

EXPRESSION OF OLFACTORY RECEPTORS DURING DEVELOPMENT IN *XENOPUS LAEVIS*

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Summary

A coordinated expression of tissue- and cell-specific genes during development is required to establish the complex functional organization of the vertebrate olfactory system. Owing to the unique features of its olfactory system and the well-characterized phases of its development, *Xenopus laevis* was chosen as a model organism to study the onset and the temporal and spatial patterns of expression of olfactory-specific genes. Using RT-PCR and *in situ* hybridization, it was found that expression of *Xenopus* olfactory marker protein and of class I receptors, which are thought to be responsible for the perception of

water-soluble odorants, was detectable as early as stage 32, less than 2 days after fertilization. In contrast, expression of class II receptors, which are thought to recognize airborne odours, was not detected before stage 49, approximately 12 days after fertilization. The results indicate that the expression of olfactory receptors and marker protein is governed by temporally regulated cues during development.

Key words: development, olfactory receptor, olfactory marker protein, gene expression, *Xenopus laevis*.

Introduction

The sense of smell enables vertebrates to recognize and discriminate, with high sensitivity and specificity, thousands of small volatile compounds. The detection of distinct odours results from interaction between odorous molecules and defined sets of olfactory sensory cells in the nasal neuroepithelium that display the appropriate chemospecificity (Sicard and Holley, 1984). The discovery of a large multigene family encoding olfactory receptors (Buck and Axel, 1991) has contributed greatly to our current understanding about the responsiveness of chemosensory neurones. These putative odorant receptors are members of the superfamily of receptor proteins with seven membrane-spanning domains which couple *via* heterotrimeric G-proteins onto intracellular reaction cascades mediating the chemo-electrical transduction process (Breer et al., 1994). Each sensory cell seems to express only one or a small subset of receptor types, which determine its chemospecificity. Cells expressing a distinct olfactory receptor subtype are segregated in a restricted zone of the nasal neuroepithelium in mammals (Ressler et al., 1993; Vassar et al., 1993; Strotmann et al., 1994a,b) and project their axons to target-specific and spatially conserved sites in the olfactory bulb (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996). The functional implication of this spatial expression pattern is not clear, but it is interesting to note that such zonal patterning may not exist in fish (Ngai et al., 1993; Byrd et al., 1996; Weth et al., 1996; Vogt et al., 1997).

In *Xenopus laevis*, two classes of olfactory receptor have recently been discovered; class I receptors are most similar to receptors from fish, and class II receptors are closely related to mammalian receptors. Both classes of olfactory receptor are expressed in the main olfactory epithelium. Each class of receptor was found to be selectively expressed in a separate compartment of the nose of the adult frog, the fish-like receptors in the lateral diverticulum (LD) and the mammalian-like receptors in the medial diverticulum (MD) (Freitag et al., 1995). Receptors related to the mammalian V2R receptors (Herrada and Dulac, 1997) are expressed in the vomeronasal organ (VNO) of *X. laevis* (J. Fleischer, unpublished observation). These specific features of the *X. laevis* olfactory system may allow unique approaches to explore some of the subtle mechanisms governing the temporally and spatially controlled expression of olfactory receptor genes during development. This approach seems to be particularly promising because *X. laevis* is a favourite model for studying various aspects of vertebrate development (Kay and Peng, 1991). Furthermore, the anatomical development of the olfactory system has been described in detail (Nieuwkoop and Faber, 1956; Reiss and Burd, 1997a). In the present study, we set out to monitor the molecular differentiation of the olfactory epithelium of the frog, with particular reference to the onset and temporal expression pattern of the olfactory marker protein and the two classes of olfactory receptor.

Materials and methods

Animals

Fertilized eggs of *Xenopus laevis* were obtained following injection of human chorionic gonadotropin (HCG; Sigma) into the dorsal lymph sac of adults, as described by Ochse (1948) and Hilken et al. (1995). The tadpoles were reared in a dechlorinated solution of 0.2% NaCl in 501 tanks and fed once a week with a suspension of nettle powder and yeast. The water was replaced and the tanks were cleaned regularly. The staging of the tadpoles was according to Nieuwkoop and Faber (1956).

For tissue preparation, animals were anaesthetized in iced water; in the embryonic stages, the vitelline membrane was removed. For experimental reasons, whole heads were collected from early stages (21–47), whereas the upper jaw was dissected in the later stages of development (from stage 48 onwards). Following preparation, the tissue samples were immediately weighed, frozen in liquid nitrogen and stored at -70°C . Either single animals were analyzed, or pools of 10 or 30 animals were processed for molecular biological analysis.

For the *in situ* hybridization studies, embryos and tadpoles were sedated on ice, decapitated and fixed in MEMFA (0.1 mol l⁻¹ Mops, pH 7.4, 2 mmol l⁻¹ EGTA, 1 mmol l⁻¹ MgSO₄ and 3.7% formaldehyde) for 1–2 h at 20 °C. The fixative was then removed, and the animals were stored in methanol at -20°C until further use. Prior to *in situ* hybridization, the animals were rehydrated in a graded series of methanol (100%, 75%) in water and in phosphate-buffered saline (PBS; 100 mmol l⁻¹ Tris, pH 7.0, 150 mmol l⁻¹ NaCl) (50%, 25%, 0%).

Molecular biological procedures

Synthesis of cDNA

The tissue was resuspended in Trizol (Gibco BRL) and minced using a high-performance sonicator (model W-385, Heat Systems Ultrasonic Inc.). Total RNA was extracted according to the recommendations of the manufacturer (Gibco BRL; Chomczynski and Sacchi, 1987). The total RNA was dissolved in 20 µl of RNAase-free water, and digestion with DNase (DNase I, Gibco BRL) was carried out as described by Freitag et al. (1995). Spectrometric determination of the total RNA concentration was followed by isolation of mRNA according to the manufacturer's specifications (Dynabeads, Dynal, Oslo); mRNA was dissolved in 10 µl of RNase-free water and reverse-transcribed using the first strand cDNA synthesis kit (Pharmacia Biotech) following the manufacturer's protocols.

Polymerase chain reaction (PCR)

To avoid bias in the data to a developmental phase, gene expression was determined in 'single animals' see below.

To compare the amount of template used in each reaction and to standardize the PCRs, empirical tests were performed as described previously (Wilson and Hemmati-Brivanlou, 1995; Wilson and Melton, 1994; Dohrmann et al., 1993; Deutsch Murphy et al., 1990; Rappolee et al., 1988). Briefly,

cDNA derived from a metamorphosing tadpole (stage 57) was diluted in steps of 1:2, so that the amount of template in the undiluted sample corresponded to 5% of the total cDNA of one animal. The dilution series was used in RT-PCRs to determine the optimal cycle number for the different primer pairs used; at the optimal number of PCR cycles, the template abundance had to correspond to the PCR output in the PCRs. The cycle number optimized for each primer set was then used for the developmental study.

