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Spatial pattern of receptor expression in the olfactory epithelium

(guanine nucleotide binding protein-coupled receptors/odor coding/olfaction/olfactory neurons/bilateral symmetry)

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ABSTRACT **A PCR-based strategy for amplifying putative receptors involved in murine olfaction was employed to isolate a member (OR3) of the seven-transmembrane-domain receptor superfamily. During development, the first cells that express OR3 appear adjacent to the wall of the telencephalic vesicle at embryonic day 10. The OR3 receptor is uniquely expressed in a subset of olfactory cells that have a characteristic bilateral symmetry in the adult olfactory epithelium. This receptor and its specific pattern of expression may serve a functional role in odor coding or, alternatively, may play a role in the development of the olfactory system.**

The olfactory system combines broad selectivity with extreme sensitivity for the detection and discrimination of a large number of odorants. A combination of two cellular mechanisms might form the basis for odor discrimination: (*i*) selectivity by a limited set of olfactory receptors in each sensory neuron and (*ii*) spatio-temporal patterns of action potentials in cells projecting from the olfactory epithelium to the olfactory bulb. In vertebrates, odorants exert their actions by modulating the excitability of sensory neurons located in an epithelium in the nasal airways (1–4). Peripheral odor-induced activity is transmitted directly to the mitral cells in the olfactory bulb where it is integrated and relayed to other regions in the central nervous system (CNS) (5). Olfactory cilia on the apical ends of neuronal dendrites are embedded in a mucus layer that separates the olfactory epithelium from the external air. Signal transduction is initiated when odorants interact with specialized receptors in the ciliary membranes (6–8). *In vitro*, odorants can rapidly stimulate guanine nucleotide binding (G) protein-coupled second messenger pathways for both cAMP and inositol trisphosphate (9), suggesting that at least two classes of G-protein-coupled receptors are expressed by olfactory neurons. A group of 10–18 candidate olfactory receptors that appear to define a class in the G-protein-coupled receptor superfamily was identified in the olfactory epithelium (10). The tissue specificity and the large number of genes for these receptors suggest that they are responsible for the detection of numerous odorants, a hypothesis that awaits confirmation. Recently, members of a putative olfactory receptor gene family have been identified in mammalian germ cells, suggesting a broader range of function for these receptors (11).

To understand the molecular and cellular basis of odor discrimination, we adopted a strategy to isolate olfactory-specific receptor genes and to study their expression in the olfactory epithelium. In this report, we have characterized a G-protein-coupled receptor (OR3)¶ and its gene expression. The OR3 pattern of expression is symmetric within and specific to the olfactory epithelium. We present evidence that the first OR3-positive cells appear to originate from the CNS, suggesting that this receptor might serve a functional role not only in odor coding but also in the elaboration of specific pathways between the olfactory bulb and olfactory epithelium during development.

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MATERIALS AND METHODS

Genomic Screening and PCR Conditions. A mouse genomic library prepared in the λ phage EMBL3 was screened at high stringency [hybridization at 68°C in 5× standard saline citrate (SSC) and washing at 68°C in 0.2× SSC] with a ^{32}P-labeled probe encoding amino acids 121–255 of rat OR3 derived from a PCR consisting of 10% of a random-hexamer-primed first-strand cDNA reaction mixture, synthesized with 1 μg of poly(A)$^+$ mRNA from rat olfactory epithelium. The primer sequences used were derived from transmembrane domain conserved regions, TMIII (I, S/A, L/I/V/F, D, R, Y) and TMVII (W, L/F, G/C, Y, V/I/L, N, S), of other G-protein-coupled receptors. An *Eco*RI restriction site was added to the 5′ end of TMIII oligonucleotides (26-mer sense primers, 5′-GGGAATTCATC/TG/TCI′I′TI′GAC/TC/AGI′TAT-3′) and a *Hin*dIII restriction site was added to the 5′ end of TMVII oligonucleotides (29-mer antisense primers, 5′-GGAAGCTTG/AGAG/AC/TTI′AI′G/ATAI′CC/AI′AG/ACCA-3′) to facilitate subcloning of the PCR-generated DNA fragments. Both pools of primers contained inosines (I′) at positions of high degeneracy. The PCR setting was designed to maximize heterologous annealing during the first 5 cycles (30 s at 94°C; 30 s at 37°C, followed by a slow temperature ramp reaching 72°C in 180 s; and 90 s at 72°C) and was continued at standard conditions during the next 35 cycles (30 s at 94°C; 30 s at 50°C, followed by a fast temperature ramp reaching 72°C in 30 s; and 90 s at 72°C). The rat OR3 sequence was isolated from the PCR mixture by subcloning the PCR products into pBluescript vectors and by DNA sequencing of the subclones. By screening the mouse genomic library with the rat PCR probe, 10 positive recombinant phages were isolated, purified, and characterized by restriction analysis and by DNA sequencing (12).

