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Two Classes of Olfactory Receptors in Xenopus laevis

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Summary

Xenopus laevis possess a gene repertoire encoding two distinct classes of olfactory receptors: one class related to receptors of fish and one class similar to receptors of mammals. Sequence comparison indicates that the fish-like receptors represent closely related members of only two subfamilies, whereas mammalian-like receptors are more distantly related, most of them representing a different subfamily. The fish-like receptor genes are exclusively expressed in the lateral diverticulum of the frog's nose, specialized for detecting water-soluble odorants, whereas mammalian-like receptors are expressed in sensory neurons of the main diverticulum, responsible for the reception of volatile odors.

Introduction

The olfactory system of vertebrates recognizes and distinguishes thousands of odors. This tremendous task is accomplished by the chemosensory neurons in the olfactory epithelium; it is generally believed that different subsets of these cells recognize different odorants and that the patterns of their projection to the bulb might constitute the elementary odor codes. The chemospecificity of the olfactory neurons is based on their having specific odorant receptors capable of interacting with structurally distinct odor molecules. Recently, a large multigene family has been identified that encodes odorant receptors (Buck and Axel, 1991). These receptors exhibit the characteristic structural features of the superfamily of G protein–coupled receptors (Buck and Axel, 1991). In mammals, the repertoire of olfactory receptors (ORs) is extremely large and may consist of as many as a thousand different subtypes (Buck and Axel, 1991; Levy et al., 1991; Parmentier et al., 1992; Raming et al., 1993). The extent and pattern of diversity among the receptor proteins encoded by the multigene family suggest that they may be capable of binding a large variety of structurally diverse odorants (Buck, 1993). Extensive in situ hybridization studies have revealed that specific subsets of receptor genes are expressed in distinct zones (Ressler et al., 1993; Vassar et al., 1993; Strotmann et al., 1994a, 1994b), and recent evidence supports the concept that sensory cells expressing the same receptor type may project to the same glomerulus in the olfactory bulb (Ressler et al., 1994; Vassar et al., 1994).

A slightly different scenario emerged when ORs in fish were studied. It was found that fish do have a gene family that encodes receptor proteins with considerable homology to odorant receptors of rat; however, the size of the fish receptor repertoire appears to be considerably smaller than in mammals (Ngai et al., 1993a). In addition, there was no evidence for any spatial expression patterns; olfactory neurons expressing specific receptor types appeared to be randomly distributed within the olfactory epithelium (Ngai et al., 1993b).

The difference in receptor diversity between rat and fish may reflect a phylogenetic trend of augmenting the receptor repertoire via gene duplication, followed by mutations and recombinations eventually leading to the large variety of receptor types in mammals. However, it is also conceivable that the small repertoire of receptor types in fish may reflect the difference in odor complexity in aquatic versus terrestrial environments; in fact, the number of odorants in water is much more limited compared with the volatile airborne odorants. The number of receptor genes in fish is much more limited than in mammals. In addition, based on sequence comparison, they form distinct families (i.e., they do not "intermix" with sequences from other species in phylogenetic trees), whereas mammalian receptor families each comprise members from several different species (Lancet and Ben-Arie, 1993). It is currently unclear whether this discrepancy is due to the larger phylogenetic distance between fish and mammals as compared with the more related mammalian species, like rat, mice, and human, or whether it reflects the fact that fish smell water-soluble molecules, like amino acids (Hara, 1994), instead of the volatile, lipophilic compounds detected by terrestrial vertebrates.

To approach some of these questions, which may provide some new insight in the evolution and the functional implication of receptor diversity, we have begun to study ORs in Xenopus laevis. Amphibia are not only ranked at an intermediate position between fish and mammals on a phylogenetic scale (Mayr, 1942), they are also adapted to both aquatic and terrestrial life; thus, they are capable of smelling airborne odors as well as water-soluble odorants (Altner, 1962).

The results presented in this study provide evidence that Xenopus laevis may have two distinct classes of genes encoding ORs; one group of receptors is closely related to those of mammals, whereas another group is similar to those of fish.

Results

The identification of genes encoding putative ORs in Xenopus laevis was based on the assumption that receptors in amphibia share significant sequence homology with the family of ORs recently explored in different species (Buck and Axel, 1991; Levy et al., 1991; Ngai et al., 1993a; Raming et al., 1993; Ressler et al., 1993). Degenerated oligonu-

cleotides corresponding to conserved sequence regions in the second, third, and seventh transmembrane domains of mammalian and fish receptors were employed in pairwise combinations as primers for polymerase chain reactions (PCRs) using genomic DNA from Xenopus laevis as template in order to amplify a set of OR-related sequences. The various primer combinations resulted in amplification products that displayed the size range expected for OR genes (Figure 1A). The resulting PCR products were analyzed for complexity by digestion with the restriction enzyme HinfI followed by agarose gel electrophoresis. Multiple bands of different size were obtained (Figure 1B). The molecular weights of the fragments add up to values greater than the original PCR product, an observation reminiscent of the original finding in rat (Buck and Axel, 1991); it indicates that the PCR products consist of several different DNA species and suggests that a family of homologous genes encoding ORs may also exist in amphibia.

Since the PCR products were obtained by amplifying genomic DNA fragments, the next step was to examine whether the amplified genes are actually transcribed and whether OR gene transcription occurs in the olfactory epithelium and not in nonsensory tissue. Poly(A)$^+$ RNA was extracted from different tissues of a frog and examined for OR expression using a reverse transcriptase–polymerase chain reaction (RT–PCR) assay. PCR products of the predicted size were obtained with RNA from the olfactory epithelium, whereas the assays with brain, testis, liver, and muscle tissue were negative (Figure 2). These results indicate that at least some of the amplified genes are in fact transcribed and that the expression is tissue specific for the olfactory epithelium. To approach the question of whether the genes are in fact transcribed in olfactory neurons, in situ hybridization experiments were performed using a PCR fragment, later designated XR2 when used as a probe. A high power view of the olfactory epithelium from an adult Xenopus laevis is shown in Figure 3. The somata of supporting cells reside in the upper layer of the pseudostratified epithelium, followed by the layer of olfactory neuron cell bodies; below, a layer of basal cells is located. Hybridization signals were seen only in the olfactory neuron cell body layer and not in the basal and supporting cells. Furthermore, hybridization with a distinct probe occurred only in a small percentage of the olfactory neurons (<1%); this observation is in line with the notion that expression of distinct odorant receptors is restricted to a small subset of olfactory neurons.