In the developmental analysis, 50 µl of the PCR mixture contained 5% of the cDNA from a single animal, 0.2 mmol l⁻¹ of each primer, 0.2 mmol l⁻¹ dNTP, 1.5 mmol l⁻¹ MgCl₂, 2.0 units of Taq (Gibco BRL) and 5 µl of 10× PCR buffer. The PCR was performed using the following conditions: 1 cycle: 2 min at 94 °C, 2 min at 50–60 °C (depending on primers), 4 min at 72 °C; 20–35 cycles: 30 s at 94 °C, 1 min at 50–60 °C (depending on primers), 90 s at 72 °C; final steps: 7 min at 72 °C, 4 °C from then on.

The numbers of cycles were: 20 cycles for ribosomal protein L8 at an annealing temperature of 60 °C; 25 cycles for elongation factor 1α (EF1α) at 60 °C; 35 cycles for *Xenopus* olfactory marker protein (XOMP) at 57 °C, and 35 cycles for *Xenopus* olfactory receptors (XORs) of class I and class II at 50 °C.

Primers

EF1α: sense: 5'-CAG ATT GGT GCT GGA TAT GC-3'; antisense: 5'-ACT GCC TTG ATG ACT CCT AG-3'.

L8: sense: 5'-TTG CAT TCC GTG ATC CTT ACA GG-3'; antisense: 5'-ATC TCT CCT GAT GGT TGA GGG-3'.

XOMP: sense: 5'-CCA AAC CCT CCA GCA AAA GA-3'; antisense: 5'-CAG CGC CAT CTA AGA AAG (AT)G-3'.

Class I olfactory receptors: X2.4 (sense): 5'-AT(ATC)G(CA)(AGC) AA(AG) TA(CT) TGG TT(CT) GG-3'; OR7.1 (antisense): 5'-A(AG)(AGCT) (GC)(AT)(GA) TA(TGA) AT(AG) AA(AGCT) GG(GA) TT-3'.

Class II olfactory receptors: X2.3 (sense): 5'-A(CT)(AC) CC(CT) ATG TA(CT) TT(GCT) (CT)T(CT) CT-3'; OR7.1 (antisense): 5'-A(AG)(AGCT) (GC)(AT)(GA) TA(TGA) AT(AG) AA(AGCT) GG(GA) TT 3'.

The primers used for amplification of XOMP were appropriate for both XOMP1 and XOMP2. Degenerate primers used for the amplification of class I and class II olfactory receptors were the same as those described by Freitag et al. (1995). Thus, the use of these primers should lead to amplification of a similar set of olfactory receptors to those described in that study.

Analysis of the PCR products and densitometry

Southern blot analysis was performed to determine the specificity of the PCR primers and for quantification purposes. PCR products were separated on 1% agarose gels and blotted onto nylon membranes (Hybond N⁺, Amersham) following standard protocols (Sambrook et al., 1989) with 0.4 mol l⁻¹ NaOH as transfer solution. DNA was fixed on the membrane, by baking for 30 min at 80 °C, and hybridized with specific

DIG-labelled probes under high-stringency conditions overnight as described by Engler-Blum et al. (1993). The filters were incubated in anti-DIG-AP Fab fragment antibody (Boehringer Mannheim) and CSPD (Tropix) as described in the *DIG System User's Guide* from Boehringer. Chemiluminescence was detected with X-ray film (Fuji AX).

DIG labelling was carried out by random hexamer priming using the DIG-DNA labelling kit (Boehringer) according to the manufacturer's protocols. Labelled DNA was dissolved in TE (10 mmol⁻¹ Tris, 1 mmol⁻¹ EDTA, pH 7.4) and diluted in prehybridization buffer to a final concentration of 2.5 ng ml⁻¹ per probe. In the case of the olfactory receptors, hybridization mixtures were used. The class I olfactory receptor mix contained probes for the XR116, XR106, XR46 and XB117 receptors (accession numbers Y08347, Y08346, Y08345 and Y08348, respectively) and therefore contained sequences of class I olfactory receptors of *Xenopus laevis* belonging to all subfamilies (Freitag et al., 1995). Under high-stringency conditions, all known class I XORs should be detected by these probes.

The class II olfactory receptor mix included probes for the XR1, XR171, XR181, XR185, XR206 and XR214 receptors (accession numbers Y08203, Y08349, Y08350, Y08351, Y08352 and Y08353, respectively). The class II receptor probes were chosen so that most of the known class II XOR families could be detected under high-stringency hybridization conditions. In control experiments, cross-hybridization of class I blots with class II probes and *vice versa* was not observed, indicating high specificity of the probes for the respective olfactory receptor class. For the detection of L8, EF1 α and XOMP, each DIG-labelled probe was used at a concentration of 2.5 ng ml⁻¹.

Gels or films were analyzed densitometrically using a personal computer with Bioprint, version 96.14, and Bio-Profil (Bio-ID), version V96.15 (Vilber-Lourmat, France). Using the DIG system for relative quantification of PCR products or in northern blots is a commonly used procedure (Hudig et al., 1994; Boelen et al., 1995). For better comparison between the blots and the ethidium-bromide-stained gels, the blots were reversed on computer (Bioprint, Vilber-Lourmat, France).

The densitometric data presented for the two housekeeping genes (L8 and EF1 α) and for XOMP are based on ethidium-bromide-stained gels. All densitometric data presented for the two classes of olfactory receptor are derived solely from Southern blots to guarantee the identity of the PCR bands as olfactory receptors.

In situ hybridization

DIG-labelled antisense RNA probes were generated by SP6 or T7 transcription of the cloned PCR fragments as specified by the manufacturer of the DIG-RNA labelling kit (Boehringer Mannheim). Briefly, 2 μ g of linearized plasmid was transcribed in the presence of 70 nmol of digoxigenin-11-uridine-5'-triphosphate, followed by partial alkaline hydrolysis of the resultant RNA, as described by Angerer and Angerer (1992), to produce fragments of approximately 200–250 base pairs in

length. General XOMP probes were used. For the detection of olfactory receptors, probes for class I receptors included XR116, XR111 and XR117, and probes for class II receptors included Xgen5 and Xgen147 (Accession numbers AJ011429, AJ011430, respectively).

Conditions for *in situ* hybridization to coronal sections of older tadpoles (starting at stage 54) were as described previously (Strotmann et al., 1994a,b; Freitag et al., 1995). Briefly, the fixed head was embedded in Tissue Tec (Miles, Elkhart, IN, USA) and rapidly frozen at -70°C . Sections were cut on a Reichert & Jung cryostat at -24°C , thaw-mounted on superfrost plus slides (Menzel-Gläser, Germany) and air-dried for 30 min. For *in situ* hybridization, slides were hybridized with DIG-antisense RNA probe for 16 h at 55°C and washed twice with $0.1\times$ standard saline citrate (SSC) for 30 min at 60°C . Slides were incubated with anti-DIG-AP antibody (1:750) for 30 min at 37°C , washed twice with TBS (100 mmol⁻¹ Tris, 150 mmol⁻¹ NaCl, pH 7.0), and the antibody was visualized with Nitroblue Tetrazolium (NBT) and bromo-chloro-indolyl phosphate (BCIP) (Biomol, Hamburg, Germany). The sections were air-dried, mounted in Euparal (Roth, Germany) and analyzed under a Zeiss Axiophot microscope.