Genomic Blot Analysis. 3T3 mouse genomic DNA was electrophoresed on a 0.5% agarose gel, transferred to nylon membrane, and hybridized at 68°C with the entire coding sequence of mouse OR3 that had been ^{32}P-labeled. The blot was washed at 68°C in 0.2× SSC, and autoradiography was performed with an intensifying screen for 7 days at −70°C.

Abbreviations: CNS, central nervous system; G protein, guanine nucleotide binding protein; 7-TMD, seven-transmembrane domains; E, embryonic day; P, postnatal day; OMP, olfactory marker protein.

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¶The nucleotide sequence reported in this paper has been deposited in the GenBank data base (accession no. M84005), and the amino acid sequence has been deposited in Swiss-Prot data base (accession no. P23275).

RNA Blot Analysis. Total RNA from diverse tissues was electrophoresed on a 0.8% formaldehyde/agarose gel and transferred to Nytran (Schleicher & Schuell). The blot was hybridized with the ^{32}P-labeled rat OR3 PCR fragment at 42°C in presence of 50% formamide and washed at 65°C in 0.2× SSC. Autoradiography was performed with an intensifying screen for 5 weeks at −70°C.

***In Situ* Hybridization.** Sections were prepared and hybridized as described (13). Single-stranded antisense RNA probes were produced by *in vitro* transcription with T3 or T7 RNA polymerase (Stratagene) from linearized pBluescript containing the entire rat OR3 PCR fragment [400 base pairs (bp)], the 5′ end (180 bp), or the 3′ end (220 bp).

RESULTS

To isolate members of the seven-transmembrane-domain (7-TMD) receptor superfamily, we used the PCR technique (14) to amplify sequences from rat olfactory epithelium cDNA. The amino acid sequences of 24 7-TMD receptors were aligned and two conserved regions in transmembrane domains TMIII and TMVII were identified. Highly degenerate oligonucleotides derived from these two domains served as sense and antisense primers in the PCR. After 40 cycles, a PCR product of 0.42 kilobase (kb) was identified in olfactory epithelium; no PCR products of this size were seen in tissue samples from tongue or cerebellum. After complete digestion with restriction enzymes *Eco*RI and *Hin*dIII, the 0.42-kb PCR fragment (OR3) was subcloned into pBluescript (Stratagene) and its nucleotide sequence was determined. The hydrophobicity profile (15) of the amino acid sequence revealed the presence of three hydrophobic domains corresponding to TMIV, TMV, and TMVI of a 7-TMD receptor. Thus the rat OR3 PCR fragment encoded amino acids 121–255 of a 7-TMD receptor (Fig. 1).

Most of the known genes encoding 7-TMD receptors are intronless (16). Therefore, to obtain the complete coding sequence of OR3, we screened a mouse genomic DNA library with the rat OR3 PCR fragment and isolated a genomic DNA fragment encoding a single open reading frame of 312 amino