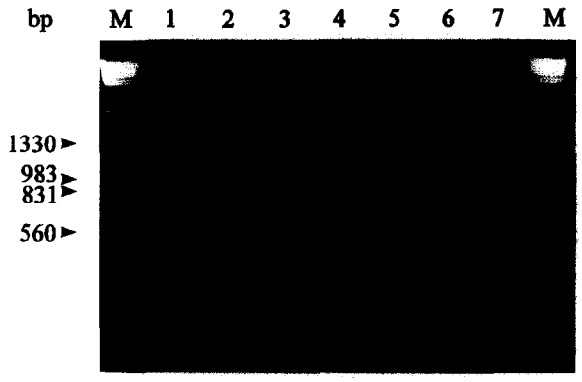

Figure 2. Tissue-Specific Expression of Receptor Genes Revealed by RT–PCR

Different tissues from Xenopus were assayed for OR expression using an RT–PCR approach. Lane 1 contained amplified genomic DNA (positive control); lane 2 contained amplified poly(A)$^+$ RNA from olfactory epithelium without reverse transcription (negative control). PCR products were obtained only with poly(A)$^+$ RNA from the olfactory epithelium (lane 3); assays with poly(A)$^+$ RNA from muscle (lane 4), liver (lane 5), brain (lane 6), and testis (lane 7) were negative. Lane M shows the position of HindIII- and HindIII/EcoRI-digested λ-DNA in base pairs.

To obtain a further identification of the amplified genes, the PCR products were cloned into a plasmid (pGem-T vector), and 19 individual clones were subjected to DNA sequence analysis. The deduced amino acid sequences of these clones revealed a diverse set of molecules (Figure 4). The encoded proteins share sequence motifs previously described as characteristic of the rat odorant receptor family (Buck and Axel, 1991). They exhibit cysteine residues at fixed positions within extracellular loops I and II, proposed to form a disulfide bridge. The highly conserved KAFXTC motif and an SY motif in putative transmembrane domains V and VI are present in most Xenopus

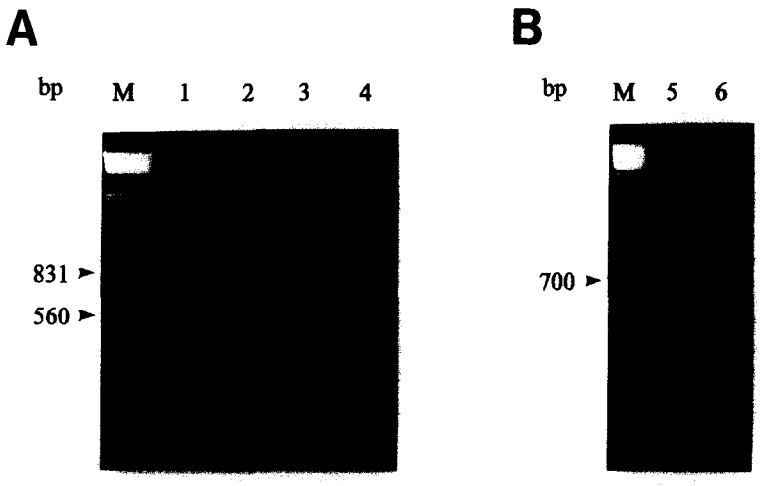

Figure 1. Amplification of Olfactory Receptor–Related Sequences from Xenopus laevis

Genomic DNA from Xenopus laevis was subjected to PCR amplification using primer oligonucleotides corresponding to conserved regions of olfactory receptors (ORs) from rat and fish. Using different primer combinations, the PCR products were size fractionated on an agarose gel, giving bands of the predicted size (A, lanes 1–4; lane M shows the positions of HindIII- and HindIII/EcoRI- digested λ-DNA in base pairs). Semipurified PCR products (B, lane 6) were digested with the restriction enzyme HinfI followed by agarose gel electrophoresis. The digestion resulted in a large number of fragments (lane 5), indicating that the PCR product may consist of several DNA sequences; lane M shows the position of the 100 bp ladder molecular weight marker (Pharmacia).

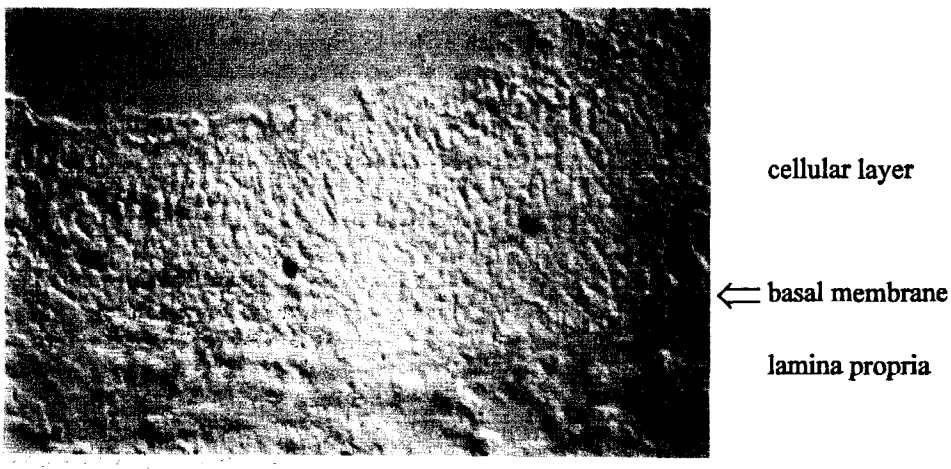

Figure 3. High Power Differential Interference Contrast Micrograph of Section through the Olfactory Epithelium

The section was incubated with a digoxygenin-labeled antisense riboprobe of clone XR2. Hybridization signals occur exclusively in the olfactory neuron cell body layer. Only a small percentage of the neurons are labeled. The asterisk indicates melanophores. Bar, 20 μm.

sequences. Furthermore, maximal homology is found in putative extracellular loops I and II as well as in intracellular loop II; in contrast, transmembrane domains IV and V show a high degree of sequence divergence. In all, there are 18 residues that are conserved in all frog sequences; most of them are also conserved in OR sequences from other species. Overall, the individual frog receptors share a 20%–98% amino acid homology with one another. They exhibit 20%–50% homology with representative rat sequences and 20%–35% homology with fish sequences.