In early-stage tadpoles (stage 23–53), whole-mount *in situ* hybridization was performed as described by Harland (1996). Briefly, for hybridization, the fixed animals were transferred into small plastic baskets and hybridized with the DIG-labelled probes. After hybridization, the animals were incubated with anti-DIG-AP-antibody (Boehringer Mannheim) and stained with NBT and BCIP as described above. Thereafter, they were dehydrated in 100% methanol for 1 min, rehydrated and embedded in Tissue Tec (Miles, Elkhart, IN, USA), and 10 μ m thick sections were cut coronally or horizontally on a Reichert & Jung cryostat (model 2800E). The sections were air-dried, mounted in Euparal (Roth, Germany) and analyzed under a Zeiss Axiophot microscope.

Results

To determine the mRNA levels of specific genes in the olfactory tissue of *Xenopus laevis* at distinct developmental stages, it was necessary to establish suitable approaches that allow sensitive and reproducible quantification of RNA from small samples of tissue. This is possible using RT-PCR techniques under conditions that ensure a linear quantitative correlation between the initial amount of template and the resulting PCR product (Deutsch Murphy et al., 1990; Dohrmann et al., 1993; Zamorano et al., 1996). RT-PCR with internal or external standards has been widely used for quantification purposes (Delidow et al., 1989; Deutsch Murphy et al., 1990; Margalit and Lancet, 1993; Wilson and Melton, 1994; Zamorano et al., 1996). EF1 α and L8 are generally used as external standards and as controls for RNA isolation and reverse transcription for determining gene expression during the development of *Xenopus laevis* (Dohrmann et al., 1993; Wilson and Hemmati-Brivanlou, 1995), since internal

standards might reduce the detection capacity for the cDNA of interest (Deutsch Murphy et al., 1990).

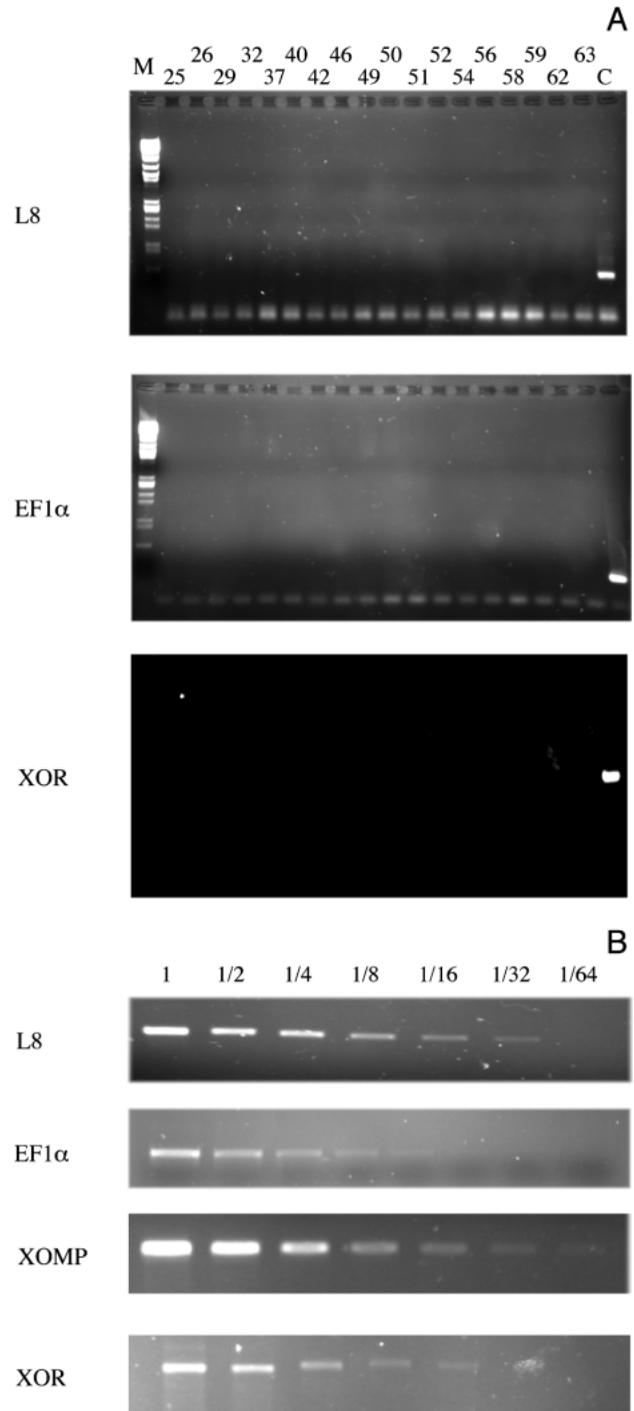
Although the RT-PCR approach is extremely sensitive (Rupp and Weintraub, 1991), it is prone to various pitfalls, one of which might be contamination with genomic DNA. To exclude this possibility, the total RNA of all samples was treated with DNAase I and, after mRNA isolation, all samples were analysed by PCR before reverse transcription. No PCR products were found in any mRNA samples, either in ethidium-bromide-stained gels or after hybridization; positive controls gave strong PCR bands (Fig. 1A). The standardization of the RT-PCRs is demonstrated in Fig. 1B (see Materials and methods). The cycle numbers were optimized for the levels of mRNA; L8 protein was amplified with 20 cycles, EF1 α with 25 cycles and XOMP or the olfactory receptors with 35 cycles of PCR. Under these conditions, even large dilutions could be analyzed in a linear fashion.

To follow the time course of developmental processes in the olfactory system and to control all steps of sample preparation, four different parameters were analyzed: tissue mass, total RNA content and the levels of expression of two housekeeping genes, L8 and EF1 α . Since the olfactory placode is not formed until stage 23/24 (corresponding to approximately 24 h after fertilization), stages 23/24 were the earliest analyzed. mRNAs for the ubiquitously expressed L8 and EF1 α (Shi and Liang, 1994; Krieg et al., 1989) served as controls for RNA extraction and reverse transcription (Fig. 2C,D). A most remarkable feature is the significant increase of all four parameters between stages 54 and 62, i.e. during prometamorphosis and early metamorphosis, and the decrease following stage 62, the metamorphic climax, corresponding to the metamorphic remodelling of the tadpole. The direct correlation between changes in total RNA content and expression of L8 or EF1 α reflect the integrity of the cDNA samples (Dohrmann et al., 1993). Stage 66 marks the end of metamorphosis and corresponds to a juvenile frog. It should be noted that sample preparation changed at stage 47 from the entire head to the upper jaw only (see Materials and methods). This procedure increases the relative fraction of the olfactory epithelium in the tissue samples from that stage onwards.

Fig. 1. Control and standardization experiments. (A) Control for genomic contamination. Reverse transcription/polymerase chain reactions (RT-PCRs) were carried out for all mRNA samples isolated (see text). The Nieuwkoop and Faber (1956) stages of animal samples used in RT-PCRs are indicated above the lanes. No amplification was found in PCRs using mRNA and primer sets for L8, EF1 α and *Xenopus* olfactory receptor (XOR) class II. Using stage 59 cDNA as a positive control (last lane, C), amplification of DNA was observed with all three sets of primers. M, bacteriophage λ markers; the numbers above the lanes indicate Nieuwkoop and Faber (1956) stages of tadpoles. (B) Standardization of the PCR cycle number. RT-PCRs were carried out with stage 57 tadpole cDNA (see text). The correlation between cDNA template concentration and resulting PCR products using specific primers for L8 cDNA after 20 PCR cycles, for EF1 α after 25 cycles and for *Xenopus* olfactory marker protein (XOMP) and olfactory receptors (XOR class II) after 35 cycles of PCR is shown. Dilutions are given above the gels.