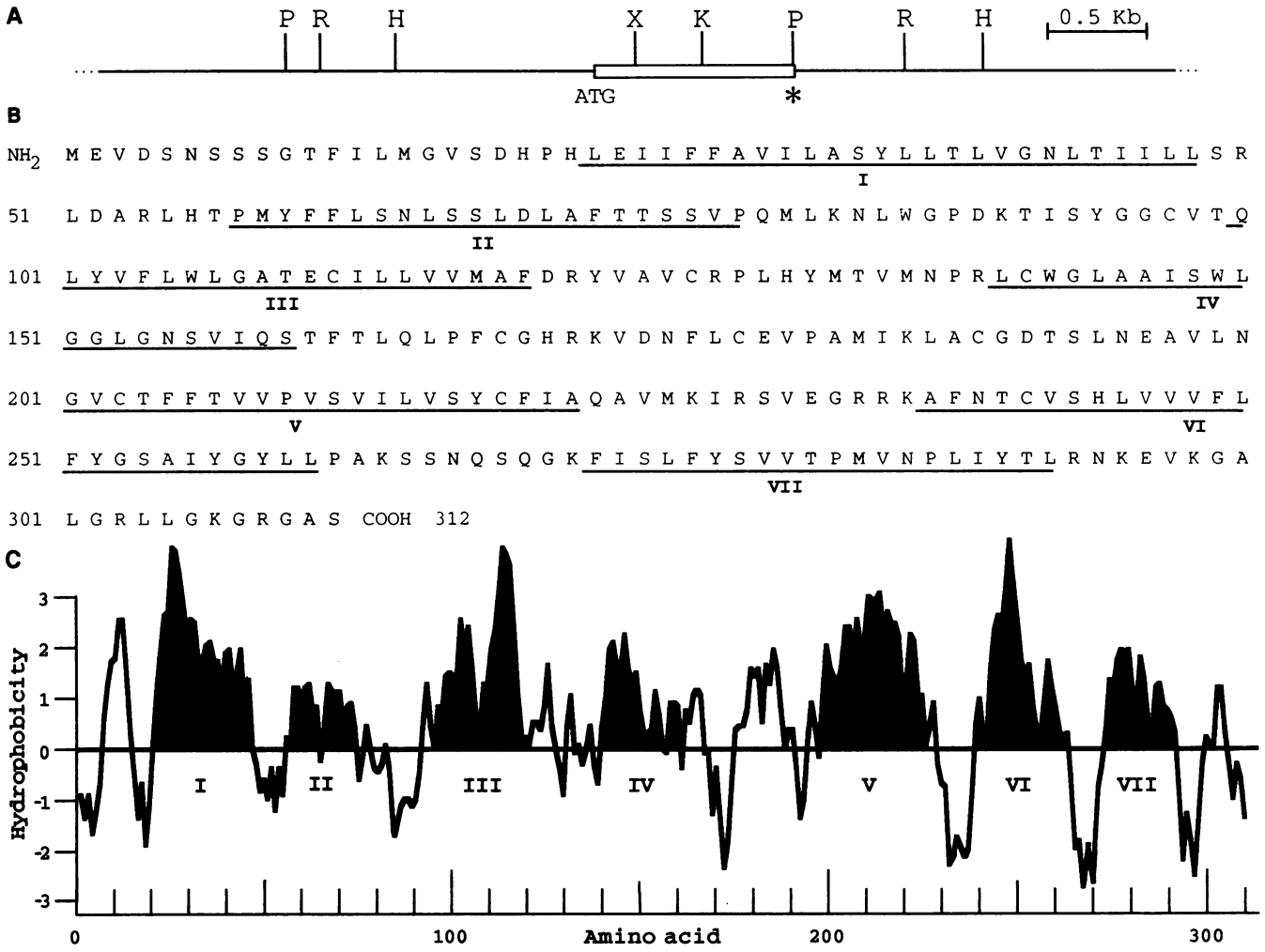

FIG. 1. Gene structure, amino acid sequence, and hydrophobicity profile for mouse OR3. (*A*) Restriction map of a mouse genomic DNA fragment encoding OR3. The positions corresponding to the beginning (ATG) and the end (*) of the coding region are indicated. H, *Hin*dIII; K, *Kpn* I; P, *Pvu* II; R, *Eco*RI; X, *Xba* I. (*B*) The deduced amino acid sequence of mouse OR3. Seven hydrophobic regions (I–VII) were assigned on the basis of hydrophobicity analysis. The calculated molecular weight of the 312-amino acid protein is 34,307; the theoretical isoelectric point of the protein is 8.65. The N-terminal deduced amino acid sequence has the same length as the other olfactory-specific receptors (9). Potential N-glycosylation sites (NXT/S) are located at positions 6, 42, 65, and 267. Potential phosphorylation sites were noted for protein kinase C (S/TXR/K) at position 291 and for casein kinase II (S/TXXD/E) at positions 49, 67, and 193. The rat OR3 PCR fragment encoding amino acids 121–255 has 97% identity with mouse OR3, differing by 4 amino acids at positions 143 (V), 208 (A), 211 (L), and 213 (I). Alignments with the amino acid sequence derived from representative of the other class of olfactory receptor family indicated that OR3 has 38% identity to clone F5, 39% to I15, 40% to F12, and 41% to I3. (*C*) Hydrophobicity profile of mouse OR3 using the Kyte–Doolittle algorithm (15). The seven shaded areas represent the putative transmembrane hydrophobic domains.

