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Random Expression of Main and Vomeronasal Olfactory Receptor Genes in Immature and Mature Olfactory Epithelia of *Fugu rubripes*

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Main olfactory receptor genes were isolated from a seawater fish, *Fugu rubripes* (pufferfish), and characterized. Two subfamilies of genes encoding seven transmembrane receptors were identified; one consists of five or more members, termed FOR1-1 to 5 of FOR1 subfamily, and the other appears to be a single copy gene, termed the FOR2 subfamily. FOR1 members show extremely high amino acid sequence similarities of about 95% to one another, and are distantly related to catfish-1 with the highest similarity of 37%. FOR2 shows 43% similarity to goldfish-A28. Phylogenically, both FOR members are categorized among pedigrees of the fish main olfactory receptor family outside the mammalian receptor family, although similarities between *Fugu* receptors and those of fresh-water fishes are lower than those among fresh-water fishes. *In situ* hybridization shows that both subfamilies of receptor genes are expressed randomly over the olfactory epithelium throughout all developmental stages, and no segregation of the signals was found. On the other hand, when three members of a vomeronasal olfactory receptor gene family, related to the Ca^{2+}-sensing receptor, were used as probes, they were also randomly expressed over the same epithelium as the main olfactory receptors. This is in contrast to the expression profiles observed for zebrafish and goldfish, where the main or vomeronasal olfactory receptors are expressed in segregated patterns. It is thus suggested that the expression pattern of fish olfactory receptors varies depending on the species, although fish olfactory receptors are highly related to one another in their primary structures, and are phylogenically distinct from those of mammals.

Key words: *Fugu*, olfactory receptor.

The olfactory systems of vertebrates are capable of recognizing and discriminating among a large number of different odorants in the environment. Olfactory transduction is initiated by the interaction of odorants with specific receptors of the olfactory sensory neurons in the olfactory epithelium; an odorant binds to an olfactory receptor(s) (OR), leading to a rapid and transient elevation in the intracellular second messenger, cyclic AMP (*1–3*).

ORs have the characteristic seven transmembrane domain structure of the superfamily of G protein–coupled receptors (*1–4*). The repertoire of OR genes expressed in ciliated receptor cells of the main olfactory epithelium (MOR genes) is extremely large, and previous studies have suggested that the gene family consists of about 1,000 members in mammals (*4*) and about 100 members in fishes (*5*). Other OR genes encoding seven transmembrane receptors not related to MORs have also been shown to be expressed in microvillous receptor cells in vomeronasal organs of mammals (*1, 3*), and can be divided into two distinct families, one related to the Ca^{2+}-sensing receptor, termed VRs or G_o-VNs, and the other termed VNRs or G_{i2}-VNs (*1, 6–9*). In fishes, both ciliated and microvillous receptor cells are observed together on the same olfactory epithelium (*10, 11*). Recently, two interesting observations have been reported. First, genes encoding mammalian VR homologs were identified in the *Fugu* genome and shown to be expressed in the olfactory organ by RT-PCR (*12*). Second, in goldfish, MORs and VRs are both expressed in the same olfactory epithelium, but the regions of MOR- and VR-positive cells are segregated (*13*), suggesting that a single organ in the olfactory epithelium of fishes functions in both main and vomeronasal olfactory reception.

The characterization of OR gene families has revealed many features of olfactory systems, leading to analyses of the olfactory coding mechanisms (*4, 6, 7, 12–17*). Especially, RNA *in situ* hybridization experiments have revealed the spatial patterns of OR expression in the olfactory epithe-

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Abbreviations MOR, main olfactory receptor; OR, olfactory receptor; ORF, open reading frame, PBS, phosphate buffer saline, PBT, PBS containing 0.1% Tween 20; PCR, polymerase chain reaction, RT-PCR, reverse transcription–polymerase chain reaction, VR, vomeronasal olfactory receptor related to Ca^{2+}-sensing receptor

lium and provide important information about olfactory reception and coding. The frequency of expression of each MOR gene suggests that, as a general rule, olfactory neurons individually express only one MOR gene out of numerous genes (*4*). Thus, each cell has a functional identity based on the MOR gene it expresses (*15*), and the brain may discriminate among many different odorants by determining which neurons have been activated. Recently, a spectrum of ligand specificities connecting multiple MORs with multiple ligands has been elucidated in rats (*18*). However, the mechanism by which OR gene expression is regulated remains to be revealed.

To seek new insight into these problems, we adopted a pufferfish, *Fugu rubripes* (*Fugu*), as a study material. Molecular analyses of OR genes from *Fugu* are very attractive for the following reasons. First, the *Fugu* genome is approximately 400 Mb, about 7.5 times smaller than that of human, while the number of *Fugu* genes is expected to be similar to that in humans (*19*); thus, the sequence analyses of OR genes may be easier than for mammalian genes and provide much information about vertebrate olfactory systems. Second, fishes are thought to respond to fewer kinds of odorants than terrestrial vertebrates (*11*), and possess a more limited OR repertoire (*5*). Therefore, fish olfactory systems probably show anatomical, cellular and molecular simplicity. Third, the olfactory organs of fishes exhibit great variety in their morphological characteristics (*11*) as a result of evolution and adaptation to habitat, and advanced studies of OR expression comparing different fish species would help in understanding the constitutive features of vertebrate olfactory systems. *Fugu* is a seawater fish belonging to an order (*Tetradontiformes*) quite different from those of the fresh-water fishes, catfish (*Ictalurus punctatus*, belonging to *Siluriformes*) (*5*, *20*), zebrafish (*Danio rerio*, belonging to *Cypriniformes*) (*14*, *15*, *21–23*), goldfish (*Carassius auratus*, belonging to *Cypriniformes*) (*13*), and medaka fish (*Oryzias latipes*, belonging to *Atheriniformes*) (*24*, *25*), whose MOR genes have been analyzed so far.

In this study, we characterized genes encoding two subfamilies of MORs in *Fugu*. These genes code for seven transmembrane receptors sharing about 40% similarity with catfish, zebrafish and goldfish MORs. One of the two MOR subfamilies, termed FOR1, contains at least 5 members that share extremely high amino acid sequence similarities of about 95% and are clustered in the *Fugu* genome; the other subfamily, termed FOR2, represents a single copy gene in the genome. We have performed *in situ* hybridization experiments and shown that each MOR gene is expressed in a small subset of olfactory sensory neurons randomly distributed on the olfactory epithelium. In addition, *Fugu* VR homologs are also randomly expressed over the epithelium in a manner similar to MORs, and no segregation of the signals between MORs and VRs was found.

MATERIALS AND METHODS

Fugu Genomic DNA—Genomic DNA of *F. rubripes* was prepared from the blood of male adult fishes by a usual method (*26*).