To analyze the homology relationship among the Xenopus sequences in more detail, a pairwise alignment that scores the similarity between every possible pair of sequences was performed. From the identity dendrogram in Figure 5, it is immediately obvious that the sequences are grouped in two distinct classes. Within class I, sequences display homology between 50% and 98%, whereas in class II the homology ranges from 30% to 66%. Between the two different classes, a sequence homology of only 20%–30% was determined. Sequence comparison

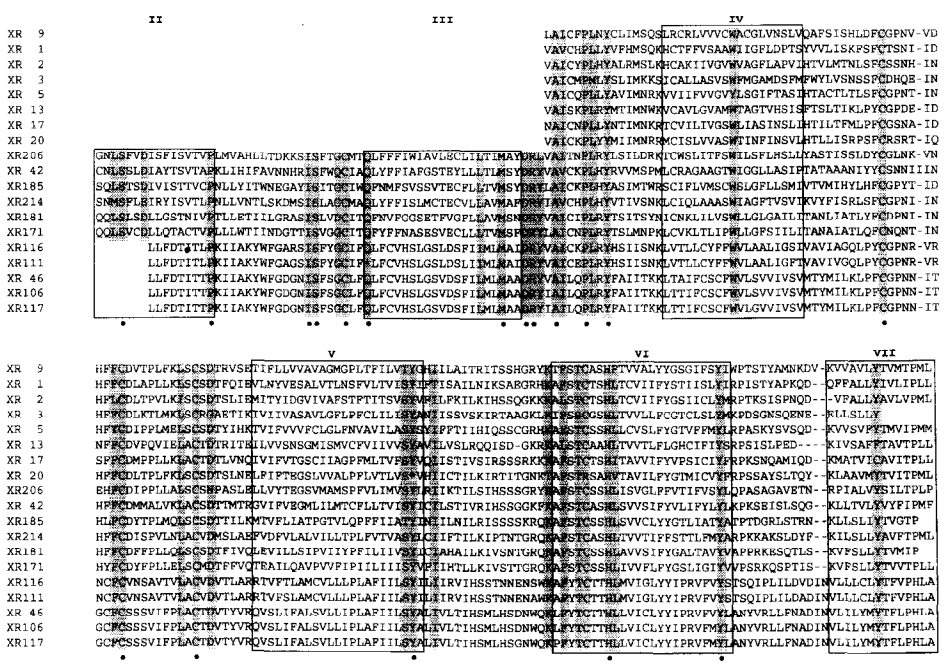

Figure 4. Deduced Amino Acid Sequences of Xenopus OR Gene Fragments

Nineteen divergent PCR products were subjected to sequence analysis, and the encoded protein sequence was determined. The predicted positions of the transmembrane domains are boxed (II–VII). Common motifs sharing >80% identity are highlighted. Amino acids conserved in all sequences are indicated by dots; XR20 and XR111 show an internal stop codon indicated by an asterisk.

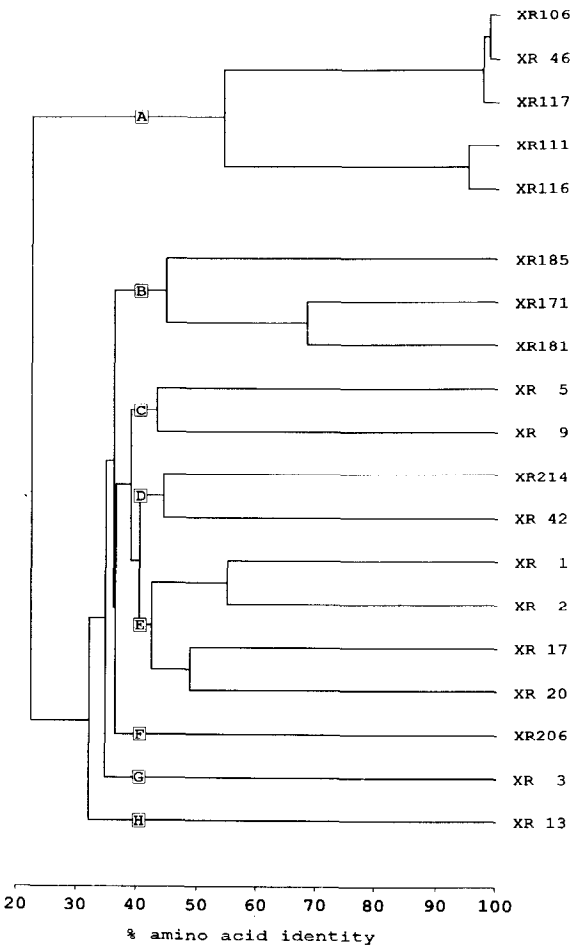

Figure 5. Identity Dendrogram of ORs from Xenopus laevis

Receptor sequences were analyzed using the dendrogram option of the GCG PILEUP program. All protein sequences were aligned between positions 124 and 279 of the rat OR12 sequence. The percentage of identity was determined by pairwise alignment. The sequences are grouped in 8 families based on the definition that a family includes sequences that share >40% identity; members of a subfamily are >60% identical (Schwartz and Dayhoff, 1979). It is immediately obvious that the sequences are grouped in two classes: class I consists of family A, whereas class II consists of families B–H.

between the two classes revealed that all class I receptor sequences exhibit an extended extracellular loop III, whereas class II receptor sequences show a deletion of 1–4 amino acid residues in this region. Apart from the homologous domains characteristic of the OR superfamily, class I sequences show a number of highly conserved motifs specific for this receptor group. Sequence domains in transmembrane domains III and V, which usually exhibit significant divergence, show a high number of identical amino acid residues. Based on the classification of homologous genes defining sequences that share >40% identity as a family and those with >60% identity as a subfamily (Schwartz and Dayhoff, 1979; Nebert et al., 1991), it is obvious that all class I receptors are members of one family (Figure 5, family A). This family could be further subdivided into 2 subfamilies, each with several members sharing >90% sequence homology. In contrast, the class II receptors define 7 families (B–H), which can be grouped in 13 subfamilies. In contrast to the multiple members of the class I subfamilies, each class II subfamily contains only 1 member, except for one B subfamily that has 2 members sharing about 65% sequence homology. The notion that there are relatively few class I subfamilies but numerous class II receptor subfamilies is supported by the results of intensive PCR and cloning experiments employing a variety of different primers and hybridization conditions, which did not lead to an identification of additional class I subfamilies.

