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A Putative Pheromone Receptor Gene Is Expressed in Two Distinct Olfactory Organs in Goats

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Abstract

Mammals possess two anatomically and functionally distinct olfactory systems. The olfactory epithelium (OE) detects volatile odorants, while the vomeronasal organ (VNO) detects pheromones that elicit innate reproductive and social behavior within a species. In rodent VNO, three multigene families that encode the putative pheromone receptors, V1Rs, V2Rs and V3Rs, have been expressed. We have identified the V1R homologue genes from goat genomic DNA (gV1R genes). Deduced amino acid sequences of gV1R genes show 40–50% and 20–25% identity to those of rat and mouse V1R and V3R genes, respectively, suggesting that the newly isolated goat receptor genes are members of the V1R gene family. One gene (gV1R1 gene) has an open reading frame that encodes a polypeptide of 309 amino acids. It is expressed not only in VNO but also in OE. *In situ* hybridization analysis revealed that gV1R1-expressing cells were localized in neuroepithelial layers of VNO and OE. These results suggest that the goat may detect pheromone molecules through two distinct olfactory organs.

Introduction

Mammals have two distinct olfactory systems (Eisthen, 1992). Volatile odorants are detected by the sensory neurons in the olfactory epithelium (OE), and these chemical signals are transmitted to the main olfactory bulb (MOB) (Kosel *et al.*, 1981). On the other hand, pheromone molecules, which elicit innate reproductive and social behavior within a species, are detected by the sensory neurons in the vomeronasal organ (VNO), and pheromonal information is transmitted to the accessory olfactory bulb (AOB) (Halpern, 1987).

Three families of receptor genes (V1R, V2R and V3R genes) have been expressed specifically in the vomeronasal neurons (Dulac and Axel, 1995; Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997; Pantages and Dulac, 2000). Each receptor gene is expressed in small subsets of vomeronasal neurons, but there is no sequence homology between V1R and V2R genes. V3R genes are distantly related to V1R genes (Pantages and Dulac, 2000). These findings suggest that these genes encode pheromone receptors, although there is no direct evidence that these receptors recognize pheromone molecules.

Recently, Leinders-Zufall *et al.* (Leinders-Zufall *et al.*, 2000) showed physiological responses in mouse vomeronasal neurons to substances reported to be pheromones in that species. Their data implied that the responsive neurons seemed to be mostly expressing V1Rs. However, the expression of the V1R family genes in VNO has been reported in only the rodent thus far (Dulac and Axel, 1995; Saito *et al.*, 1998). Thus, we attempted to identify the V1R family genes and analyze the expression of these genes in goat, in which activation of the pheromone-induced neuroendocrine secretion has been well documented (Chemineau, 1987). We found that one of the genes was expressed not only in the sensory neurons of VNO but also in those of OE, implying that two distinct olfactory systems are involved in goat pheromone sensing.

Materials and methods

Histology

VNOs were dissected from adult goats and mice [Crj: CD-1 (ICR)]. Cryostat sections of 10 μm thickness were stained

with Mayer's hematoxylin. To estimate the volumes of VNOs and neuroepithelia in goats and mice, we assumed that the VNOs had a columnar structure. The volumes were calculated as follows: the cross-sectional area × rostral–caudal length of the VNOs. The cross-sectional area was measured using NIH image software (available on the Internet at http://rsb.info.nih.gov/nih-image/).

Cloning of goat V1R homologue genes

To isolate goat V1R homologue genes, polymerase chain reaction (PCR) was performed using degenerate primers derived from the conserved regions of gene families of rat and mouse V1Rs, with goat genomic DNA as the template (Dulac and Axel, 1995; Saito *et al.*, 1998). The degenerate primers used were S1 (5′-CACIIGAAIYARIYCYRRY-AYYTTCA-3′), S2 (5′-CTCARYSYYARAARMTCCTG-TTT-3′), AS1 (5′-AWACAAAKAARSTCAYDAGC-3′) and AS2 (5′-ACARAARGRCTKAYDGTRGCATA-3′), which corresponded to the amino acid sequences of H(R/K)(K/N)Q(T/I/A)(R/Q)HL(Q/H), LSP(R/K)(S/K/N)SCL, LM(S/T)(F/L)FV and YAT(V/L)S(P/L)(F/S)V, respectively.