Isolation and Characterization of Fugu Olfactory Receptor Sequences—Genomic DNA encoding parts of the *Fugu* main olfactory receptors were amplified from the genomic DNA by PCR using degenerate primers corresponding to conserved regions of MORs from catfish and zebrafish (*5*, *14*). The 5′ primers were primer A, 5′-CC(CT)A(AT)GTA-CCT(CG)GC(CT)GT(CG)TTCAACCT-3′; and primer B, 5′-GC(CT)ATCTGCCT(CG)CC(CT)CT(CG)AG(AG)TACCA-3′, corresponding to the amino acid sequences, P(KM)Y(IL)-AVFNL and AICLPLRY(HQ), respectively. The 3′ primer was primer C, 5′-TAGATGAT(AG)GGGTT(CG)AGCAT(A-G)GG(GATC)GG-3′, corresponding to the amino acid sequence, PPMLNPIIY. PCR was performed under the following conditions: 95°C for 45 s, 60°C for 2 min, and 72°C for 2 min (40 cycles). The reaction mixture was then analyzed on a 1.2% agarose gel and DNA fragments with the expected lengths were recovered from the gel. The DNA fragments were used as templates for a second PCR under the same conditions, and the reaction products were treated with the Klenow fragment of DNA polymerase I and T4 polynucleotide kinase, subcloned into pUC18, and subjected to DNA sequencing.

Southern Blot Analysis—*Fugu* genomic DNA was digested with *Eco*RV, *Hin*dIII, *Pvu*II, and *Sac*I, electrophoresed in 1% agarose gels, and blotted onto Hybond-N membranes (Amersham). The membranes were hybridized with ^{32}P-labeled DNA probes under high stringency conditions at 65°C in hybridization buffer containing 6× SSPE, 5× Denhardt's solution, 0.5% SDS and 100 μg/ml denaturated salmon sperm DNA. The final wash was carried out at 65°C in 0.1× SSC containing 0.1% SDS. The probes used were DNA inserts of PCR clones amplified with primers A and C for the FOR1 probe and amplified with primers B and C for the FOR2 probe.

Isolation of Genomic Clones—A *Fugu* genomic library amplified from $>1 \times 10^6$ pfu independent clones were purchased from Clontech Laboratories Inc. and about 1×10^5 pfu of recombinant phage clones corresponding to approximately 4 times the *Fugu* haploid genome were screened under high stringency conditions at 65°C with each of the same ^{32}P-labeled probes as described above. The inserts of positive phages were subcloned into pUC18, and subjected to DNA sequencing. As a result, ten clones for FOR1 and one clone for FOR2 were obtained and subjected to further analyses. Linkage of the FOR1-positive clones was first determined as shown in Fig. 2. The FOR2-positive clone was digested with *Sal*I into two fragments of 3.2 and 15 kb, and only the shorter fragment of 3.2 kb hybridized with the FOR2 probe was used for further analyses. DNA regions containing ORFs of FOR1 and FOR2 subfamily genes were sequenced.

Isolation of Genomic DNA Fragments Encoding VN-ORs—Among many *Fugu* VRs reported (*12*), three representative members belonging to three major pedigrees of the phylogenic tree were isolated. The primers used were as follows: AAGATCACAGCCAGTGCGTT and GCTCTTT-GGCATGAGCGGTA for fCa12 of type II, TAGCTGTGT-TCTCAGTCGGT and TCGTTGAAATTATCGGGGAG for fCa02.1 of type III, and CTGGGTATCTGTCTGACAGC and AGATTCCGTGCAAGAAATGC for fCa09 of type IV (*12*). These primers correspond roughly to the C-terminal regions of the receptors lacking introns, and the expected lengths of PCR amplification were 400–600 bp. PCR fragments amplified using the genomic DNA were cloned into pBS-SK and confirmed by sequencing.

In Situ Hybridization of Olfactory Epithelium Using Cryosections—Adult fishes (about 30 cm in length) were used.

PCR fragments of three VN-ORs and full length of ORFs of FOR1 subfamily members and FOR2 were cloned into pBS-SK vectors and the digoxigenin-UTP-labeled antisense RNA probes were synthesized by T3 or T7 RNA polymerase. Sense probes for each gene were also synthesized and used as controls, but none of them gave specific signals (data not shown).

In situ hybridization was performed as described previously (*25*, *27*, *28*). Briefly, the olfactory epithelium was removed, frozen in O.C.T. compound, and sectioned into 9 μm slices. The sections were post-fixed for 10 min in 4% paraformaldehyde in PBS, pH 7.0, treated with 0.1% diethylpyrocarbonate in PBS two times for 15 min, washed in 5× SSC for 15 min, and prehybridized in prehybridization solution of 50% formamide and 5× SSC containing 40 μg/ml of denatured herring sperm DNA for 2 h at 58°C. Then, 0.2 ml of hybridization buffer containing 50% formamide, 5× SSC, 5× Denhardt's solution, 500 μg/ml sonicated, denatured herring sperm DNA, 250 μg/ml torula tRNA, 1 mM DTT, and digoxigenin-labeled antisense probes for *Fugu* ORs at about 50 ng/ml was added to the slide. The slide was covered with a siliconized coverslip and hybridized overnight at 58°C. The coverslips were removed by soaking in 5× SSC at 58°C, and the slides were washed two times in 0.2× SSC for 30 min at 58°C. The sections were washed in TBS for 5 min, blocked in 0.5% blocking reagent (Boehringer) in TBS for 1 h at room temperature, and incubated in the same solution containing a 1:1,000 dilution of anti-digoxigenin Fab fragments conjugated with alkaline phosphatase for 1 h. Then the slides were washed three times in TBS for 15 min and once in alkaline phosphatase buffer containing 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 50 mM $MgCl_2$ for 5 min. The color reaction was performed by adding a color developing solution containing nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in alkaline phosphatase buffer onto the slide.

Whole Mount In Situ Hybridization—For whole mount *in situ* hybridization, the same probes as for the cryosections described above were used. *In situ* hybridization was carried out essentially as described previously (*29*). In brief, juvenile *Fugu* fish (just before hatching to 4 weeks after hatching) and the heads of young fish (6–7 months) with olfactory rosettes were fixed in 4% paraformaldehyde in PBS overnight, washed, and soaked in 70% ethanol at –20°C until use. The fish and rosettes excised from the heads were rehydrated in a series of graded methanol concentrations, and finally in PBT (PBS containing 0.1% Tween 20). The samples were treated with 10 μg/ml proteinase K in PBT for 4 min at room temperature, re-fixed in 4% paraformaldehyde in PBS for 20 min, and washed 4 times for 5 min in PBT. The samples were then prehybridized in hybridization buffer containing 50% formamide, 5× SSC, 500 μg/ml tRNA, 50 μg/ml heparin, 0.1% Tween 20, pH 6.0, adjusted with 1 M citric acid, for 4 h at 65°C. After prehybridization, the solution was replaced with hybridization buffer containing digoxigenin-labeled antisense RNA probes for *Fugu* ORs at about 100 ng/ml. After hybridization for 20–44 h, the samples were washed as described and immunoreacted with anti-digoxigenin Fab fragments conjugated with alkaline phosphatase. The signals were visualized as described above. Whole-mount samples from the *in situ* reaction were dehydrated through a series of graded methanol concentrations and xylene and embedded in Paraplast plus (Sigma Co.). Then, 9 μm paraffin sections were prepared.