To analyze further the complexity of the two receptor classes in Xenopus, Southern blot analyses were performed with genomic DNA employing different PCR fragments as probes. Using stringency conditions that prevent cross-hybridization, the probes were annealed to Southern blots of DNA from an individual frog after digestion with various restriction endonucleases (EcoRI, PstI, HindIII, and ApaI). A probe specific for class I receptors labeled a large array of 10–12 bands, whereas a probe specific for class II receptors visualized only 1–2 bands (Figure 6). Since the OR genes supposedly do not contain introns within the coding region (Ben-Arie et al., 1994), the number of hybridizing bands should approximate the number of receptor genes. The results of additional Southern blot analyses using different probes indicate that, for the class I receptors, 1 subfamily comprises about 10 genes, the other about 4. Therefore, each subfamily of class I seems to be composed of several genes exhibiting >90% sequence identity. In contrast, the class II receptors can be grouped in 14 subfamilies, each having only 1 or a few members. Thus, class II receptors represent a large array of families and subfamilies, reminiscent of the mammalian ORs (Lancet and Ben-Arie, 1993). However, in contrast to subfamilies of rat receptors, which have up to 17 genes (Buck and Axel, 1991), a subfamily of class II receptors in Xenopus consists only of 1 or a few genes (Figure 6).

To determine the sequence relatedness of the two classes of Xenopus receptors to those from other species, a phylogenetic tree was constructed for the Xenopus sequences together with a variety of published OR sequences from human, rat, mouse, and fish. As shown in Figure 7, the class I sequences are closely related to fish-derived gene sequences, though without "intermixing." In contrast, the class II sequences appear to be very closely related to the mammalian ORs; in fact, they do not form a distinct group, but rather represent new members of receptor families previously considered to be characteristic for mammals. These observations suggest that Xenopus laevis may have two distinct classes of genes encoding odorant receptors: one encoding receptor proteins that resemble those of fish and one encoding ORs similar to those of mammals. The identification of two distinct classes of genes encoding fish- and mammalian-like OR proteins in Xenopus raises the question of whether the different receptor types are expressed in different regions of the frog's nose.

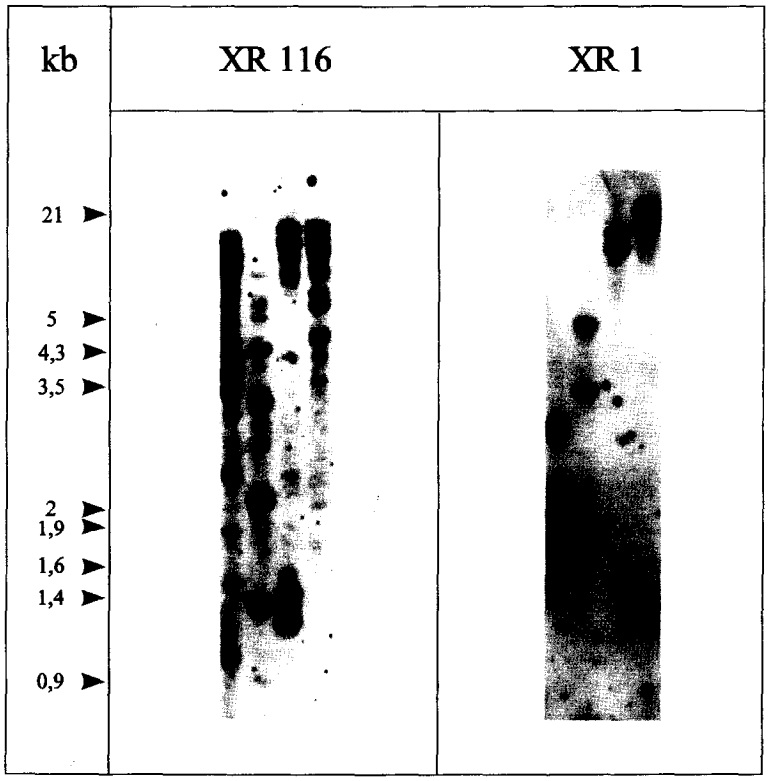

Figure 6. Southern Blot Analysis with PCR Fragments Representing Class I and Class II Receptors

Xenopus genomic DNA isolated from muscle tissue was digested with restriction endonucleases EcoRI (lane 1), PstI (lane 2), HindIII (lane 3), and ApaI (lane 4), electrophoresed on 1% agarose gels, and blotted to nylon filters. Blots were annealed with digoxygenin-labeled probes corresponding to clones XR116 (class I) and XR1 (class II). The high stringency conditions prevent any cross-hybridization; hybridizing fragments share a sequence homology of >90%. The position of HindIII and HindIII/EcoRI digested λ-DNA is shown on the left in kilobase pairs. XR116 shows an internal recognition sight for ApaI; XR1 shows one for PstI.

