was expressed only in the olfactory bulb of 1^+ (Fig. 1). We named this partial clone 2 "Sockeye salmon Olfactory system Imprinting related Gene" (SOIG).

3.3. Northern blot analysis for SOIG mRNA expression

Expression of SOIG mRNA in the brain and body tissues (telencephalon, hypothalamus, optic tectum, cerebellum, medulla oblongata, olfactory epithelium, gill, liver, heart, head kidney, spleen, muscle, intestine and testis) from 1^+ lacustrine sockeye salmon was analyzed by northern blotting (Fig. 2). The SOIG probe to 1.7 kb transcript in the olfactory epithelia. No signal was detected by northern blot analysis in other tissues.

3.4. Localisation of SOIG mRNA in the olfactory system

To determine the distribution of SOIG mRNA expression in the olfactory system (olfactory epithelium and olfactory bulb), we performed in situ hybridization using DIG-labelled sense and anti-sense RNA probes (Fig. 3). The signals for SOIG were

observed mainly in the olfactory receptor cells and basal cells in the olfactory epithelium of 1^+ lacustrine sockeye salmon. In addition, the hybridization with labeled sense control probe showed no detectable hybridized signals in adjacent sections. On the other hand, in the olfactory bulb, no SOIG probe was detected (data not shown).

3.5. cDNA cloning and characterization of SOIG

To isolate full length SOIG cDNA, we constructed a cDNA library from 1^+ lacustrine sockeye salmon olfactory epithelium. A cDNA library was screened using the cDNA probe, which was synthesized from the same template clones as for generation of northern blot probes. The screening of approximately 1.2×10^6 plaques from the cDNA library yielded positive clones for SOIG, and the clone was subjected to sequence analysis.

SOIG cDNA was 1700 bp in length, having an open reading frame of 759 bp encoding 252 amino acids. A putative polyadenylation signal was located 19 and 29 nucleotides upstream of the poly (A) tail. The first ATG codon was located at 7 nucleotides. The partial SOIG cDNA isolated from forward subtraction cDNA library was located at nucleotide 318 to 487 of the full length cDNA (Fig. 4). The deduced amino acid sequence had a calculated molecular mass of 27 kDa and contained a

lymphocyte antigen-6 (Ly-6) domain that consists of 10 conserved cysteine residues with the characteristic spacing pattern (positions 153-223). Motif search showed that Ly-6 superfamily motif CCXXXXXCN was included in putative amino acid residues of SOIG (positions 134-141, 212-219). Fasta search revealed SOIG identity 31.8 and 30.3% similarity, respectively, with the amino acid sequence of u-PAR from cattle (Kraetzschmar et al., 1993) and monkeys (Engelholm and Behrendt, 2001) (Fig. 5).

4. Discussion

In the present study, we isolated four partial cDNAs from a forward subtractive cDNA library and found that clone 2 (SOIG) was expressed only in the olfactory bulb of 1^+ lacustrine sockeye salmon. The predicted open reading frame of SOIG encodes a protein of 252 amino acids and displayed low amino acid sequence similarity with u-PAR. SOIG has a Ly-6 superfamily domain that consists of 10 conserved cysteine residues and Ly-6 superfamily motif. The u-PAR being a member of Ly-6 superfamily, has an important role in proteolysis of extracellular matrix proteins (Tarui et al., 2001). The u-PAR is a heavily glycosylated signal-chain protein with a molecular weight of 50-60kDa (Roldan et al., 1990), and organized into three Ly-6 superfamily domains, differently folded domains of approximately 90 amino acids, with four to five

disulphide bonds (Ragno, 2006). Ly-6 superfamily members have been identified in several different organisms, including human and mouse (McKenzie et al., 1977; Bickmore et al., 1993; Suh et al., 1994). Although Joseph et al. (2001) reported that Ly-6-related protein is associated to olfaction in C elegans, the detailed biological function of the Ly-6 superfamily members, except for u-PAR, is not known. Ly-6 superfamily has a unique structure domain, showing 8-10 conserved cysteine residues with a characteristic spacing pattern and shares the consensus sequence motif CCXXXXCN at the carboxy-terminal end (Palfree, 1996). SOIG has single Ly-6 superfamily domain with 10 conserved cysteine residues and calculated molecular weight of 27 kDa, but contained Ly-6 superfamily motif. Thus, SOIG may be a novel member of the Ly-6 superfamily other than uPAR, or a distant relative of this suprefamily that shares similar structure.