RESULTS

Isolation and Analysis of Fugu Genomic DNA Encoding Main Olfactory Receptors—Since MORs from *Fugu* were expected to exhibit similarity to known MORs such as those from catfish and zebrafish, we designed degenerate oligonucleotides primers corresponding to highly conserved regions among catfish MORs (*5*) and zebrafish MORs (*14*), and used them for PCR amplification of the genomic DNA. The PCR products were cloned and sequenced, and two different types of clones showing the characteristic seven transmembrane structure in their deduced amino acid sequences were isolated. One of the two clones, obtained from a pair of primers corresponding to transmembrane domains 2 and 7 and termed FOR1, showed about 40% amino acid sequence similarity to catfish-1 (*5*) in the cloned region. The other clone, obtained from primers to transmembrane domains 3 and 7, encoded another OR, termed FOR2, which showed about 40% similarity to zebrafish-9 (*14*) and 45% to the recently cloned goldfish-A28 (*13*). These clones were used as probes to screen a *Fugu* genomic library, and ten genomic clones that hybridized strongly with the FOR1 probe and one clone hybridizing with the FOR2 probe were obtained. Linkage of the FOR1-positive clones and the positions of the ORFs were determined as described below, and the nucleotide sequences around the ORFs of the FOR1- and FOR2-positive clones were determined.

Complexity of Genes Encoding Fugu Main Olfactory Receptors—To analyze the complexity of *Fugu* MOR genes, we performed Southern blot hybridizations to genomic DNA using the two olfactory receptor PCR clones as probes. Since MOR genes are reported not to contain introns within the coding regions, the number of genomic DNA fragments detected by Southern analysis was expected to approximate the number of similar genes if restriction

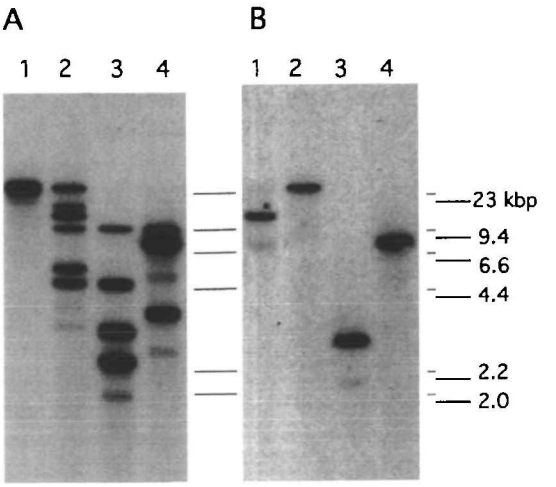


Fig 1 **Genomic Southern analysis of *Fugu* MOR genes.** Five micrograms of each *Fugu* DNA was digested, electrophoresed, blotted and hybridized with probes for PCR clones FOR1 (A) and FOR2 (B). Restriction enzymes used are *Eco*RV (lane 1); *Hin*dIII (lane 2), *Pvu*II (lane 3), and *Sac*I (lane 4)

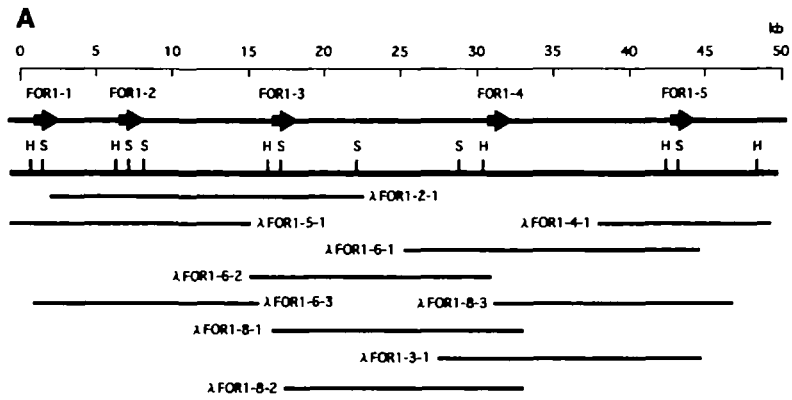
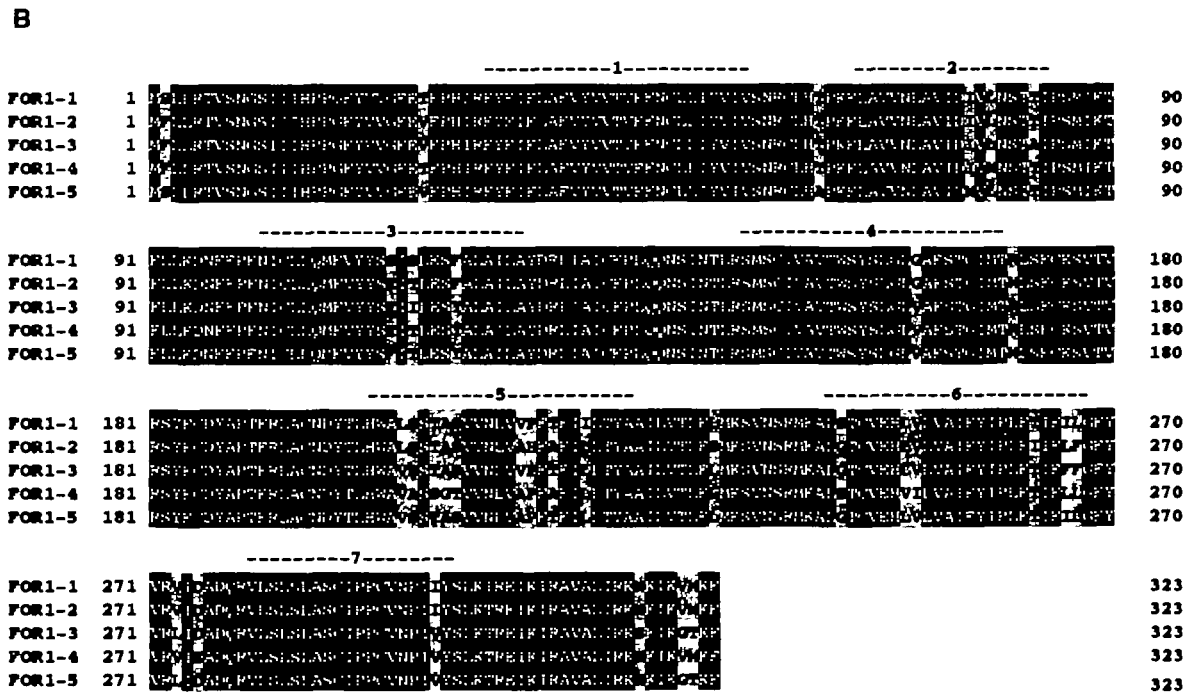
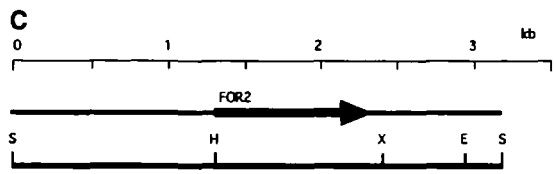