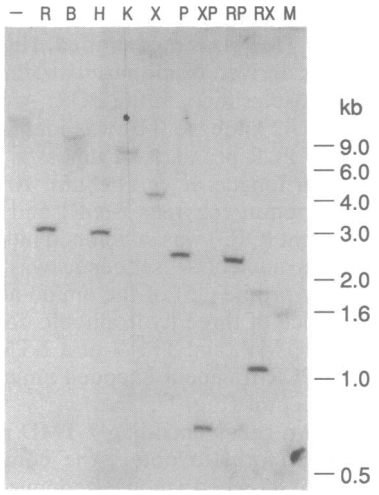

FIG. 2. OR3 genomic DNA analysis. 3T3 mouse genomic DNA (10 μg) was digested with restriction enzymes *Eco*RI (R), *Bam*HI (B), *Hin*dIII (H), *Kpn* I (K), *Xba* I (X), *Pvu* II (P), *Xba* I and *Pvu* II (XP), *Eco*RI and *Pvu* II (RP), *Eco*RI and *Xba* I (RX), or without restriction enzymes (–). Positions of the size markers (lane M) are indicated.

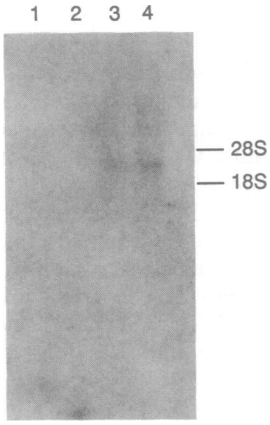

FIG. 3. Tissue distribution of OR3 mRNA in rats. Total RNA (20 μg) from liver (lane 1), brain (lane 2), and olfactory epithelium from a newborn (lane 3) and an adult (lane 4) was hybridized with the ^{32}P-labeled rat OR3 PCR fragment. The positions corresponding to 28S (4785 bases) and 18S (1869 bases) rRNA are indicated.

acids (Fig. 1). The mouse OR3 coding sequence from the start codon (ATG) (17) to the stop codon (TGA) was 939 bases and, as anticipated, had no introns. The hydrophobicity profile of the deduced amino acid sequence revealed the presence of seven hydrophobic domains. The mouse OR3 amino acid sequence exhibits similarity to other G-protein-coupled receptors such as putative olfactory receptors (38–41% identity) (10), the serotonin 5HT2 (21%), 5HT1C (22%), and 5HT1A (24%) receptors, and opsin (22%). The amino acid positions at the beginning of TMII (residues 55–71) and at the end of TMIII (residues 115–129) and TMVII (residues 286–295) show the highest score of sequence identity in alignments with other G-protein receptors. If the correct topological organization of the OR3 receptor consists of seven transmembrane regions, then these conserved domains face the cytoplasmic side of the cell membrane and could interact with common regulatory proteins (G proteins, kinases, or phosphatases) present in the olfactory epithelium.

The olfactory system might produce numerous odor receptors by genetic recombination mechanisms similar to those used by the immunoglobulin system (1). Genomic DNA analysis, however, reveals that a single gene hybridized to the OR3 probe in the mouse (Fig. 2) and rat (data not shown) genome. The similar restriction patterns deduced from the mouse genomic DNA blot (Fig. 2) and the recombinant DNA phage analyses (Fig. 1*A*) suggest an intronless receptor gene. A preliminary chromosomal mapping experiment demonstrated that OR3 does not map on either the human X or Y chromosomes.