Northern blot analysis and in situ hybridization revealed that SOIG mRNA was expressed in the olfactory receptor cells of the olfactory epithelium (Fig 2, 3). Recently, several olfactory-specific proteins have been identified, including olfactory marker protein (Margolis, 1972; Kott et al., 1992) and salmonid olfactory system-specific protein (N24) (Shimizu et al., 1993). An olfactory marker protein (OMP) has been isolated from olfactory bulbs of mice and rats (Margolis, 1972; Keller

and Margolis, 1975, 1976). OMP is expressed almost exclusive in mature olfactory receptor cells and used as a molecular marker for recognizing theses cells in mammals (Johnson et al., 1993; Levai and Strotmann, 2003). Electrophysiological studies using OMP knockout mice suggest that OMP contributes to olfactory sensitivity (Buiakova et al., 1996; Ivic et al., 2000; Youngentob et al., 2001). In teleost, olfactory receptor cells of rainbow trout showed immunoreactivities of the OMP using anti-mammalian OMP sera (Riddle and Oakley, 1992). However, there is few report on the relation between olfaction and OMP in salmonid. The salmonid olfactory system-specific protein, N24, was also identified in the lacustrine sockeye salmon (Shimizu et al., 1993), and was observed in the axons of olfactory receptor cells (Kudo et al., 1996; Yanagi et al., 2004). Although the detailed function of N24 is still unknown, N24 possesses glutathione S-transferase class pi-like structure (Kudo et al., 1999). Moreover, Shimizu et al. (1993) reported that N24 was higher in fish in the natal river than those in seawater at both the period of imprinting to the natal river and during homing to the natal river. The present study demonstrated that SOIG mRNA was expressed in the olfactory receptor cells of the olfactory epithelium. No SOIG mRNA signal was detected in other tissues or organs. These finding suggest that SOIG may play a particular role in the olfaction or imprinting function of the lacustrine sockeye salmon.

Thyroxine (T_4) surge is observed during smoltification (Dickhoff et al., 1982; Yamauchi et al., 1984; Dickhoff and Sullivan, 1987). The period for olfactory imprinting in salmon is closely connected with changes in T_4 that occur during smoltification (Hasler and Scholz, 1983). Dittman et al. (1996) reported that imprinted memory is formed in the during smolt stage. One can deduce from these reports that the imprinting function of salmonids may be related to thyroid hormone. The development and turnover of olfactory receptor neurons in the olfactory epithelium occurs by proliferation of multipotent basal stem cells (Huard et al., 1998; Jang et al., 2003). Proliferation of these cells stimulated by thyroid hormones (Mackay-Sim and Beard, 1987; Paternostro and Meisami, 1989, 1994). In teleosts, the proliferation of basal cells in the olfactory epithelium are induced by thyroid hormone in salmon during smolt stage (Sean et al., 2004). We found that SOIG mRNA was expressed in the basal cells of olfactory epithelium in smolts, suggesting that SOIG expression may be related to the proliferation of basal cells.

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Table 1. Sequence of primers used for cDNA-RDA

Primer	Sequences
R-Bgl-12	5´-GATCTGCGGTGA-3´
R-Bgl-24	5´-AGCACTCTCCAGCCTCTCACCGCA-3´
J-Bgl-12	5´-GATCTGTTCATG-3´
J-Bgl-24	5´-ACCGACGTCGACTATCCATGAACA-3´
N-Bgl-12	5´-GATCTTCCCTCG-3´
N-Bgl-24	5´-AGGCAACTGTGCTATCCGAGGGAA-3´

Table 2. Sequences of primers used for Semi-quantitative RT-PCR. S, Sense primer; AS, Anti-sense primer.