Fig 2 **A: Organization of the FOR1 gene cluster.** A genomic DNA region encompassing five members of the FOR1 subfamily was obtained by linking the 10 FOR1-positive phage clones shown at the bottom Scale, positions of ORF denoted by arrows, restriction sites of *Hin*dIII (H) and *Sal*I (S), and assignment of the 10 clones are shown in this order. **B: Amino acid sequence similarity among five members of the FOR1 subfamily.** Residues common to all members and to three or four members are represented in reversed and shaded letters, respectively The seven transmembrane domains, 1–7, are shown above the alignment The nucleotide sequences of ORFs of FOR1-1 to FOR1–5 are available in DDBJ/EMBL/GenBank databases with the accession numbers AB31380 to AB38314 C: Restriction map of FOR2 gene. Scale, positions of the ORF denoted by an arrow and restriction sites for *Hin*dIII (H), *Sal*I (S), *Eco*RI (E), and *Xba*I (X) are shown in this order





endonucleases that do not cleave the probe are used. Accordingly, for the digestion of *Fugu* genomic DNA, we used *Eco*RV, *Hin*dIII, *Pvu*II, and *Sac*I, which do not cut the two PCR clones. Hybridization was performed under high stringency conditions in order to detect genes with high similarity to each probe. As shown in Fig. 1A, the FOR1 probe detected 5–7 bands in most lanes of the blot, indicating that 5–7 closely related genes exist in the *Fugu* genome and constitute a subfamily, the FOR1 subfamily. In contrast, the FOR2 probe detected a single strong signal in each lane, although another weak signal was observed in lanes 1, 3, and 4 (Fig. 1B). It was thus indicated that FOR2 is essentially a single copy gene in the genome, but there might be other genes with limited similarities to FOR2.

Genomic Organization of FOR1 Subfamily Members and Their Amino Acid Sequences—To determine the linkage of the FOR1 subfamily genes (ORFs) in the *Fugu* genome, we analyzed ten genomic clones obtained by screening a *Fugu* genomic library with the FOR1 probe. These clones were all linked and covered a genomic region of about 50 kb containing five ORFs of FOR1 subfamily members reiterated tandemly in the same orientation (Fig. 2A). However, considering the number of bands detected by genomic Southern blot analysis (Fig. 1A), there may be more FOR1 subfamily genes outside the genomic region isolated. When genomic DNA was digested with *Hin*dIII (lane 2), a strong signal (>23 kb) positive for FOR1 was observed in addition to the expected bands of 5.6, 6, 10, 12.5, and 14 kb from the restriction map, although this signal might be caused by partial digestion of genomic DNA. The intergenic distances between members of the FOR1 subfamily were elucidated from the assignment of ORFs shown in Fig. 2A, and found to fall in the range of 5–12 kb (on average, 10 kb). This is shorter than the average intergenic distance (15 kb) identified in a human olfactory receptor gene cluster (*30*), and is comparable to those of zebrafish (*23*) and medaka (*25*) OR

genes.

In terms of similarity within a subfamily, five members of the FOR1 subfamily showed very high similarities of about 95% (Fig. 2B). This value is much higher than those of known fish OR subfamily members, such as a zebrafish subfamily (*23*) and two medaka subfamilies (*25*), and as high as that of the catfish-32 subfamily (*5*). However, significant diversity was observed in transmembrane domains 3, 5, and 6, which are important for the ligand specificity of ORs and other rhodopsin-type seven transmembrane receptors (*31*, *32*). The 5′-upstream regions of five FOR1 subfamily members showed relatively low similarities (data not shown) and cis-elements for the gene expression or translation of the FOR1 members could not be identified.

Phylogenic Analysis of FOR1 and FOR2 with Other MORs—Complete sequences of five olfactory receptors for FOR1 subfamily members and one receptor of the FOR2 subfamily member (Fig. 2C) were obtained First, two representatives (FOR1-1 and FOR2 proteins) were compared with known vertebrate MOR proteins by database search and sequence alignment. FOR1-1 showed the highest similarity to catfish-1 (*5*) and zebrafish-6 (*14*) (Fig 3A), and close values to other catfish and zebrafish MORs such as catfish-3, 32A, and 47 and zebrafish-2. On the other hand, FOR2 showed the highest similarity to goldfish-A28 (*13*) with a value of about 43%, which is comparable to, or somewhat higher than that between FOR1-1 and catfish-1 (Fig. 3B), but showed much lower similarities to other MORs.

As shown in Fig. 3C, two *Fugu* MORs are phylogenically located within the fish MOR family and distantly related to mammalian MORs. FOR1 appears to be included in a major set of pedigrees containing many fish MORs such as catfish-1, 3, 32A, and 47, but the distance between FOR1-1 and these MORs is larger than those among MORs of known fresh-water fishes. On the other hand, FOR2 constitutes an independent pedigree together with goldfish-A28. However, the similarity between FOR2 and goldfish-A28 is lower than those between catfish MORs and goldfish MORs, *e.g.* catfish-47 *vs.* goldfish-A2 and catfish-202 *vs.* goldfish-A25.

Expression of Fugu OR Genes in Adult Olfactory Epithelium—Next, we carried out *in situ* hybridization using digoxigenin-labeled antisense RNA probes. The nucleotide sequences of the five FOR1 members are very similar and efficiently cross-hybridize with each other; as a result, no difference was observed among profiles using either a single probes or combinations of probes for the five FOR1 members (data not shown). The results shown in Figs. 4 and 6 were recognized as the summation of the expression of all FOR1 members. As shown in Fig. 4, A and B, the olfactory epithelium of *F. rubripes* protrudes from the body surface and forms a rosette-like structure. When either a FOR1 or FOR2 probe was used, positive olfactory cells could be observed randomly distributed over the epithelium on both the apical and basal surfaces (Fig. 4, B–H). The frequency of the FOR1 probe signals was estimated to be 3–5% of epithelial cells, about 10 times higher than for FOR2, which correlates roughly with the gene numbers of the subfamilies (FOR1:FOR2 = >5:1).