A comparison between Fig. 2A and Fig. 2B reveals that the total RNA content is approximately $1 \mu\text{g mg}^{-1}$ tissue mass during all developmental stages. This level is in line with the concentration of total RNA usually found in brain tissue (Chomczynski and Sacchi, 1987).

The olfactory marker protein (OMP), which is a specific indicator for mature olfactory sensory cells (Margolis, 1980), has been an invaluable tool for studying the mammalian olfactory system. Although previous immunocytochemical studies have suggested that OMP may also exist in lower vertebrates (Rama-Krishna et al., 1992), it was only recently



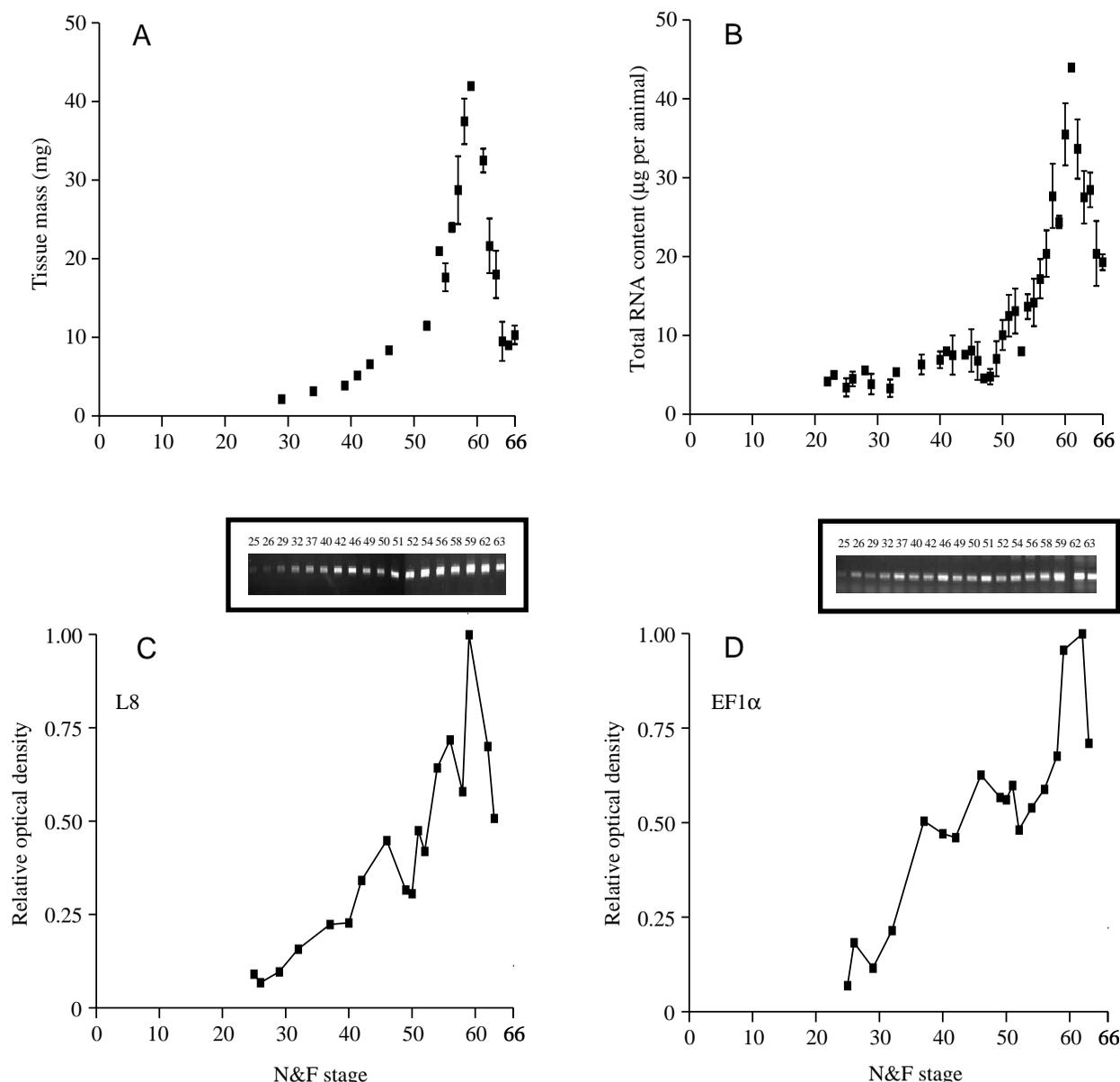


Fig. 2. Tissue fresh mass, total RNA content and housekeeping gene expression during development. (A) Fresh mass of the collected tissue plotted against N&F (Nieuwkoop and Faber, 1956) stage. (B) Amount of total RNA in different developmental stages from stage 21 to stage 66 (juvenile frog). Bars indicate the standard error of the mean (S.E.M.). $N=3-50$ for single stages; at certain stages, pools of 3–10 animals were processed simultaneously. (C) Developmental expression of L8. One representative RT-PCR is shown. The corresponding N&F stages are indicated. The amount of L8 RT-PCR end-product (in arbitrary units) is plotted against stage of development (N&F stage). A value of 1 corresponds to the maximal value of expression; in this case at stage 59. (D) Developmental expression of EF1 α plotted as in C. The maximal value of expression is at stage 62 (see text).

that two OMPs were identified from *X. laevis* (XOMPs) (Rössler et al., 1998). The sequence information allowed us to use specific primers in PCRs to monitor the onset and time course of XOMP expression in *X. laevis* nasal tissue during critical developmental stages. PCR primers were used that allowed us to amplify both XOMPs equally well. As depicted in Fig. 3, mRNA encoding XOMP was first detectable at stage 32. The gradual increase in the level of mRNA until stage 47 was followed by a two- to threefold increase between stage 49 and 51. This two- to threefold increase may be partly due to

the change in tissue preparation from the whole head to the upper jaw at stage 47. The high plateau level until the metamorphic climax at stage 62 was followed by a reduced level during the late metamorphic stages. Experiments using primers specific for XOMP 1 or XOMP 2 indicated the same time course of expression for both XOMPs (data not shown).