Primer	Sequences
1-S	5´-ACAGCTGGTCCAGATGTTCA-3´
1-AS	5´-GCCTTCCATGTAGTCCTCAT-3´
2-S	5´-ACACTCAAGTCCATTGTGGG-3´
2-AS	5´-GGACGACCATTTTTGTCAGTC-3´
3-S	5´-TTGGCGTTACCCTACCGTTA-3´
3-AS	5´-ACTGATCATGGTCACCTTCAG-3´
4-S	5´-ATCCGTCGTATCATGAGACC-3´
4-AS	5´-TTGAGGAGCTTGCTGACCACAT-3´
β-actin-S	5´-TGTAACATGGGCAGTATCCTT-3´
β-actin-AS	5´-GATGTCGGAACACATGTGCACT-3´

1

Table 3. Genes obtained from forward subtractive cDNA library using olfactory bulbs of 1^{+} lacustrine sockeye salmon.

Clone No.	Size of fragment	Identity	Gene name	Accession No.	Species
1	204 bp	98%	TAT-binding protein-1 mRNA, partial cds.	**AF281342**	Oncorhynchus mykiss
2	170 bp	60%	DNA sequence from clone RP23-17616 on chromosome 11	**AL596212**	Mus musculus
3	397 bp	67%	Glutamate carboxypeptidase-like protein 1 mRNA, partial cds.	**AY188285**	Xenopus laevis
4	167 bp	82%	cDNA clone MGC: 64169IMAGE: 6797400, complete cds.	**BC053296**	Danio rerio

Figure captions

Fig. 1. Results of 3% agarose gel electrophoresis of semi-quantitative RT-PCR products. 1, 2, 3 and 4 indicates clone number. Specific products for clone 1, 2, 3, 4 and ß-actin mRNAs were amplified from aliquots of the same mRNAs of the olfactory bulb of 1-year-old (1^+) and 3-year-old (3^+) fish. RT-PCR was performed using pooled samples (20 fish per each group).

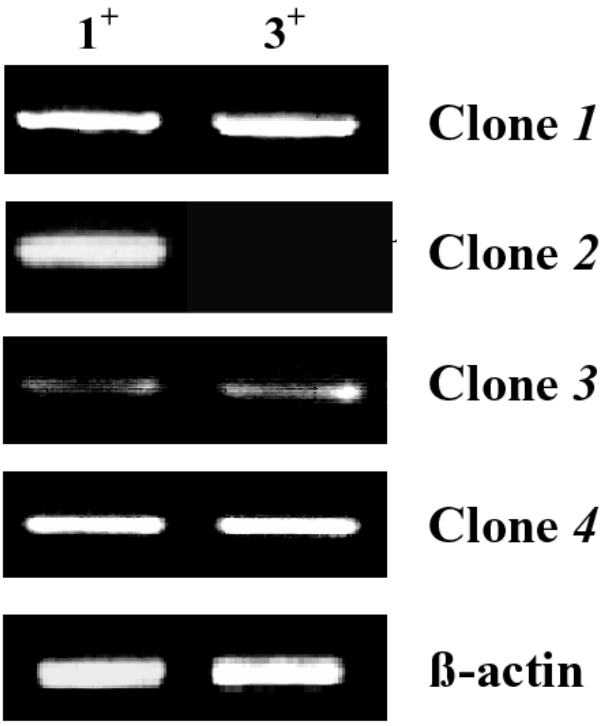
Fig. 2. Northern blot analysis of 1^+ lacustrine sockeye salmon SOIG mRNA in the brain tissues (A) and body tissues (B). Lane A-1, telencephalon; lane A-2, hypothalamus; lane A-3, optic tectum; lane A-4, cerebellum; lane A-5, medulla oblongata; A-6, olfactory epithelium; lane B-1, olfactory epithelium; lane B-2, gill; lane B-3, liver; lane B-4, heart; lane B-5, head kidney; lane B-6, spleen; lane B-7, white muscle; lane B-8, intestine; and lane B-9, testis. Total RNA (10μg) isolated from brain and body tissues was blotted to nylon membranes. The positions of SOIG transcripts are indicated by arrows. The relative positions of 28S and 18S ribosomal RNA are indicated. Northern blot analysis was performed using pooled samples from 5 fish.