Since RT-PCR has shown that some types of VRs are also expressed in *Fugu* olfactory epithelium (*12*), we conducted *in situ* hybridization using these *Fugu* VRs obtained by

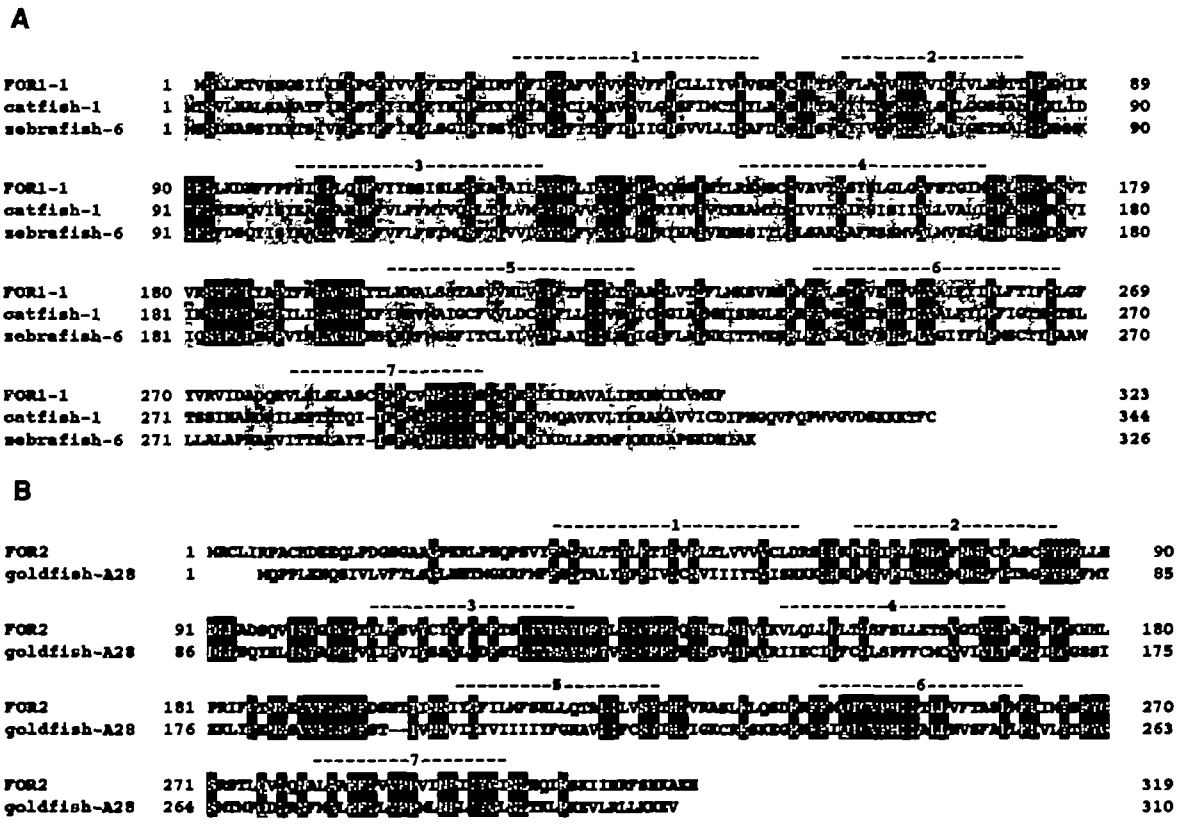


Fig 3, A and B

PCR as probes in order to examine whether segregation or a specific profile is observed for MORs and VRs in *Fugu*. As shown in Fig. 5, patterns similar to those of FOR1s and FOR2 were obtained using *Fugu* VRs; positive signals were randomly distributed throughout and on both the apical and basal surfaces of the epithelium, although the frequencies varied and were dependent on OR species.

Expression of Fugu OR Genes in Developing Olfactory Epithelium—*Fugu* olfactory epithelium shows a unique anatomical structure in that the olfactory rosette protrudes from the head surface and appears not to have internal structures buried under the body surface; this is different from other fishes having olfactory epithelia located under the skin and connected to the environment by the entrance and exit of water. Since it is possible to consider that the expression patterns of MORs and VRs in adults result from the unique structure that changes during development, and so might show some specific profile in immature epithelium, we carried out *in situ* hybridization using developing fishes. As shown in Fig. 6, A, B, C, F, G and H, positive signals of FOR1 and FOR2 probes were detected randomly distributed in the olfactory placode of hatching period fishes. The olfactory placode is buried under the skin, round-shaped, and lacks the rosette-like structure. In addition to the ramdom distribution, the signals were oriented randomly, which also shows that the olfactory neurons had not gained an orderly arrangement. From 2 weeks after hatching, the anatomical structure of the epithelium apparently changes from round-shaped buried epithelium to the protruding waved morphology characteristic of *Fugu*, in which all olfactory neurons are oriented in an orderly fashion toward the apical surface; this transformation is completed at 4 weeks (Fig. 6, D, E, I, and J). At this stage, the expression profiles of FORs are the same. The situation is also the same for VRs; the signals are observed randomly