To obtain the entire coding sequences of goat V1R homologue genes, the inverse PCR method was used (Triglia *et al.*, 1988). The specific PCR primers used for the first PCR were 5′-ACCCAGACCATTCTATTCCTC-3′ and 5′-TTCTCAAGAATGTTGTGGGATTA-3′ for gV1R1, and 5′-GAAGAAAGGACCACTGAGACC-3′ and 5′-GAC-CACTGAGACCGTCCTGC-3′ for gV1R2. PCR primers used for the second PCR were 5′-ATACCTAAGGA-AGTGAATCACG-3′ and 5′-GGAACCATTGATGAAC-ATGTTG-3′ for gV1R1, and 5′-GGATGCTTTTGGAGA-AAGCTT-3′ and 5′-TTTGGAGAAAGCTTGGTGCTG-3′ for gV1R2. The generated PCR products were cloned and sequenced.

Southern hybridization of reverse transcription–PCR (RT–PCR) products

Total RNAs were extracted from various tissues using ISOGEN (Nippon gene), and were treated with DNase I. The poly(A)$^+$ RNAs were purified using an Oligotex-MAG mRNA purification kit (Takara). The cDNAs were synthesized from these poly(A)$^+$ RNAs by AMV RT-XL (Takara), and were used as templates for RT–PCR. The specific PCR primers used were 5′-TAATCCACA-TAGTCATGCCTA-3′ and 5′-TTCAGTACTGATTAGCA-CCA-3′ for gV1R1, and 5′-TAATCCACATAGTCATG-CTA-3′ and 5′-TAATACGACTCACTATAGGG-3′ for gV1R2.

To analyze the expression of V1R family genes in mouse VNO and OE, RT–PCR was performed. The specific primers used were mouse V1R-S primer (5′-TAGAAG-CTCCTGTTTARCAA-3′) and mouse V1R-AS primer (5′-GAAGATGCYGGGYCTGWTT-3′), which corresponded to the amino acid sequences of RSSCL(A/T) and (K/N)Q(T/A)(Q/R)HL, respectively. The PCR products were electrophoresed, blotted and hybridized in 4× sodium citrate/chloride buffer (SSC) containing 50% formamide, 50 mM phosphate buffer (PB) pH 7.5, 10× Denhardt's solution and 0.1% sodium dodecyl sulfate (SDS) at 42°C. The filters were washed in 0.2× SSC containing 0.1% SDS at 65°C. In some cases, the filters were hybridized under relaxed conditions in 4× SSC containing 25% formamide, 50 mM PB, pH 7.5, 10× Denhardt's solution and 0.1% SDS at 42°C, followed by relaxed washing with 2× SSC containing 0.1% SDS at 42°C. The probes used for hybridization were derived from the coding sequences of gV1R1 and gV1R2 genes, and the entire coding sequence of the mouse V1R gene (clone 2–4) (Hagino-Yamagishi *et al.*, 2001), respectively.

The specific primers used for the detection of the olfactory marker protein (OMP) gene were 5′-ACRGGC-ACCTCGCAGAACTG-3′ and 5′-CCAAAGGTGAYGAG-GAARTACAT-3′, and those for the detection of β-actin were 5′-CAATGGATCCGGTATGTG-3′ and 5′-CGTTGT-AGAAGGTGTGATGCC-3′.

In situ hybridization

VNOs and OEs of male goats were collected and fixed in Bouine's solution (Sigma). The OE was decalcified in 10% ethylenediamine tetraacetic acid (EDTA) for 2 days. These fixed tissues were processed through a standard paraffin embedding protocol. Sections were cut into 15 μm thickness and attached to silanized slides. A riboprobe was prepared and labeled with digoxigenin as described previously (Schaeren-Wiemers and Gerfin-Moser, 1993). The 710 bp DNA fragment of gV1R1 was used for the preparation of the riboprobe. *In situ* hybridization procedures were essentially the same as those described previously (Schaeren-Wiemers and Gerfin-Moser, 1993). Hybridization was carried out in a hybridization solution containing 50% formaldehyde, 10 mM Tris–HCl pH 7.6, 200 μg/ml tRNA, 1× Denhardt's solution, 600 mM NaCl, 0.25% SDS and 1 mM EDTA pH 8.0 at 55°C overnight. Hybridized RNAs were detected with an alkaline phosphatase-conjugated anti-DIG Fab fragment antibody (Roche), and signals were visualized using 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (Roche) as chromogenic substrates.

Southern hybridization of goat genomic DNA

Ten micrograms of goat genomic DNA, which was digested with *Eco*RI, *Bam*HI and *Hin*dIII, was blotted and hybridized with probes specific for gV1R1 and gV1R2 genes. Hybridization was carried out under the relaxed conditions described above.