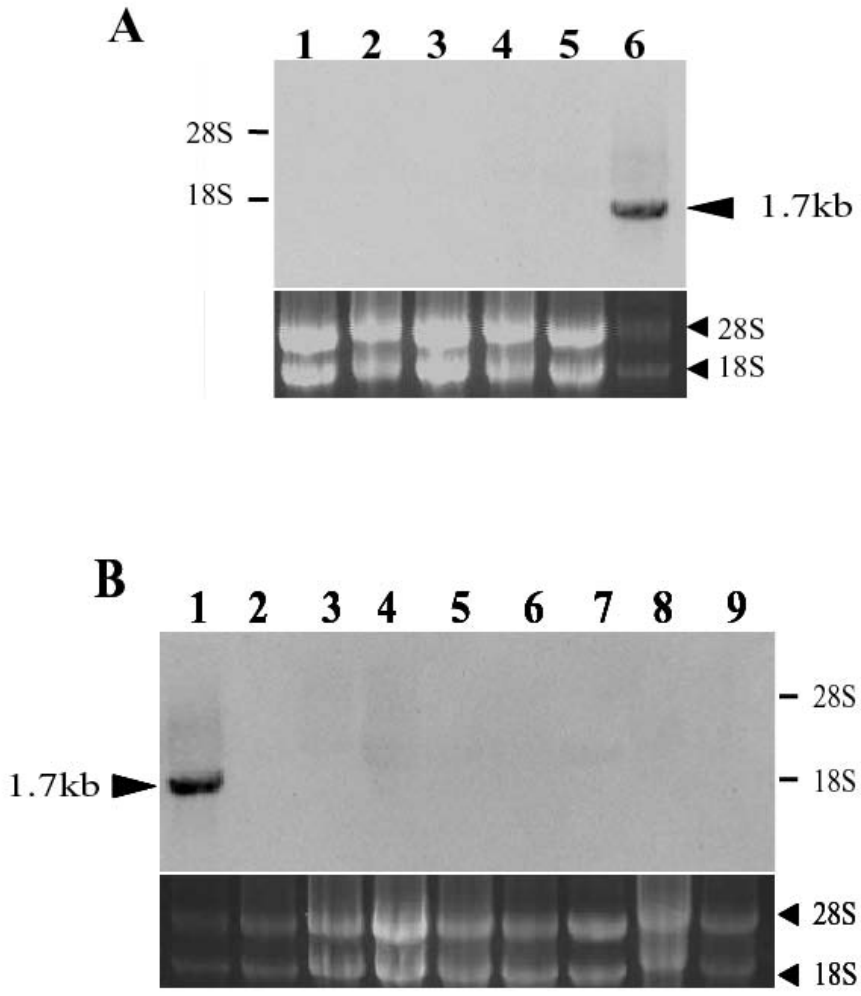
Fig. 3. Expression of SOIG mRNA demonstrated by in situ hybridization in the olfactory epithelium of lacustrine sockeye salmon. (A) Adjacent sections were stained with Carazzi's hematoxylin and eosin counter-stain. (B) Section hybridized with antisense probes. (C) Section hybridized with sense probes as a negative control. Olfactory receptor cell and basal cell are indicated a and b. Scale bar: 100 μm.

Fig. 4. Nucleotide sequence and deduced amino acid sequence of full length SOIG cDNA. The stop codon (ATG) and polyadenylation signal (AATAAA) are indicated by an asterisk and thick underlines. The nucleotide sequence of partial SOIG cDNA isolated from forward subtractive cDNA library are shaded.