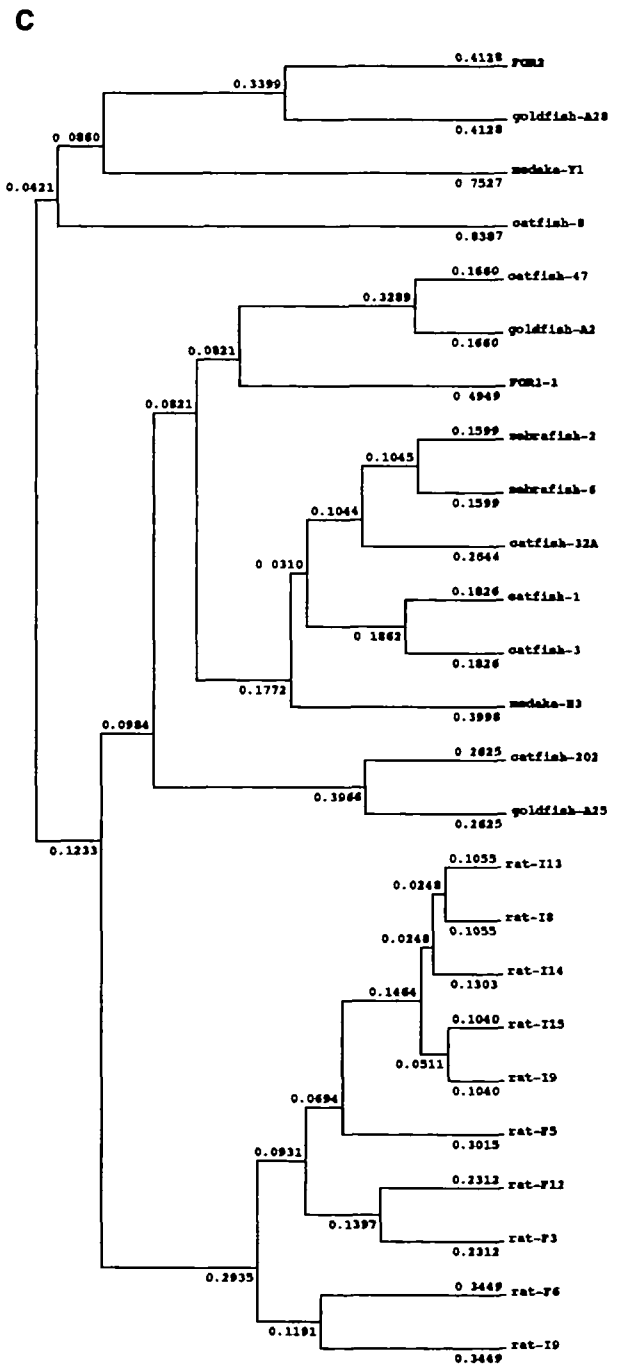


Fig 3. **Amino acid sequence similarity of fish MORs.** A: Alignment of FOR1-1 with catfish and zebrafish MORs. Two MORs, catfish-1 (*5*) and zebrafish-6 (*6*), were obtained because their sequences showed the highest scores in a database search using FOR1-1 as a query. Residues common to all and to two sequences are shown in reversed and shaded letters, respectively. Gaps are inserted to maximize similarity. B: Alignment of FOR2 with goldfish-A28, obtained as the most similar sequence by database search. Residues common to two sequences are shown by reversed letters. Gaps are inserted to maximize the similarity The nucleotide sequence of the ORF of FOR2 is available in DDBJ/EMBL/GenBank databases with the accession numbers AB31385 C Phylogenic tree of fish and mammalian MORs. Two *Fugu* MORs, FOR1-1 and FOR2, and representative fish and rat MORs were aligned and analyzed into a phylogenic tree by the UPGMA method using GENETYX-MAC software, ver. 10 (Software Development) Both FOR1-1 and FOR2 are positioned distantly to other fish MORs; FOR1-1 shows nearly equal similarity to several fish MORs including catfish-1, zebrafish-6, and catfish-47. FOR2 shows high similarity to goldfish-A28, and these two MORs constitute an independent pedigree distant from others. The accession numbers of the genes in this figure are as follows: catfish-1, -3, -32A, -202, -47, and -8, L09217 to L09222 (*5*); zebrafish-2 and -6, AF012755, and AF012762 (*14*); goldfish-A2, 25, and 28, AF083076, AF083078, and AF083079 (*13*); medaka E3 and Y1, AB029477 and AB029479 (*25*); rat-F3, 5, 6, 12, -I7, 8, 9, 13, 14, and 15 , P23265, P23266, P23267, P23268, P23270, P23271, P23272, P23269, P23273, and P23274 (*4*).

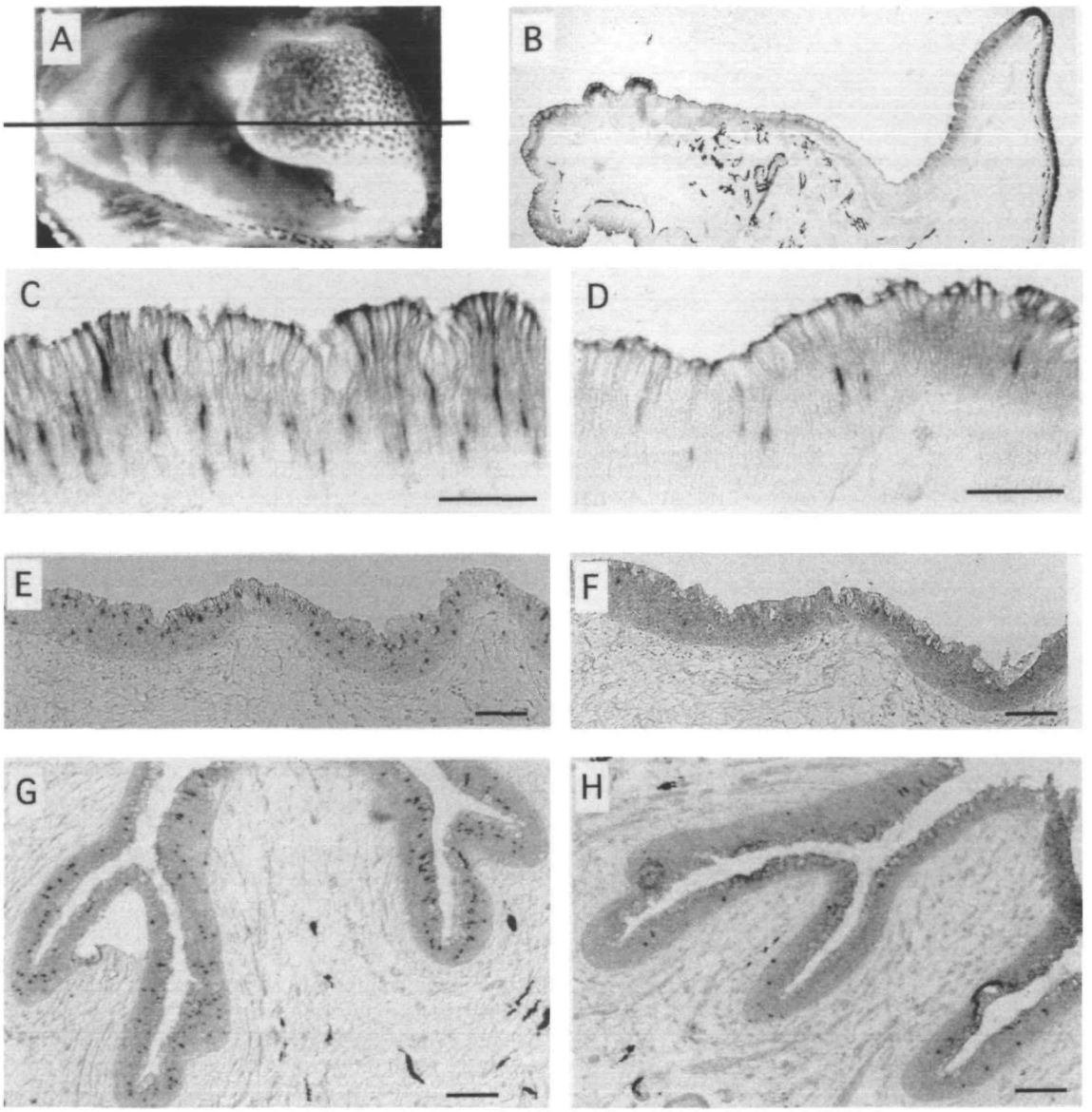


Fig 4 *In situ* hybridization of FOR1 and FOR2 subfamilies. A, Anatomy of the *Fugu* olfactory rosette of a young fish (about 10 cm in length, 6–7 months after birth), which protrudes from the body surface and lacks internal structure allowing the entrance and exit of water. The solid-line denotes the rough orientation of the cut shown in B–D. B–D: The results of whole mount *in situ* hybridization using a young fish similar to that in A, probed with FOR1-1 (B and C) and FOR2 (D). E–H: *In situ* hybridization using cryosections of olfactory rosette from an adult fish (about 30 cm in length, about 20 months after birth). Sections from two separate regions (E and F *vs.* G and H) were subjected to hybridization using probes for FOR1-1 (E and G) and FOR2 (F and H). In each case, the positive signals are distributed randomly throughout the epithelium and no specific pattern is apparent, although the frequencies of FOR1-positive signals (C, E, and G) are much higher than those of FOR2-positive signals (D, F, and H). Bars represent 50 μm.

distributed throughout the epithelium (Fig. 6, K–P). In conclusion, both MORs and VRs of *Fugu* seem to be expressed randomly over the olfactory epithelium with no specific pattern observed.