Results

Goat vomeronasal organ

Goat VNO is a columnar structure located at the base of the

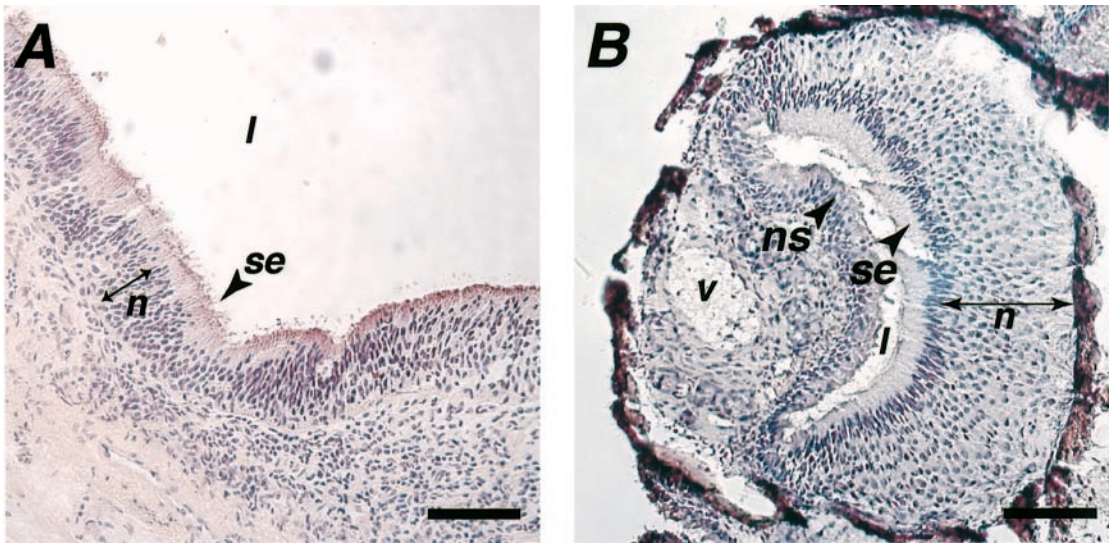

Figure 1 Goat and mouse vomeronasal organs Coronal sections of part of Shiba goat VNO **(A)** and whole mouse VNO **(B)**. se, sensory epithelium; ns, nonsensory epithelium; n, neuroepithelium; l, lumen of VNO; v, vein. Bars: 100 μm.