To examine the issue of chemospecificity, the onset and temporal pattern of olfactory receptor (XOR) expression were studied. In addition, the expression patterns of the two classes of olfactory receptors were compared. Degenerate primers

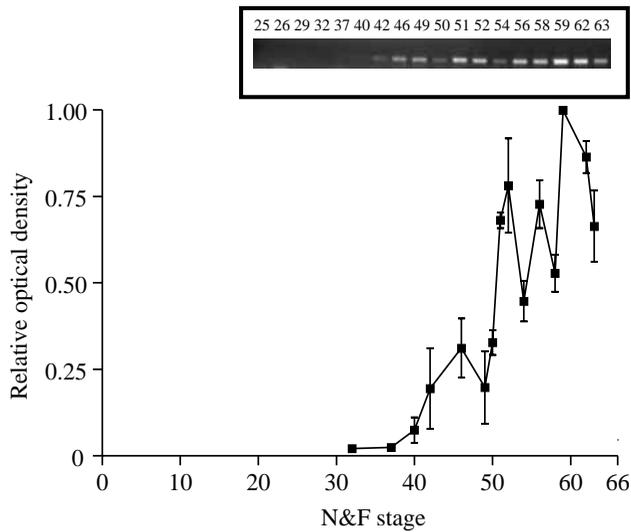


Fig. 3. Expression of *Xenopus* olfactory marker protein (XOMP) at different developmental stages. Arbitrary units of optical density are plotted against Nieuwkoop and Faber (1956) stage (N&F). The maximal value is reached at stage 59 and is given a value of 1. Three different sets of data from two sets of cDNAs are summarized in the graph. The inset shows one representative RT-PCR. The corresponding N&F stages are indicated above the RT-PCR bands. The densitometric data are based on direct measurement of the PCR bands in ethidium-bromide-stained gels. Values are means \pm S.E.M. ($N=3$).

were used to distinguish between class I (fish-like) and class II (mammalian-like) olfactory receptors. PCR approaches have previously been used to monitor the onset of olfactory receptor expression during development in the rat (Margalit and Lancet, 1993). To reinforce the specificity of PCR products, they were hybridized with receptor probes in all cases (see Materials and methods). All densitometric data concerning the XORs are derived from Southern blots. Such Southern blot data (depicted in Fig. 4) indicate that the onset of expression differs significantly between the two receptor classes. For class I receptors, expression was first observed at stage 32. This corresponds to an age of 40h after fertilization and coincides with the onset of XOMP expression (Fig. 3). In contrast, mRNA for class II receptors was not detectable before stage 49, approximately 10 days later. During the prometamorphic phase (stages 54–57), the level of mRNA for both receptor classes rises slightly, whereas during metamorphosis the increase in mRNA levels is steep and reaches its peak at approximately stage 59. This rise is significantly delayed compared with the rise in XOMP expression, which occurs at approximately stage 50. The problem of false negative results, particularly for the class II XORs, was ruled out by using higher template concentrations in control PCRs and by *in situ* hybridization experiments. None of these procedures revealed an expression of class II XOR before stage 49.

The *in situ* hybridization technique was also employed to explore whether the increasing mRNA levels for XOMP and XORs coincide with an increasing number of cells expressing

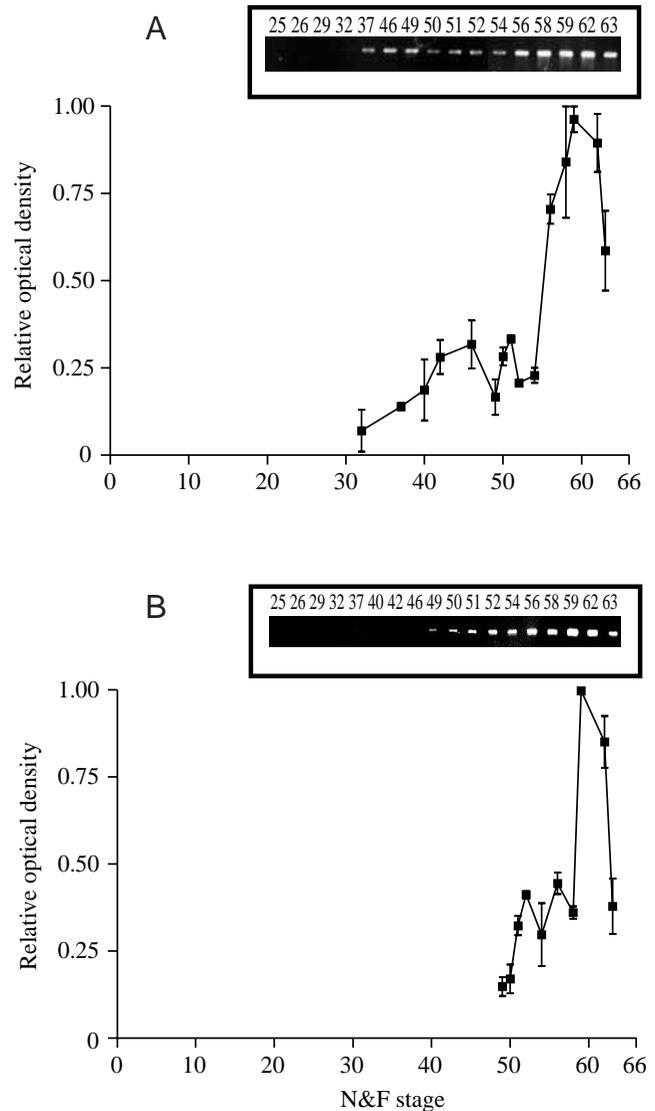
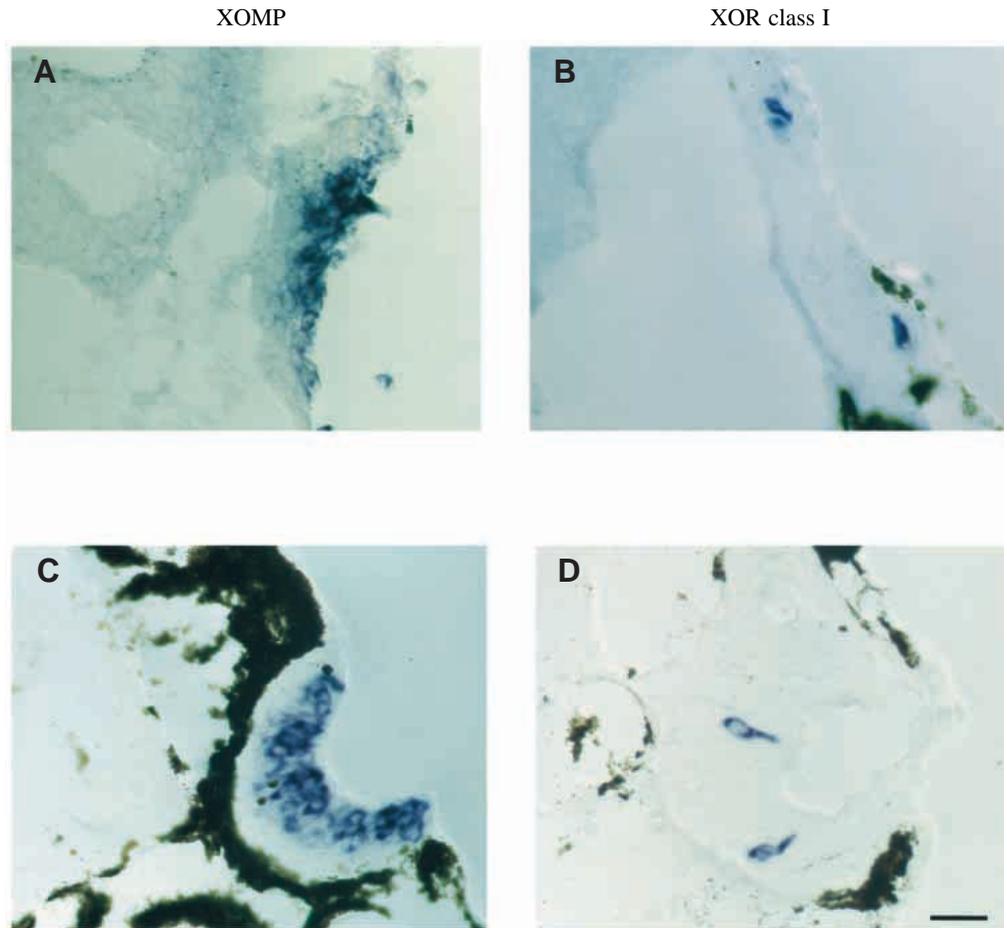