The specificity of expression of OR3 mRNA was examined in various tissues. An RNA species of ≈3 kb was detected on a blot of total olfactory epithelium RNA (Fig. 3, lanes 3 and 4) after long exposure times, suggesting that OR3 expression is very low. No signal was observed with RNA from liver (lane 1) or brain (lane 2). The olfactory-specific expression of OR3 mRNA was analyzed by *in situ* hybridization (Figs. 4 and 5). Sagittal, coronal, and horizontal sections were prepared from mouse embryonic stages embryonic day (E) 8 through E19, postembryonic stages postnatal day (P) 0, P6, P12, and P21, and adult (16). Adjacent sections were hybridized with (*i*) the antisense RNA (400 bases) derived from the entire rat OR3 PCR fragment, (*ii*) the antisense RNA (180 bases) derived from the 5′ OR3 PCR, and (*iii*) the antisense RNA (220 bases) derived from the 3′ OR3 PCR. They produced comparable results. The OR3 transcripts are present only in the olfactory epithelium and exhibit a bilateral symmetric pattern (Figs. 4 *E* and *H* and 5). The same pattern is reproducible across animals (10 subjects, male or female, were tested). No signal was detected in any other tissue at embryonic or postembryonic stages, except from bone and cartilage, which is presumably background (compare Figs. 4*H* and 5).

OR3 was first detected at E10 (Fig. 4 *A–C*) in a compact group of cells located below the lateral ventricles of the telencephalon (only one side shown). At E11 the labeling had a similar distribution but was more intense (data not shown). This region of the telencephalon corresponds to the primordium of the olfactory bulb (18). At E10 and E11, the OR3-positive cells are distributed along the curvature of the base of the telencephalic epithelium and are not in the primitive olfactory epithelium (or nasal pit). From E12–E13 until adult stages, OR3 transcripts were detected only in the developing or mature olfactory epithelium (Fig. 5). In contrast to the growing number of olfactory neurons (reaching 10–100 million cells at P12), the increase of OR3-positive olfactory neurons was not substantial (Fig. 4 *D–G*). We estimated that ≈10^3 cells were OR3-positive in a P12 mouse by counting a complete series of adjacent coronal sections (P0 or P21 sections contained comparable numbers of OR3-positive cells). In contrast to the restricted distribution of OR3, the entire population of olfactory neurons shows hybridization with olfactory-neuron-specific probes such as OMP (19) (Fig. 4*K*) or the olfactory-specific G protein G_{olf} (20) (data not shown).

Histological studies (21) have shown that the olfactory epithelium contains three cell types: (*i*) the sustentacular (or supporting) cells whose ovoid nuclei reside mainly in the upper layer of the epithelium, (*ii*) the olfactory neurons whose round nuclei are mainly in the middle layer, and (*iii*) the basal (or precursor) cells whose nuclei form the deepest layer. OR3 transcripts were detected primarily in the middle layer of the epithelium corresponding to olfactory neuron nuclei (Fig. 4 *I* and *F*; for control with OMP, see Fig. 4*L*). These data suggest that OR3-positive cells are olfactory neurons, but they could also represent a subpopulation of supporting cells or basal cells whose nuclei are displaced from their normal location. Additional evidence for a neuronal expression of this receptor was obtained by promoter analysis of the 5′ flanking region of the OR3 gene. DNA sequence analysis of a 3000-bp fragment upstream of the ATG start codon (data not shown) indicated that in addition to canonical TATA (at position −170) and CCAAT (at position −590) box consensus sequences, the OR3 promoter