In detailed morphological studies of Xenopus, it has been shown that the nasal cavities consist of three separate subsystems; one is the vomeronasal organ (VO), whereas the other two, the lateral and medial diverticulum (LD and MD, respectively), are subdivisions of the main chamber (Föske, 1934; Altner, 1962; Hofmann and Meyer, 1991). To determine whether the two receptor classes are expressed selectively in one of the distinct subsystems of the nose, spatial expression of the two receptor classes was analyzed by in situ hybridization. Coronal sections through the noses of adult Xenopus specimens (Figure 8A) were probed with antisense RNA of the fish-like receptor clone XR46. This resulted in labeling of the sensory epithelium in the LD of the main chamber (Figure 8B), whereas the MD and VO were devoid of any hybridization signals. Two other fish-like probes (XR106 and XR117) were analyzed with virtually the same results. Experiments using antisense probes of the mammalian-like receptor XR2 gave hybridization signals exclusively in the MD of the main chamber (Figure 8C). Similar experiments were performed with probes representing other mammalian-like receptors (XR3, XR17, XR42, XR171, and XR181); all the probes gave hybridization signals only in the MD. Ten different animals, with equal numbers of males and females, were studied. About ten sections were analyzed for each clone. These comprehensive hybridization studies indicate that fish- and mammalian-like receptors are expressed in different anatomical compartments.

To scrutinize the notion of a spatially restricted expression of the two receptor classes in more quantitative approaches, tissue-specific RT–PCR experiments were performed with olfactory epithelium from the medial and lateral diverticulum. Primer pairs leading to amplification of either fish- or mammalian-like receptors were employed. The resulting PCR fragments were analyzed by Southern blot hybridization employing a mixture of several labeled probes that covered the whole spectrum of both class I and class II sequences. The results depicted in Figure 9 show that in the MD only PCR products derived with primers specific for mammalian receptors were recognized by the mixed probe. In contrast, in the LD reactive PCR products were obtained only with fish-specific primers. These data confirm the results of the in situ hybridization experiments and emphasize that expression of each receptor class is spatially restricted to a particular compartment of the frog's nose.

The compartmentalization of the olfactory system in Xenopus is thought to be an adaptation to life in aquatic and terrestrial environments. As first described by Altner (1962), underwater a valve-like skin flop in the nostril closes the MD, while the LD maintains access to the waterborne odors. In contrast, when the animal is outside of the water and while breathing at the water surface, the LD is closed and the MD is in contact with outside air. The role of the VO remains unclear. These observations suggest that the MD is specialized for airborne odors, whereas the LD may detect water-soluble odorants. Based on this information, it is conceiveable that the spatially restricted expression of the two receptor classes may have immediate functional implications; the fish-like receptors

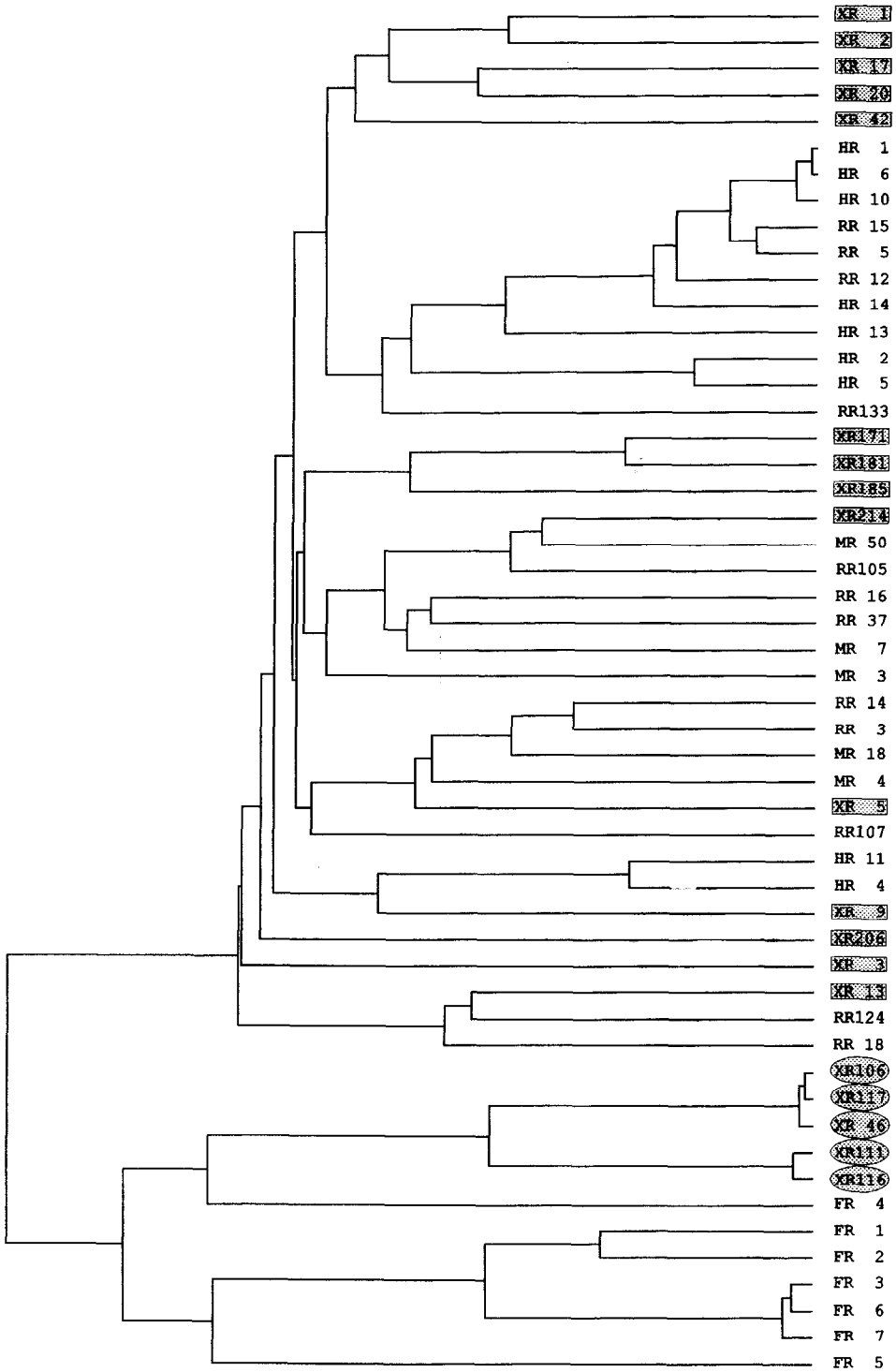

Figure 7. Evolutionary Tree of the OR Superfamily

Using the Phylogeny Interference Package 3.5c (J. Felsenstein, University of Washington), the UPGMA method (program NEIGHBOR) was applied to a matrix of maximum-likelihood distances (program PRODIST). The initial alignment of sequence data was obtained by calculation of all pairwise similarities between the sequences and a final progressive alignment (CLUSTAL W; Thompson et al., 1994). The sequences analyzed are from Xenopus laevis XR (this study), fish FR (Ngai et al., 1993a), rat RR (Raming et al., 1993; Strotmann et al., 1994a), mouse MR (Ressler et al., 1993), and human HR (Ben-Arie et al., 1994). Xenopus-derived sequences are highlighted; class I receptors are circled, and class II receptors are boxed.