Fig. 4. Expression of class I and class II olfactory receptors at different developmental stages. Three sets of data corresponding to two sets of cDNA preparations are included in the graphs. One representative Southern blot each is shown in each inset. For convenience, the blot is presented as a negative image. The Nieuwkoop and Faber (1956) stages (N&F) are indicated. The densitometric data are based on Southern blots only to verify the identity of the PCR bands as olfactory receptors. (A) Expression of class I (fish-like) olfactory receptors. Class I *Xenopus* olfactory receptor (XOR) expression is first detected at stage 32. A slight increase in expression between stages 32 and 54 is followed by a steep increase in expression during prometamorphosis and early metamorphosis up to the metamorphic climax (see text). (B) Expression of class II olfactory receptors. Orientation and arrangement as in A. In contrast to class I expression, the onset of class II expression occurs at stage 49. The steep increase in expression occurs at stage 58. Values are means \pm S.E.M. ($N=3$).

these genes. In agreement with the low mRNA levels (Fig. 3), only a few XOMP-positive cells exist at stage 37 (Fig. 5A) when the olfactory placode is shallow and small (Koo, 1996).

Fig. 5. *In situ* hybridization of representative stages during early development. Horizontal sections through the olfactory organ at stages 37 (A,B) and 46 (C,D) are shown. The sections in A and C were incubated with a DIG-labelled antisense riboprobe encoding *Xenopus* olfactory marker protein (XOMP). In both stages, specific expression was detectable, with the intensity and number of signals (representing mature olfactory receptor neurones) increasing with the age and size of the tadpole. (B) A representative section of a stage 37 tadpole incubated with an antisense riboprobe against XR117, which represents a class I *Xenopus* olfactory receptor (XOR). Two distinct olfactory neurones are labelled. The dendrite and cell body are clearly labelled. (D) Section of a stage 46 animal also incubated with an XR117 antisense riboprobe. The probe labelled two cells in the sensory epithelium each with a clearly stained cell body and dendrite. Scale bar, 30 μ m.



In agreement to the higher level of expression revealed by RT-PCR, many more XOMP-positive cells were found at stage 46 (Fig. 5C). At both stages, XOMP reactivity can be found throughout the whole epithelium. In contrast, as shown for example for XR117, class I receptor subtypes were expressed in only very few cells, both at stage 37 (Fig. 5B) and at stage 46 (Fig. 5D). However, owing to the larger surface area of the epithelium, there are more XR117-positive cells in stage 46 than in stage 37 animals. Interestingly, in these early stages, mRNA was also detected in the cellular processes, notably in the dendrites.

To explore the spatial distribution patterns of XOMP and XORs during the critical phase between stage 51 and 59, when the lateral and medial diverticulum of the nasal cavity are formed (Weiß, 1986), serial sections of these stages were analyzed by *in situ* hybridization. Two different probes were used for both the class I receptors (XR116, XR117) and the class II receptors (Xgen5, Xgen147). Fig. 6 shows consecutive sections through the nasal cavity of a stage 56 tadpole. An invaginating fold develops at the medial axis of the ventral epithelium (indicated by arrows), eventually separating the two compartments. XOMP-positive cells can be found throughout the entire olfactory epithelium in this section, and the ventral area of the epithelium is lightly labelled (Fig. 6A). As shown here for XR116, cells expressing class I receptors are restricted

to the lateral region of the epithelium; the developing lateral diverticulum (Fig. 6B, arrowheads). Neurones expressing class II receptors, as indicated for Xgen147, are restricted to the medial part, close to the developing nasal septum (Fig. 6C).

The expression patterns of the two classes of olfactory receptor during the segregation of the main nasal cavity from a single principal cavity into the two compartments (lateral diverticulum, LD; medial diverticulum, MD) is shown schematically in Fig. 7. Three representative stages during this developmental process are shown. The schematic drawings represent two-dimensional reconstructions of the right three-dimensional nasal cavity, viewed dorsally. Only the main olfactory epithelium, lining the principal cavity, is depicted; the vomeronasal organ is located more ventrally and is not shown here. Fig. 7A displays the two-dimensional projection of XOMP-positive regions. In the early stages of development, until stage 48, XOMP-expressing olfactory cells are distributed throughout the entire nasal epithelium. Between stages 51 and 59, the olfactory epithelium undergoes considerable growth and remodelling; the ventral region of the epithelium, particularly, is expanding. In this zone, a thin, XOMP-negative epithelium is apparent, possibly a proliferation zone. As the whole olfactory epithelial surface grows, this region broadens. From the caudal to the rostral limits, the cells in this new zone mature and express XOMP and XORs; the initially spacious