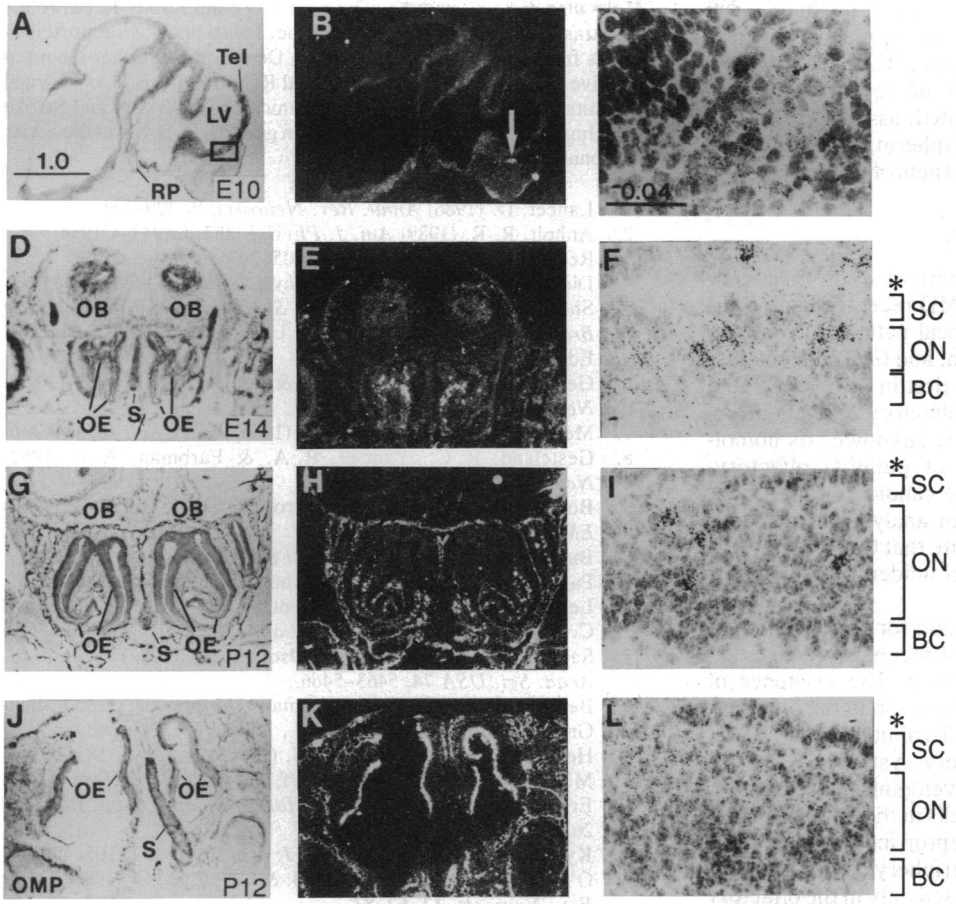

FIG. 4. OR3 *in situ* hybridization to the murine olfactory epithelium during development. Thick sections (10 μm) were cut from mouse heads at E10 (sagittal) (*A–C*), E14 (coronal) (*D–F*), and P12 (coronal) (*G–I*) and were processed for *in situ* hybridization with rat OR3 antisense RNA probes. OR3 in the adult gave comparable results to P12. Control sections from P12 (*J–L*) were prepared with OMP antisense RNA. Bright-field (*A*, *D*, *G*, and *J*) and dark-field (*B*, *E*, *H*, and *K*) photomicrographs of the same sections at the same magnification are shown. (Bar in *A* = 1.0 mm.) Bright-field images from each section (*C*, *F*, *I*, and *L*) at higher magnification illustrate the distribution of silver grains. (Bar in *C* = 40 μm.) The enlarged view of the rectangle in *A* is shown in *C*. The white arrow in *B* indicates the group of OR3-positive cells (also enlarged in *C*). The nuclear layer of the supporting cells (SC), olfactory neurons (ON), and basal cells (BC) and the surface of the epithelium exposed to air (*) are indicated. Cartilage and bone presumably contribute to the background (*H* and *K*). Tel, telencephalon; LV, lateral ventricle; RP, Rathke's pouch; OB, olfactory bulb; S, nasal septum; OE, olfactory epithelium; OMP, olfactory marker protein.