In addition, the data suggest that the frequencies of OR expression vary during the development; in the early stage before 1 week, the relative frequency of FOR2 expression appeared to be about one-half that of FOR1, but fell to about one-tenth in later stages from 4 weeks to adult (Figs. 4 and 6).

DISCUSSION

In this study, we isolated and characterized genes coding for MORs from a seawater fish, *F. rubripes*. The expression patterns of the FORs genes obtained here, together with those of VRs, are shown to be randomly distributed over the olfactory epithelium of both juvenile and adult fishes.

The organization and structure of FORs are essentially similar to those of other vertebrates, and phylogenic analysis showed that FORs fall among pedigrees of fish MORs, and are distantly related to mammalian MORs. In a recent

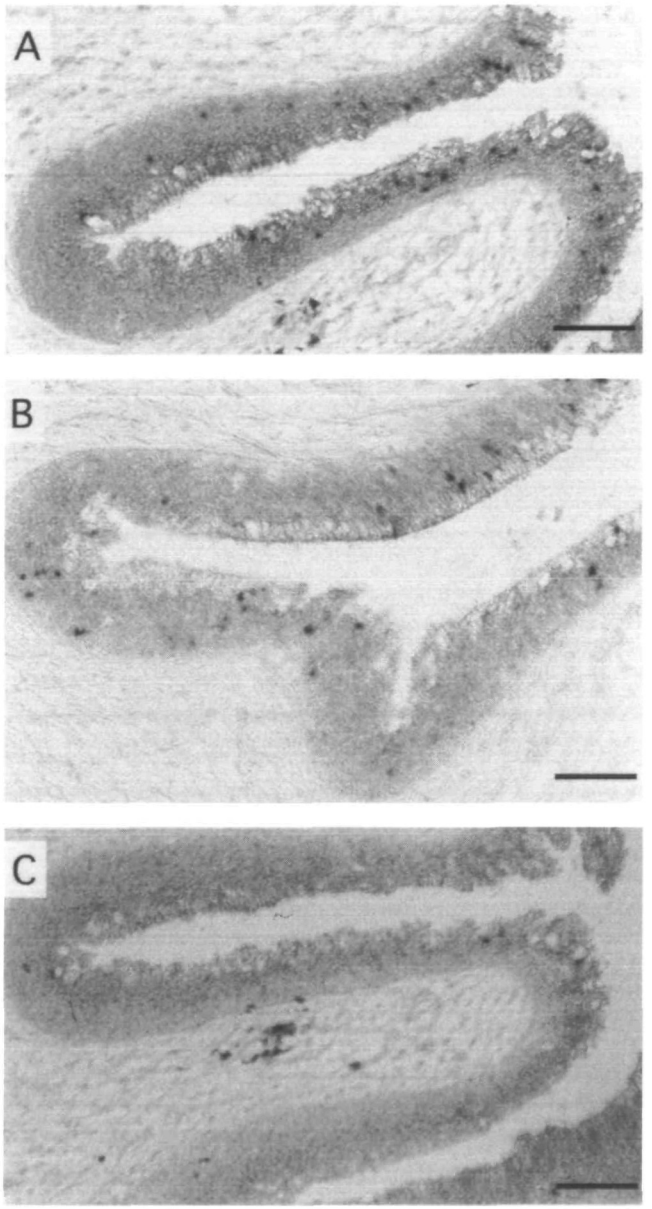


Fig. 5. ***In situ* hybridization of *Fugu* VRs.** Cryosections from the rosette near the position shown in Fig. 4, G and H, were subjected to hybridization using probes for fCa02.1 (A); fCa09 (B); and fCa12 (C) (*12*) Positive signals were randomly distributed over the olfactory epithelium, although the frequencies vary and are dependent on the probe. Bars represent 50 μm.

analysis of medaka MOR genes (*25*), we showed that fish MORs have more widely diverged amino acid sequences compared with mammalian MORs, although the number of MOR genes is much smaller than those in mammals. This conclusion is supported by the present analysis of *Fugu*, which belongs to another order, *Tetradontiformes* (Fig. 3C). The FOR1 and FOR2 subfamilies appear to constitute independent pedigrees within the fish MOR family. FOR1 is included among major pedigrees of fresh-water fish MORs that belong to a limited number of orders related to each other; on the other hand, FOR2 constitutes a pedigree with a goldfish MOR, goldfish-A28 (*13*). Considering that there should be more MOR genes not yet identified in *Fugu*, medaka fish, and goldfish, it is conceivable that several MOR subfamilies are common to most fishes, such as subfamilies comprising goldfish-A2 and catfish-47, or FOR2 and goldfish-A28. However, the similarities between FORs and related fresh-water fish ORs are lower than those among fresh-water fishes belonging to the orders *Cypriniformes* or *Siluriformes*. In addition, MOR subfamilies unique to a species (or an order), such as catfish-8 and Medaka Y (Fig. 3C), may also exist.

As for the similarity among FOR1 subfamily members, very high values of about 95% were observed, higher than for most other subfamilies reported previously (*23*, *25*) and as high as those for catfish-32 subfamilies (*5*). In the case of catfish-32, the differences are observed mainly in the 3rd and 4th transmembrane domain. On the other hand, FOR1 members show significant differences in the 5th transmembrane domain as well as the 3rd and 4th domains. These three domains should be important for the ligand-binding specificity as described for rat-I7 and adrenoceptors, where single amino acid substitutions in these transmembrane domains confer ligand-binding specificities and/or abilities (*31–33*). Thus, FOR1 members probably show similar but distinct ligand specificities. Taken together with the phylogenic analysis discussed above, each FOR subfamily member probably shows a unique ligand-binding spectrum against odorants specific for *Fugu* or common to some related fishes. Further analyses of ligand-binding would clarify the physiological importance of these ORs.

Analyses of OR gene expression by *in situ* hybridization have revealed that each *Fugu* OR gene, including both MORs and VRs, is expressed in a subset of olfactory neurons scattered randomly over the epithelium. The signal frequencies vary depending on the gene subfamily and the developmental stage; the expression frequencies of FOR1-positive cells is several times higher than that of FOR2-positive cells in adult fishes, a finding that correlates with their gene numbers. The frequencies of FOR1 and FOR2 expression in juvenile fishes are close, which might be a result of the noncoordinate expression of FOR1 members during development, as has been reported for zebrafish, in which the timing of MOR expression is dependent on the gene (*23*). VR expressions also vary with frequencies roughly between those of FOR1s and FOR2.

In addition, all of the MOR and VR genes examined here are expressed from the early stages of development upon hatching when the olfactory epithelium has not morphologically matured and become exposed to the environment. The early onset of MOR gene expression has also been described previously in zebrafish (*14*, *22*), and in each case, OR gene expression appears to be initiated before the onset of their function as external sensors.