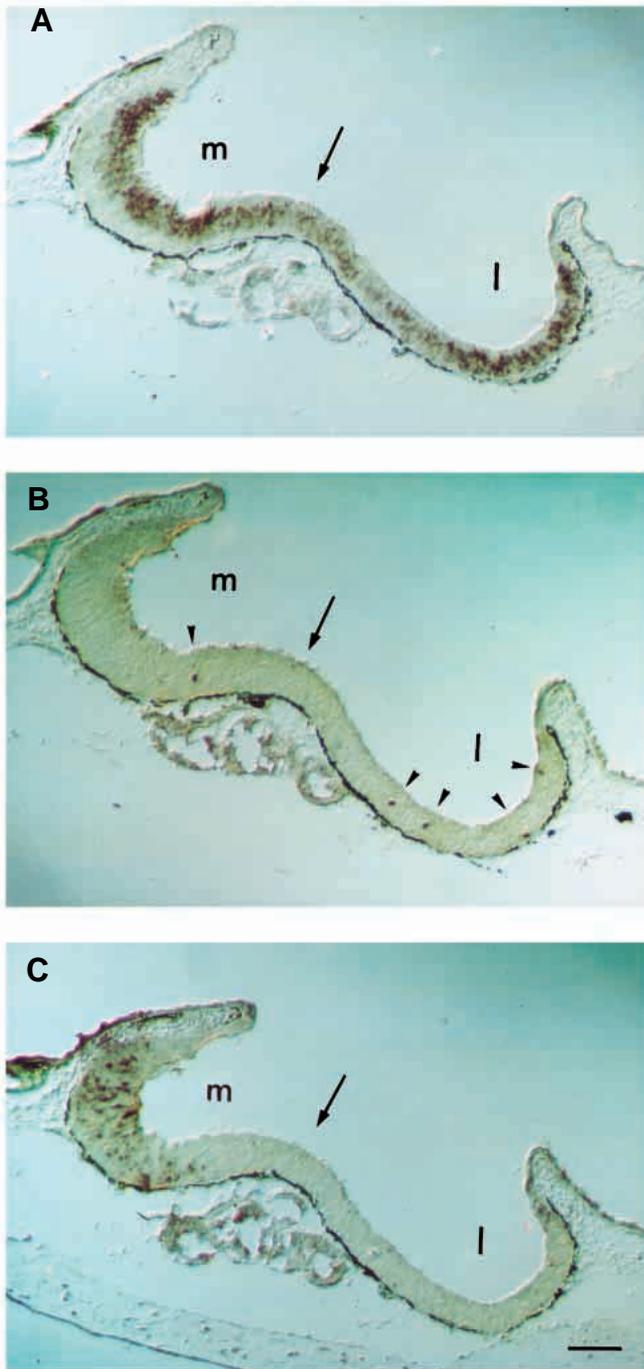


Fig. 6. *In situ* hybridization of a stage 56 tadpole. Three consecutive coronal sections through the olfactory epithelium of a stage 56 tadpole, incubated with *Xenopus* olfactory marker protein (XOMP) (A), XR116, a representative class I *Xenopus* olfactory receptor (XOR) (B), and Xgen147, a representative class II XOR (C) are shown. The developing fold in the epithelium is highlighted by an arrow. (A) The whole olfactory epithelium is labelled with XOMP, representing mature olfactory receptor neurones. The expression of XORs is segregated into different zones at this stage. (B) Class I XOR expression is located in the ventrolateral region of the epithelium (arrowheads). (C) Class II XOR expression is located in the medial zone of the epithelium. In this stage, an overlap of receptor expression does not exist. m, medial part of the principal cavity; l, lateral part of the principal cavity. Scale bar, 50 μ m.

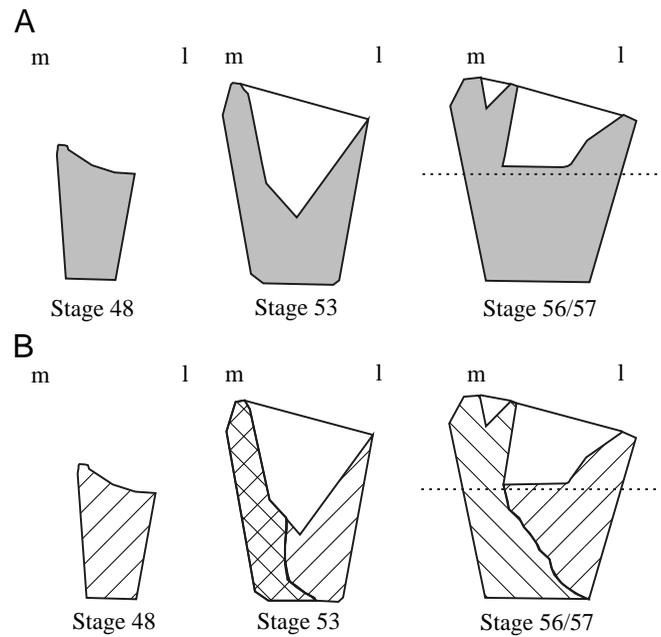


Fig. 7. Morphological changes in the olfactory epithelium during the reorganization into the adult system during pre- and prometamorphosis. (A) Dorsal view of schematic two-dimensional representations of the three-dimensional right nasal cavity at three representative developmental stages (N&F stages 48, 53 and 56/57) showing the expression of *Xenopus* olfactory marker protein (XOMP) (dotted). (B) Same projection as in A, but showing the expression of class I (top right to bottom left hatching) and class II (top left to bottom right hatching) *Xenopus* olfactory receptors (XORs). An overlap of expression of the two classes can be seen at approximately stage 53 in the medial zone of the epithelium, whereas the expression is clearly segregated in stage 56/57 tadpoles. m, medial, l, lateral. The dotted line drawn at stage 56/57 indicates the approximate location of the coronal sections shown in Fig. 6. At stage 56/57, the principal cavity has partially segregated into the prospective lateral diverticulum and the prospective medial diverticulum. The vomeronasal organ remains essentially unchanged and is not depicted here.

XOMP-negative region is largely replaced by a sensory epithelium.

Fig. 7B shows the XOR-positive areas. At stage 48, only class I XOR-expressing cells are found, and they are distributed throughout the whole olfactory epithelium. From stage 49 onwards, class II olfactory receptors are expressed. Cells expressing class II XORs are located in the medial region of the epithelium, close to the developing septum. During the earlier stages (up to stage 53/54), a co-localization of both XOR classes can be found in the medial region of the PC. In the following stages, cells expressing class I receptors disappear from this medial zone and are subsequently restricted to the lateral region. The olfactory epithelium grows considerably following stage 48, and many of the novel cells seem to express class II receptors.

An overall picture of the onset and time course of expression of olfactory-specific genes during development of *X. laevis* is