Fig. 5. Amino acid sequence comparison of SOIG, cattle uPAR (accession number **L03545)** and monkey uPAR (accession number **AF302074)**. This alignment was performed using CLUSTAL W (Thompson et al., 1994). Conserved cysteine residues are shown in bold letters. Identical amino acids are indicated by asterisks. Dash represents a gap in the sequence introduced to maximize alignment. The CCXXXXCN motif is shaded. The three positions of LY-6 superfamily domains in cattle and monkey uPAR are indicated by dot lines (DI-DIII).

501

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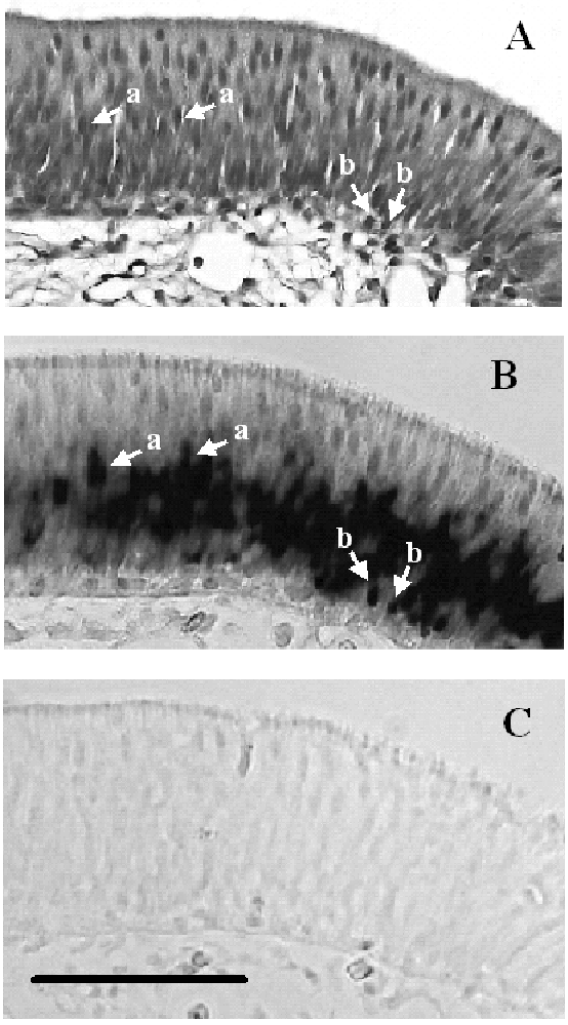
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C

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  91  TGTAAAGATCTGGCAGACACCCACTGCTTAGAACAAACCCTGGAGACCTGCAGTGATGGACAAGTGTGTTCCACAAACTCCAGC
       C  K  D  L  A  D  T  H  C  L  E  Q  T  L  E  T  C  S  D  G  Q  V  C  S  T  N  S  S   56

 175  AGCTCTCTGCGATCCTCCGTAGTTGTTAATGACAGAGGACGCATCCATTTTAGCATTTGGGACGTTGACATTGATATCCGCTGG
       S  S  L  R  S  S  V  V  V  N  D  R  G  R  I  H  F  S  I  W  D  V  D  I  D  I  R  W   84

 259  GGAAAAGACCGTACAGCAACCAACCCCAACGTTGTTTCAAACTCAAGTATCAATGAGAGGATCGTGAAGGGATGCATGGACGAC
       G  K  D  R  T  A  T  N  P  N  V  V  S  N  S  S  I  N  E  R  I  V  K  G  C  M  D  D  112

 343  CATTTTTGTCAGTCAAGCCCCAAAATGTCTTTGAATATCGGTTTCCATCAATTCACTTCTTCGTGCTGCAACTCATCTGGCTGC
       H  F  C  Q  S  S  P  K  M  S  L  N  I  G  F  H  Q  F  T  S  S  C  C  N  S  S  G  C  140