The expression of each MOR and VR gene in *Fugu* seems random and no signal segregation was found throughout development. This is somewhat in contrast previous reports that have shown specific patterns of OR expression; in zebrafish (*21*), each MOR gene is expressed as a circular region, and in goldfish (*13*), MORs and VRs are found segregated on the basal and apical surface of the olfactory epithelium, respectively. Meanwhile, the random expression of MORs has recently been reported in medaka fish (*25*). In terms of their anatomical characteristics, zebrafish appear to possess olfactory epithelium with a well-developed rosette closely arranged in orderly fashion, while other

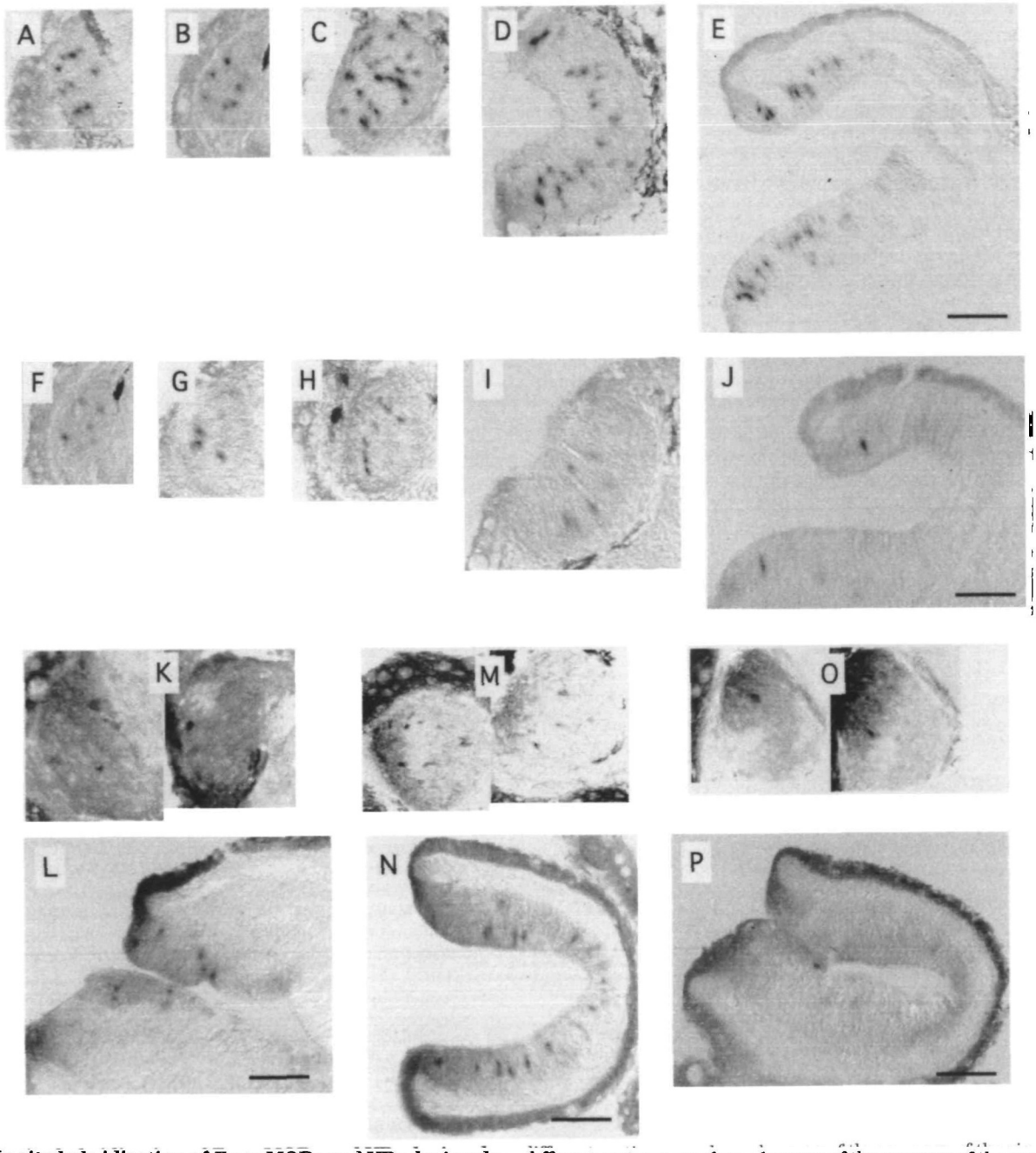


Fig 6. ***In situ* hybridization of *Fugu* MORs and VRs during development.** Whole mount *in situ* hybridization was carried out as in Fig 4 using probes for FOR1-1 (A-E), FOR2 (F-J), fCa02.1 (K and L), fCa09 (M and N), and fCa12 (O and P). Fish stages are as follows: A and F, just before hatching; B, G, K, M, and O, 2 days after hatching; C and H, 1 week after hatching; D and I, 2 weeks after hatching; and E, J, L, N, and P, 4 weeks after hatching. In panels K, M, and O, two different sections are shown because of the rareness of the signals. In all cases, positive signals are found in various regions over the epithelia, although the frequencies of the signals are dependent on the probes. At the early stages before 1 week, the frequency of FORs-positive signals compared with FOR1-positive signals is higher than that in later stages. Bars represent 50 μm.

fishes show simpler structures such as the relatively smooth epithelium in medaka fish, or the less-developed rosette-like surface in *Fugu*. Each olfactory receptor cell on such simple surfaces is exposed widely to the nasal cavity or external environment, and probably shows equivalent odorant reception in the flow of water. In contrast, well-developed rosette structures, such as that of zebrafish, might display some difference in the efficiency of odorant reception depending on the position of the cells along the water flow, but the wide and deep surface and the large number of olfactory cells enable more complicated and sensitive odor reception. Thus, there might be a correlation between the structure of the olfactory epithelium and the zonal expression of MORs as a result or process of evolutionary and adaptational strategies. Similarly, the segregated pattern of MORs and VRs observed in goldfish can be ascribed to the depth along the apical-basal axis, which confers different positions of the nuclei of microvillous and

ciliated cells as described in catfish (*13*, *34*). There are few anatomical descriptions in other species more distant from *Cypriniformes*. It is thus assumed that the expression profiles of MORs and VRs in fishes are dependent on species and may not show a common profile, a feature that is also true for the anatomical structures of the olfactory epithelia, which show various morphologies specific for each species (*11*, *25*), even though the primary structures of the ORs are related.

In mammals, MORs show zonal expression (*3*), and, in addition, MORs and VRs are expressed separately in different organs, the main olfactory epithelium and the vomeronasal organ (*1*). It is possible that there are two types of segregation of OR expression, one among MORs and the other between MORs and VRs. This might allow, for example, a presorting for axonal targeting or provide an advantage for responsive properties by lateral interaction. In these terms, our observations suggest that the spatial segregation of OR expression would not be the basic or intrinsic design of olfactory systems, but a mechanism acquired during the process of evolution and adaptation. More precise analyses of the individual genes, including identification of the ligand-binding spectrum of each OR using various fish species, is needed to elucidate the physiological importance of variable OR gene structures and expression patterns.

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