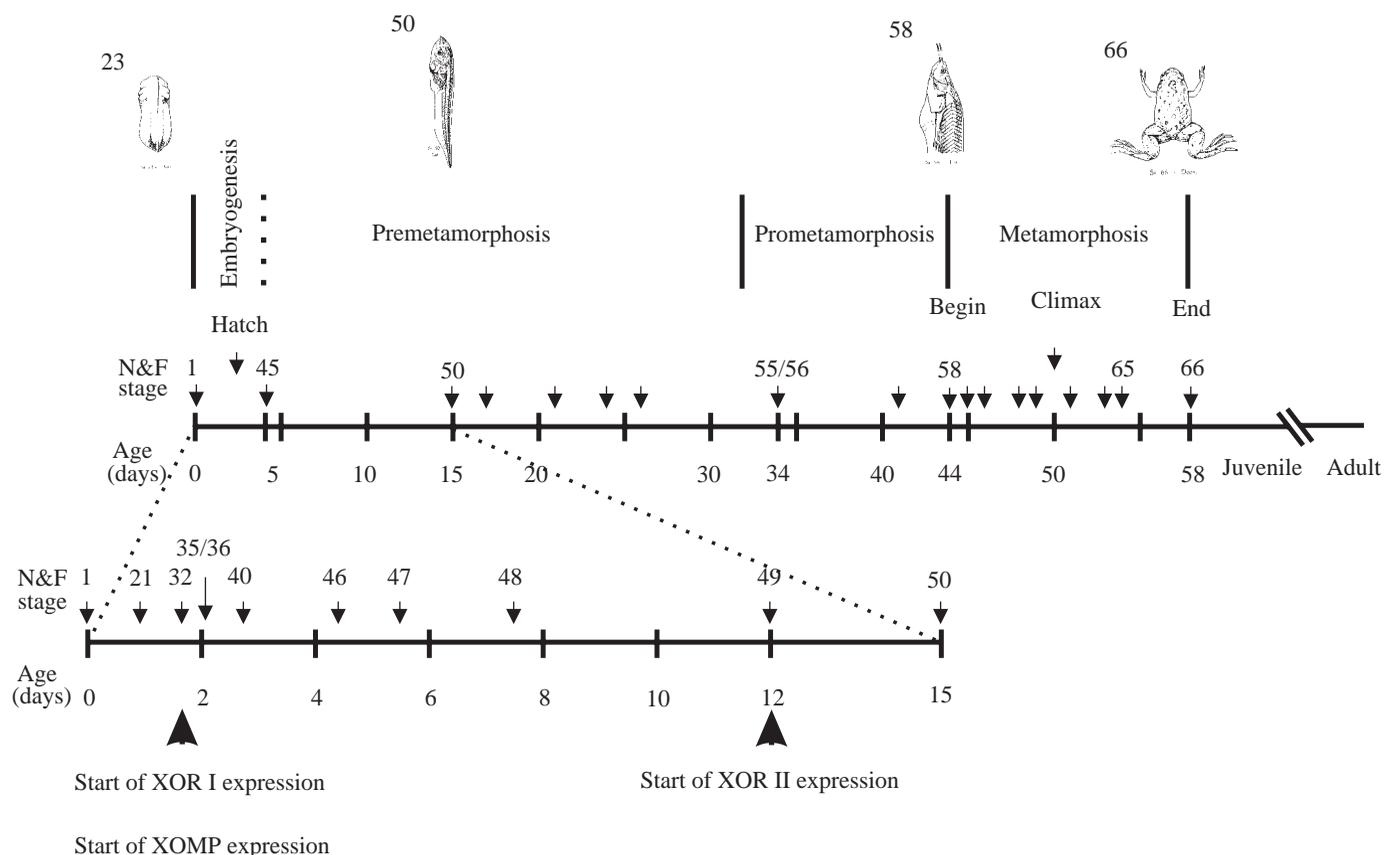


Fig. 8. Onset and time course of expression of olfactory-specific genes during development of *Xenopus laevis*. Schematic drawings of tadpoles (stages 23, 50, 58 and 66) are shown (adapted from Nieuwkoop and Faber, 1956). The drawings are not to scale. The time scale is delineated in days, such that one section in the upper scale represents 5 days, unless otherwise indicated. The age of the animals is shown below the time scale (age in days). The age corresponds to laboratory conditions at a rearing temperature of 20 °C. Small arrows above the time scale represent Nieuwkoop and Faber (1956) stages (N&F stages). A magnification of the time scale during early development is shown below. Representative N&F stages are indicated. The large arrowheads delineate the beginning of class I olfactory receptor expression, *Xenopus* olfactory marker protein (XOMP) expression and class II *Xenopus* olfactory receptor (XOR) expression, as explained in the text.

depicted in Fig. 8. The Nieuwkoop and Faber stages (N&F) are correlated with ‘normalized days’. The small arrows symbolize N&F stages; from stage 50 on, each stage is indicated by an arrow, some also highlighted with the stage number. The early developmental phase, from stage 1 (fertilization, begin of embryogenesis) to stage 50 (early premetamorphosis), is magnified on a separate time scale. First analyses were performed using stage 23/24, corresponding to 24 h after fertilization. The expression of XOMP and of class I XORs is initiated quite early in development, at stage 32. The expression of class II XORs, which are probably responsible for the detection of airborne odorants, occurs some 10 days later, at approximately stage 49.

Discussion

In adult *Xenopus laevis*, the peripheral olfactory system shows a tripartite organization (Altner, 1962) consisting of the vomeronasal organ (VNO) and the main olfactory system, which is itself subdivided into the medial (MD) and lateral

(LD) diverticula. In adult *X. laevis*, two classes of olfactory receptor (XOR) are expressed, each in a distinct diverticulum. Class I XORs are expressed in the LD, whereas class II XORs are found only in the MD (Freitag et al., 1995). The results of the present study indicate temporal and regional differences in the pattern of expression for XOMP and for the two classes of XOR during embryogenesis. XOMP expression and class I XOR expression can be detected for the first time at approximately stage 32 (Figs 3, 4), when the olfactory placode develops into a functional olfactory organ and the first axons reach the forebrain (Koo, 1996). As has been demonstrated previously (Nef et al., 1992; Strotmann et al., 1995) in rodents, the expression of olfactory receptors precedes the innervation of the bulb. In contrast, in zebrafish, the first expression of olfactory receptors has been demonstrated 30–31 h after fertilization (Byrd et al., 1996; Barth et al., 1996, 1997); this is after the axons have reached the bulb primordium (Hansen and Zeiske, 1993), which occurs approximately 24 h after fertilization. Receptor expression in *X. laevis* coincides with the innervation of the bulb primordium at stage 32 (Koo,

1996). The sensory cells reach morphological maturity at approximately stage 37/38 in *X. laevis* (Nieuwkoop and Faber, 1956).

The late expression of mammalian-like receptors at stage 49 may be part of the developmental program preparing the animal for its 'amphibious' lifestyle. Receptor expression slightly precedes the formation of choanae at stage 50 (Nieuwkoop and Faber, 1956) and the segregation of the principal cavity (PC) into the MD and LD, which begins at approximately stage 51 (Weiß, 1986). Whereas cells in the lateral region of the olfactory epithelium express exclusively class I XORs, cells in the medial zone expressing class I and class II XORs are intermingled until stage 54. The lateral region remains positive for XOR class I and never shows reactivity for XOR class II during the entire restructuring process. Later, cells expressing class I XORs disappear from the medial region, the prospective MD. Previous studies employing horseradish peroxidase (Weiß, 1986) and soybean agglutinin staining (Key, 1986; Meyer et al., 1996; Reiss and Burd, 1997b) have shown that the projections also gradually segregate during this developmental phase. At stage 52, when the first axons from the LD reach the bulb, an overlap of the projections from the LD and MD was observed. At stage 59, all neurones in the MD send their axons to the newly formed dorsomedial part of the bulb, whereas the LD neurones still send their axons to the developmentally older ventrolateral part of the olfactory bulb (Fritz et al., 1996; Reiss and Burd, 1997a,b).

Previous studies led to conflicting views concerning the origin of the LD. While some data support the idea that the PC is segregated into the LD and MD by a ridge in the lumen of the olfactory organ (Nieuwkoop and Faber, 1956; Venus, 1996), some investigators have stressed that the LD develops *de novo* from cells of undetermined origin (Altner, 1962; Föske, 1934). In a recent study, it has even been suggested that the LD may derive from the VNO, on the basis of soybean agglutinin labelling similarities between the two compartments (Meyer et al., 1997). Our findings do not support the idea that the LD develops *de novo* or that it may derive from the VNO. Rather, the results of this study suggest that the prospective MD of the adult frog develops anew during the prometamorphic and metamorphic stages, whereas the LD represents the former principal cavity of the early tadpoles. The two compartments are separated between stages 49 and 59 by a semicircular ridge developing in the PC, and a functional reconstruction of the medial part (MD) occurs at the same time.

Similar to the different onset of expression revealed for the two classes of XORs in *X. laevis*, a temporal pattern of olfactory receptor expression at different developmental stages has also been found in zebrafish and chick (Barth et al., 1996, 1997; Nef et al., 1996). Some olfactory receptor genes are expressed early, whereas others are not expressed until later stages. In all cases, the onset of expression requires time-related cues, which have different effects on the expression of distinct receptor types. A major difference between the development of zebrafish and of *X. laevis* is the specific role

of thyroid hormones during the metamorphosis of amphibians. The regulatory mechanisms may differ between these two animal species, particularly with regard to class II XORs, the appearance of which coincides with the first detection of thyroid hormone in the blood of the tadpole (Tata et al., 1993). This observation supports the idea that thyroid hormones might play a key role in controlling the expression of XORs in *X. laevis*.

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