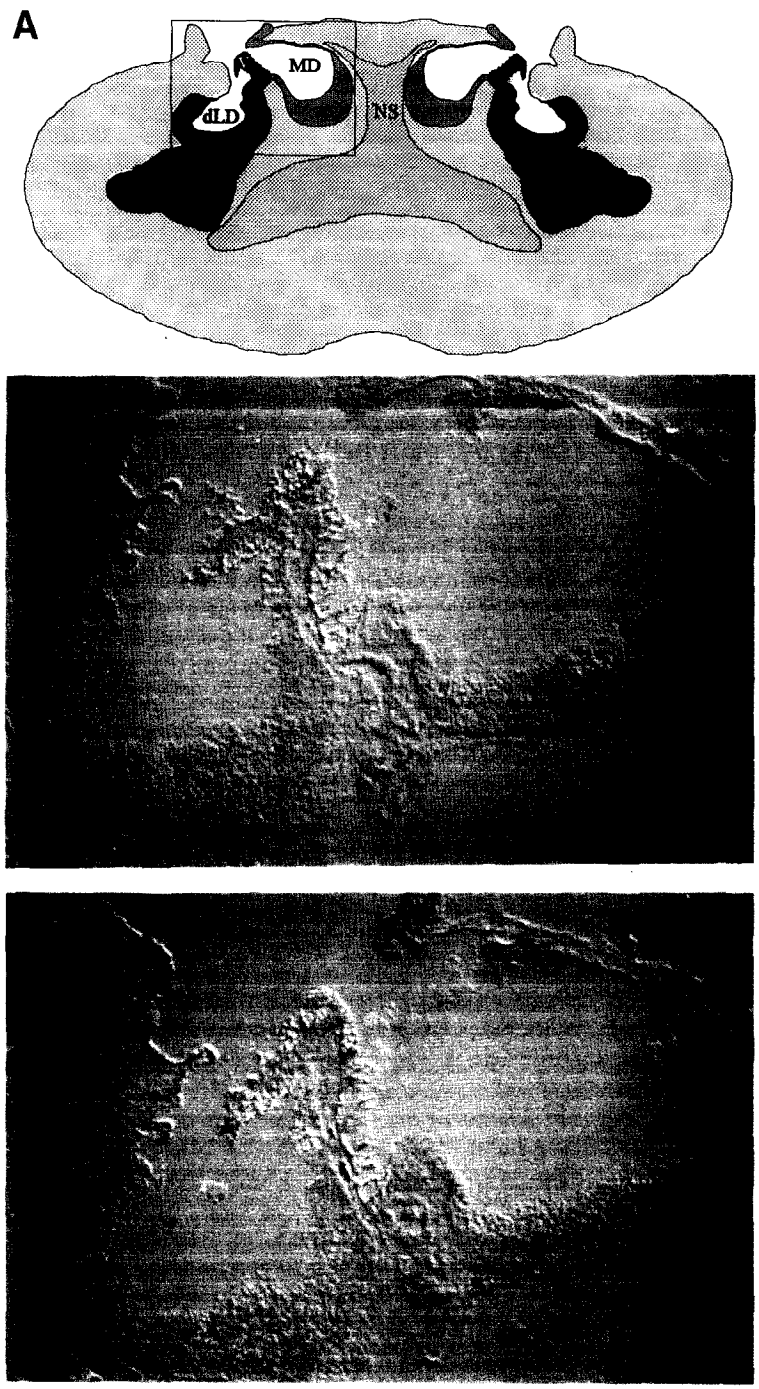

Figure 8. In Situ Hybridization of an Adult Xenopus Coronal Section Using a Digoxygenin-Labeled Antisense Riboprobe

(A) Schematic representation of a coronal section through the nose of an adult Xenopus showing the two subcompartments of the nose. dLD, dorsal portion of the lateral diverticulum; vLD, ventral portion of the lateral diverticulum; MD, medial diverticulum; NS, nasal septum; V, valve-like structure that closes the LD or MD depending on the environment. The boxed area is shown in (B) and (C).

(B) Hybridization with the fish-like receptor clone XR46 led to a labeling of cells in the sensory epithelium of the LD only; no hybridization signals were found in the MD.

(C) An adjacent section probed with the mammalian-like receptor clone XR2. This riboprobe labeled only cells in the MD.

Bars, 100 μm.

in the LD may be responsible for odor detection in water, whereas the mammalian-like receptors in the MD detect airborne odors.

Discussion

Previous studies have shown that the multigene family encoding ORs is much more complex in mammals than in the phylogenetically more primitive fish (Buck and Axel, 1991; Ngai et al., 1993a). All hitherto identified receptor subtypes from different mammalian species can be categorized into four families. The relatively few receptor sequences from fish also constitute four distinct families (Lancet and Ben-Arie, 1993), indicating much more subtle differences among the related receptors in mammals than in fish. Exploring the repertoire and diversity of ORs in Xenopus revealed that in amphibia the OR family is not merely an intermediate between fish and mammals, according to their position on the phylogenetic scale; rather, the frog seems to have a gene repertoire encoding two