 427  AACAGTGACACTTTCTCTGACGACCCCCACAATGGACTTGAGTGTTTTTCTTGCACTGATCCAGAAGATGAGGTTTGTAATCAG
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 595  TGTGTCTCCAGGAGTATGTGTGGTTCCGGTTCTGGTTCTGTGTCCTGCTGTGAGGGAAGTCTCTGCAACAGAAACAGGGCTATG
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 679  AATTCCGCTCAGCCTGTAGCACTTACAGTCCTCACTCTGCTAGTTGGGATAACAACCACCACACTTCTACAATCAGTCGATCAA
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 763  TGAATATCTTGATTGGGGTTTCTTGAGGATTTGCATATTTTTCTCATGAGGATTAGTTTTGTGGGCATCTACTTCTGGGGCTTT
       *

 847  CACACTCTGTATTGTCCAAAGTGTAACGACCTTGTCACTCCCTGACCCACTAGAGTACTGTGGGATCTGTTGCTTGTTGCTCAT
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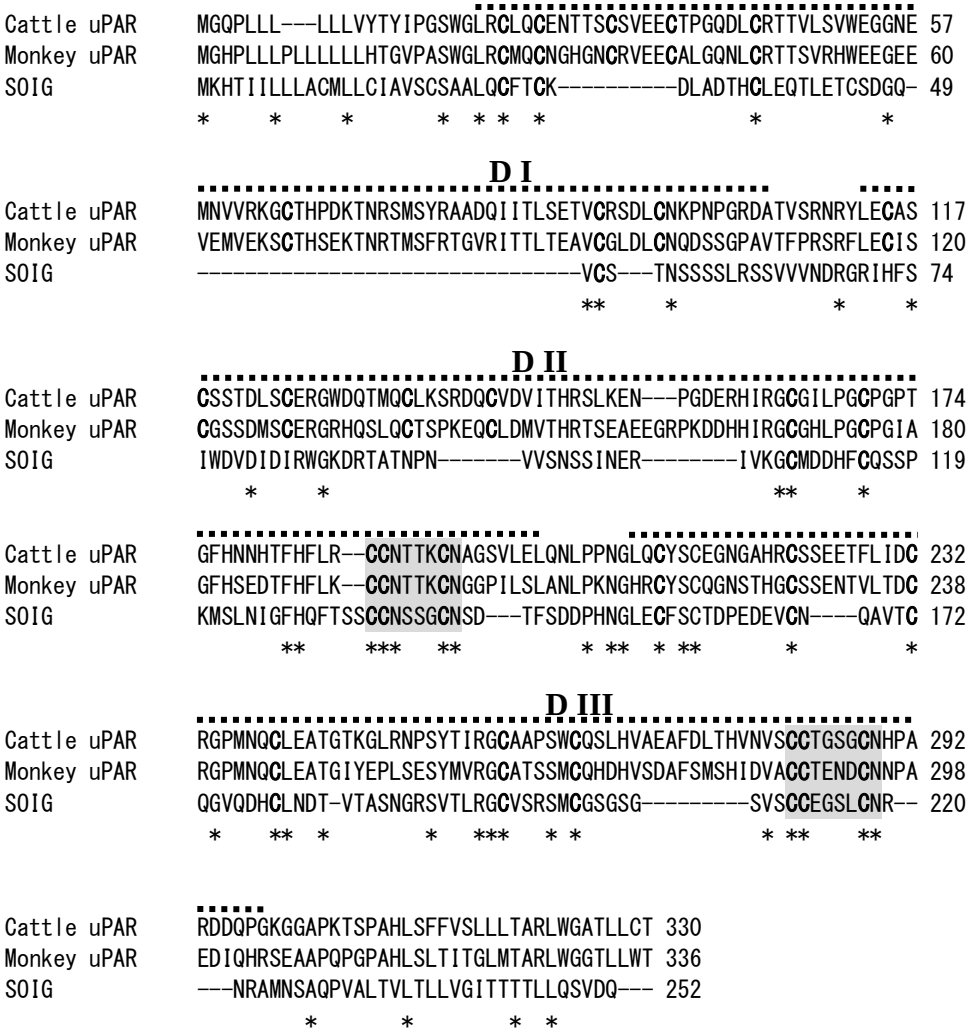
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