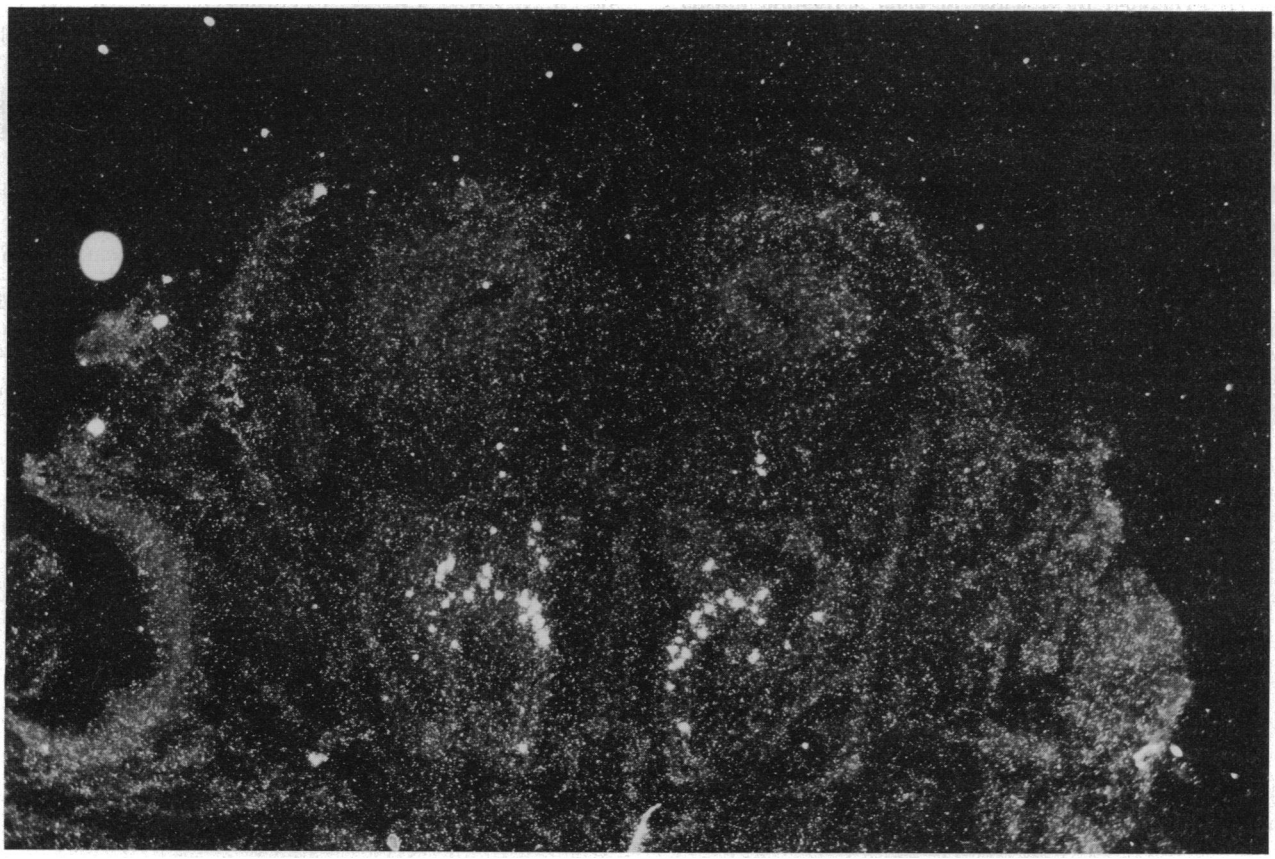

FIG. 5. Coronal section of a mouse head hybridized with OR3 antisense RNA. This is an enlarged view of Fig. 4*E* showing OR3-positive cells and the low background at E14.

fragment contains a neural-specific motif at position −800 from the start codon. This conserved sequence motif is centered around a core of 7 nucleotides (CCAGGAG) and is common to the 5′ flanking regions of several neuronal-selective genes, e.g., the 43-kDa growth-associated protein GAP-43, type II sodium channel, peripherin, superior cervical ganglion cDNA clone SCG-10, and neurofilament (22–24).

DISCUSSION

The three principal findings in this report are (*i*) the discovery of another member (OR3) of the 7-TMD receptor family, (*ii*) the discovery of a specific symmetrical pattern of receptor expression in the olfactory epithelium, and (*iii*) evidence that mRNA for the OR3 receptor occurs first in cells associated with the CNS and only later in the olfactory epithelium.

The function of the OR3 receptor is unknown. Its homology (38–41%) to the large family of putative olfactory-specific receptors makes it a possible candidate for an odor receptor; however, our Southern blot analysis and preliminary genomic library screening indicate that OR3 may belong to a small group of receptors with yet unidentified functions in the olfactory epithelium.

The bilateral symmetry of expression for OR3 within the olfactory epithelium reveals a level of receptor organization not previously recognized in this tissue. The existence of specific patterns of receptor gene expression in the olfactory epithelium, in contrast to a random distribution, may reflect an important property of the olfactory system to establish symmetric contacts between the developing olfactory neurons in the periphery and the mitral cells in the olfactory bulb.

The cells expressing the OR3 receptor may migrate from the CNS (or neural crest) to the periphery, explaining the finding that in the adult OR3 is expressed only in the olfactory epithelium and not in the brain. Alternatively, our results could be explained by a wave of gene activation during embryonic development that turns on receptor genes of different nonmigrating cells. Certain neurons of the CNS migrate into the brain from the olfactory epithelium (25, 26), and a dual origin (central and peripheral) for sensory neurons has been observed in the dorsal root and other ganglia (27, 28), but it had not previously been observed in the olfactory epithelium. If this description is correct, OR3 may be involved in targeting olfactory cells to their final destination in response, for example, to tropic factors released by the primary epithelium (18).

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