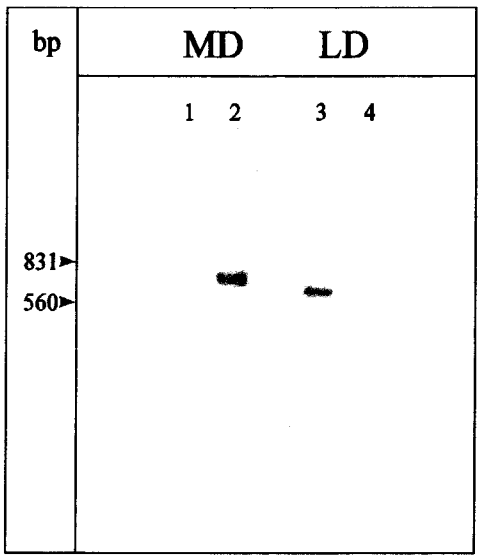

Figure 9. Selective Expression of Receptor Subtypes in the Medial and Lateral Diverticulum

RNA from different compartments of the frog nasal cavity was subjected to RT–PCR analysis using primer pairs corresponding to class I and class II receptors. The resulting PCR products were analyzed on Southern blots using labeled probes representing all class I receptors as well as all class II receptors. The amount of probe was similar for class I and II probes. In the MD, only PCR products derived with primers specific for class II receptors were recognized. In the LD, OR-related PCR products were obtained with class I–specific primers only. Lanes 1 and 3, X2.4/OR7.1; lanes 2 and 4, X2.3/OR7.1.

distinct classes of receptors, one related to fish receptors and another similar to mammalian receptors. Since Xenopus laevis is well adapted to life in both aquatic and terrestrial environments, the coexistence of two receptor classes may have immediate functional implications and shed some new light on the functional/structural differences of ORs in catfish and rat (Ngai et al., 1993c). Terrestrial animals smell airborne odorants, which are volatile and hydrophobic, whereas aquatic animals smell water-soluble molecules, like amino acids. It is therefore conceivable that the structural differences between ORs in mammals and fish reflect, at least in part, the different physicochemical features of the ligands to which they are tuned. The idea that receptors of each class may be specialized for detecting either volatile or water-soluble odorants, respectively, is further emphasized by the observation that fish-like receptors are expressed in the LD of the frog's nose, which is specialized for detecting water-soluble odorants, while mammalian-like receptors are expressed in the MD, responsible for the reception of volatile odors (Altner, 1962). Sequence comparison revealed the catfish- and fish-like receptors from Xenopus have an extended extracellular loop III compared with mammalian-like receptors; it is therefore tempting to speculate that the length and the structure of extracellular loops may play a functional role in recognizing odorous ligands. More detailed comparative analyses may allow us to elucidate the critical structural domains responsible for detecting odorous ligands in general, including the discrimination of hydrophobic versus hydrophilic ligands.

The observation that the fish-like receptors from Xenopus represent only two subfamilies with many closely related members, while almost every one of the mammalian-like receptors represents a different subfamily, may shed some new light on the evolution of the OR genes. Although originating from a common ancestor, fish- and mammalian-like receptors in amphibia may have evolved differently. The limited number of fish-like subfamilies in Xenopus may be a reflection of the limited number of odorants in the aquatic environment (Hara, 1994); the closely related genes within a subfamily may be the basis for a relatively high acuity toward the small odorant repertoire. The more distantly related mammalian-like receptors may represent an adaptation that allows the animals to deal with terrestrial life. The notion that most of the class II receptors of Xenopus represent members of families previously considered as characteristic for mammals (Lancet and Ben-Arie, 1993) suggests that class II receptors in Xenopus may already cover the whole range of volatile odorants, though representing only a coarse-mesh screen. During vertebrate evolution, the recognition system may have been refined by increasing the number of subtypes in each family. Thus, the two classes of odorant receptors in Xenopus may reflect different levels of phylogenetic differentiation. Studies in progress analyzing the olfactory gene families in phylogenetically interesting species, such as Latimera, will contribute to better understanding of the phylogenetic origin and evolution of the OR multigene family in vertebrates.

Experimental Procedures

Tissue Preparation

Adult Xenopus laevis were purchased from a local supplier (Kähler, Hamburg). For tissue preparation, animals were cooled on ice and subsequently killed by decapitation. Tissues were dissected at 4°C and rapidly frozen in liquid nitrogen.

Preparation of Genomic DNA

High molecular weight DNA was prepared from muscle tissue of an individual adult Xenopus laevis according to Ausubel et al. (1987).

PCR

To amplify Xenopus OR sequences, degenerated oligonucleotide primers were designed against conserved regions of transmembrane domains II, III, and VII of ORs from rat (Buck and Axel, 1991; Raming et al., 1993), catfish (Ngai et al., 1993a), or clawed toad (this report). The 5′ primers were: X2.2, 5′-TT(CT)AA(CT)(CT)T(AGCT)GC(AT)(GCT) T(AGCT)TC(ACT)GA-3′; X2.3, 5′-A(CT)(AC)CC(CT)ATGTA(CT)TT(GCT)(CT)T(CT)CT-3′ ; X2.4, 5′-AT(ACT)G(CA)AGCT)AA(GA)TA(CT)TGGTT(CT)GG-3′; OR3.1, 5′-GC(AGCT)ATGGC(AGCT)TA(CT)GA(CT)(AC)G(AGCT) TA-3′. The 3′ primers were: OR7.1, 5′-A(AG)(AGCT)(GC)(AT)(GA)TA(AGT)AT(GA)AA(AGCT)GG(AG)TT-3′; X7.1, 5′-TA(AGT)AT(AG)A(CT)AGT)GG(AG)TT(AGT)A(AT)CAT -3′.

For amplification of OR sequences from genomic DNA, 100 ng of DNA was used as template. Amplification was carried out in 50 μl of 10 mM Tris–HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, with 200 μM of each dNTP, 100 pmol of each degenerated primer, and 3 U of *Taq* DNA polymerase (GIBCO–BRL) using a crocodil II-Thermocycler (Appligene). PCR was performed according to the following schedule: 94°C for 4 min, 50°C for 2 min, and 72°C for 5 min (1 cycle); 94°C for 40 s, 50°C for 1 min, and 72°C for 1.30 min (40 cycles); 72°C for 10 min. Following PCR, 10 μl of the reaction products were analyzed on 1.5% agarose gels.

RNA Isolation

For the analysis of tissue-specific expression of the obtained sequences and for the specific amplification of distinct OR sequences from the different subsystems of the nasal cavity of Xenopus, mRNA from various tissues was isolated. Poly(A)$^+$ RNA was obtained using oligo(dT)$_{25}$ magnetic beads (Dynal). All steps were performed according to the manufacturer's specifications, except for the following modifications. A total of 20 mg of tissue was disrupted and homogenized in 200 μl of buffer using a micropotter. Poly(A)$^+$ RNA was hybridized to 75 μl of oligo(dT)$_{25}$ beads. To exclude contamination of poly(A)$^+$ RNA with genomic DNA, RNA coupled to the magnetic beads was incubated for 15 min at 37°C in 50 μl of 10 mM Tris–HCl (pH 8.0), 150 mM LiCl, 1 mM $MgCl_2$, with 3 U of fast protein liquid chromatography–pure DNase I (Pharmacia). After two washing steps, the poly(A)$^+$ RNA was eluted in 11 μl of RNase-free water. DNases were inactivated by incubation for 10 min at 70°C.

RT-PCR

Poly(A)$^+$ RNA was used as template for reverse transcription to cDNA using a first-strand cDNA synthesis kit (Pharmacia). RNA solution (8 μl) was primed with 0.2 μg of NotI d(T)$_{18}$ primer. First-strand cDNA synthesis was performed in a final volume of 15 μl; 3 μl of the cDNA solution was used for further PCR amplification. To recognize a possible contamination of poly(A)$^+$ RNA with genomic DNA, 1.6 μl of poly(A)$^+$ RNA solution was used as template in a control PCR experiment.

Cloning and Sequencing

PCR products were subcloned into pGEM-5Zf(+) vector using the pGEM-T vector System I (Promega). Recombinant plasmids were subjected to DNA sequencing using the T7 sequencing kit (Pharmacia), following standard annealing procedures of SP6 and T7 primers to double-stranded templates.

Analysis of Sequence Data

Analysis of sequence data was performed using the HUSAR 3.0 software package based on the sequence analysis software package 7.2 from the Genetic Computer Group (Madison, WI) and the PHYLIP 3.5c software (J. Felsenstein, Department of Genetics, University of Washington, Seattle, WA).

Southern Blot Analysis

For Southern blot analysis, DNA of an individual animal was digested with EcoRI, PstI, HindIII, or ApaI, respectively, size fractionated on 1% agarose gels, and blotted onto Hybond N$^+$ membranes (Amersham) using standard protocols (Sambrook et al., 1989). Xenopus OR probes were labeled using the Dig DNA labeling kit (Boehringer). Blots were hybridized under high stringency conditions to Dig-labeled DNA probes as described by Engler-Blum et al. (1993), following the optimized hybridization and detection protocol (blocking reagent and anti-Dig AP-Fab fragments were obtained from Boehringer). For the hybridization of RT–PCR products to Dig-labeled XR DNA, RT–PCR products were size fractionated by gel electrophoresis, blotted, and hybridized to a mixture of probes encoding Xenopus ORs as described above. To eliminate positive signals arising from single-stranded PCR products, PCR products (10 μl) were pretreated for 15 min at 37°C with 5 U of S1 nuclease (GIBCO–BRL) in 5 mM sodium acetate (pH 4.6), 1.25 M NaCl, 5 mM $ZnSO_4$, 25% glycerin.

In Situ Hybridization

RNA Probe Synthesis

Antisense RNA probes were generated using the SP6/T7 RNA transcription system according to the manufacturer's (Boehringer) specifications with recombinant pGEM vectors as template. In brief, 2 μg of linearized transcription vector were transcribed in the presence of 70 nmol of digoxigenin-11-uridine-5′-trisphosphate, followed by partial alkaline hydrolysis of the RNA according to Angerer and Angerer (1992), thereby producing fragments ~ 200 bp in length. The antisense RNA was collected by ethanol precipitation followed by centrifugation (30,000 × g; 30 min); the pellet was then resuspended in 20 ml of hybridization buffer containing 50% deionized formamide (Amersham).

Tissue Preparation and Hybridization

Adult Xenopus laevis were cooled on ice and decapitated, and the frontal part of the head was embedded in Tissue Tek (Miles, Elkhart, IN) and rapidly frozen at −70°C. Sections (10 μm thick) were cut on a Reichert & Jung cryostat (model 2800 E) at −24°C, thaw mounted on silanated slides, and air dried for 30 min. Slides were sequentially treated with 4% formaldehyde in 0.1 M phosphate-buffered saline for 5 min, 200 mM HCl for 10 min, and 1% Triton X-100 for 2 min at room temperature. Sections were subsequently dehydrated in a graded series of ethanols (60%, 80%, 95%, 100%, and 100% for 1 min each) and stored in 95% ethanol at 4°C. For in situ hybridization, tissue sections were air dried and covered with 10 μl of hybridization solution containing Dig-labeled antisense RNA. A coverslip was then placed on the solution and surrounded with rubber cement. Sealed sections were placed in closed humid boxes; hybridization was carried out at 55°C for 16 hr. Following incubation, sections were washed twice for 30 min in 0.1 × SSC at 60°C. Hybridization was visualized using an anti-Dig-AP antibody (1:750) for 30 min at 37°C, followed by two washes in Tris-buffered saline (100 mM Tris–HCl [pH 7.0], 150 mM NaCl) for 15 min. Bound antibodies were visualized using nitro-blue tetrazolium and bromo-chloro-indolylphosphate as substrates. The reaction was stopped after 12 hr by a 10 min rinse in 20 mM Tris–HCl [pH 8.0], 5 mM EDTA. Subsequently, sections were mounted in Euparal (Roth) and examined under a Zeiss Axiophot microscope.

Controls

In control experiments, sense RNAs were transcribed and hybridized to tissue sections as described for the antisense probes. Alternatively, sections were treated with RNase A (20 μg/ml in 10 mM Tris–HCl [pH 8.0], 500 mM NaCl, 1 mM EDTA) for 30 min at 37°C prior to in situ hybridization. Signals were abolished in all these cases.

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