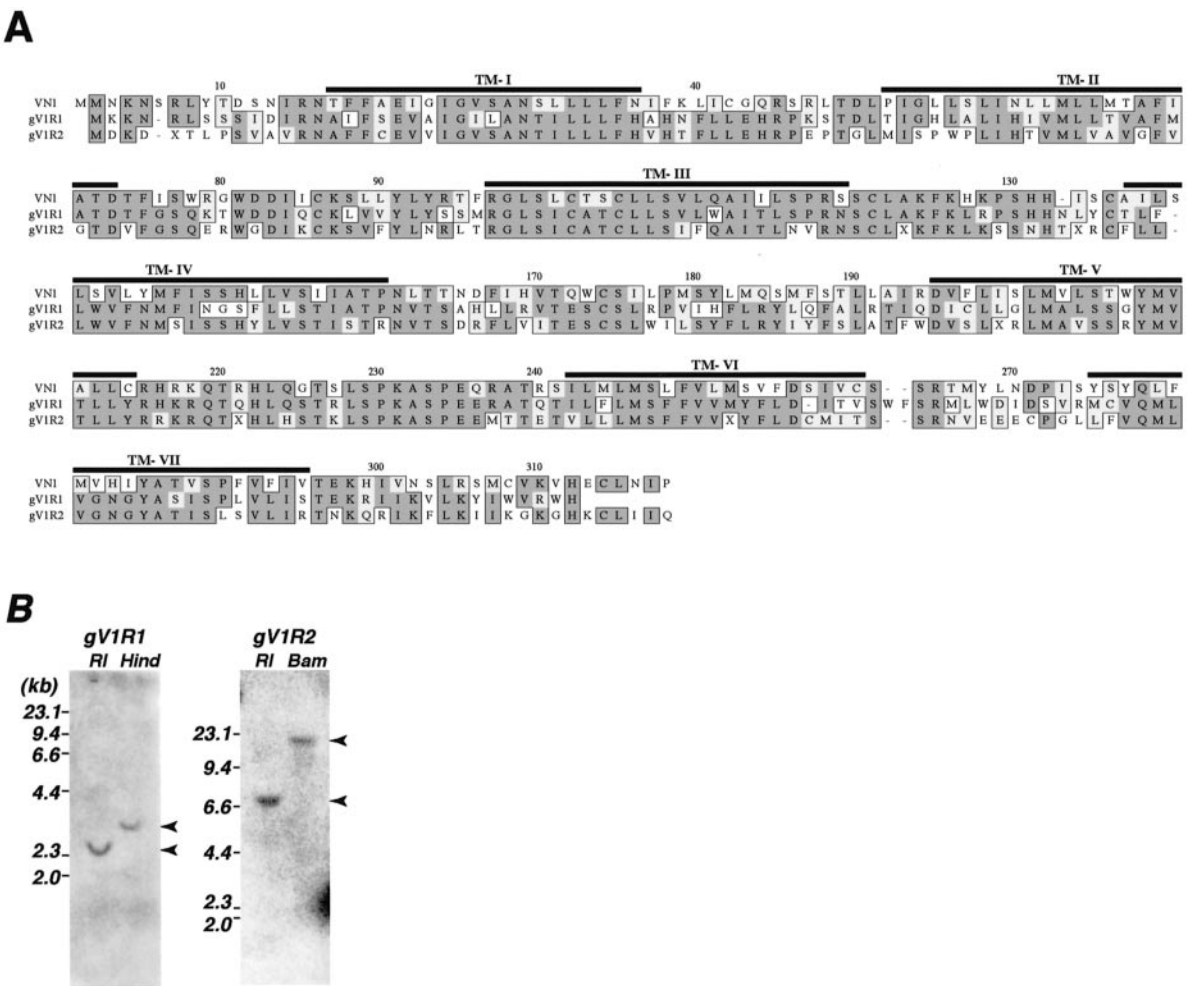

Figure 2 **(A)** Alignment of deduced amino acid sequences of goat V1Rs and rat V1R. Deduced amino acid sequences of goat V1R homologue genes (gV1R1, gV1R2 genes) and the rat V1R gene (VN1 gene) were aligned. Bars indicate seven putative transmembrane domains (TM I–VII). GenBank accession numbers for gV1R1 and gV1R2 genes are AB064662 and AB064663, respectively. **(B)** Southern hybridization. Goat genomic DNA was digested with *Eco*RI, *Hin*dIII and *Bam*HI. The digested DNA was hybridized with probes specific for gV1R1 and gV1R2 genes, respectively.

nasal septum. One feature of goat VNO is that the neuroepithelial layer (indicated by 'n' in the figures) is thinner than that in mouse VNO (Figure 1A,B). The ratio of the volume of the neuroepithelial layer to that of the whole VNO was ~2% in the goat and 25% in the mouse. These results show that the ratio of the volume of the neuroepithelial layer to that of the whole VNO in the goat is ~8% of that in the mouse.

Cloning of goat V1R genes

We identified two V1R homologue genes (gV1R1 and gV1R2 genes) from goat genomic DNA using degenerate primers derived from highly conserved regions in rat and mouse V1R family genes (Dulac and Axel, 1995; Saito *et al.*, 1998). The deduced amino acid sequences of the gV1R gene show ~40–50% and 20–25% identity to those of rat and mouse V1R and V3R family genes, respectively (Dulac and Axel, 1995; Pantages and Dulac, 2000), suggesting that the newly isolated goat VNO receptor genes are members of the V1R gene family rather than the V3R gene family. The gV1R1 gene has an open reading frame that encodes a polypeptide of 309 amino acids (Figure 2A). On the other hand, the gV1R2 gene has multiple frameshifts and stop codons in its coding sequence (Xs in Figure 2A), thereby indicating that gV1R2 does not function as a receptor protein.

Hybridization analysis of goat genomic DNA shows that even under relaxed conditions, only a single band can be detected using probes specific for gV1R1 and gV1R2 genes (Figure 2B). This result is inconsistent with the previous notion that multiple bands can be detected when genomic DNAs from the rodent are hybridized with probes specific for V1R family genes under relaxed conditions (Dulac and Axel, 1995; Saito *et al.*, 1998). Our data imply that the size of the V1R gene family in the goat is relatively smaller than that in the rodent.

Expression of gV1R1 and gV1R2 genes

As our first trial, we examined the expression of V1R homologue genes in goat VNO by Northern hybridization analysis, but could not obtain positive signals (data not shown). We assumed that the relative amount of gV1R-expressing cells in the entire VNO preparation was too low to allow detection of gV1R transcripts, because the ratio of the volume of the neuroepithelial layer to that of the whole VNO in the goat was only 8% of that in the mouse (Figure 1). Thus, we adopted a more sensitive method, the Southern hybridization analysis of RT–PCR products, and found that the gV1R1 gene was expressed not only in VNO and AOB, but also in OE and MOB (Figure 3A, top panel). It should be noted that we obtained OE RNA from the lateral side of OE to eliminate possible contamination by vomeronasal neurons that project their axons along the nasal septum to the AOB. The gV1R1 gene was also expressed in the testis but not in other tissues (Figure 3A, top panel). The same

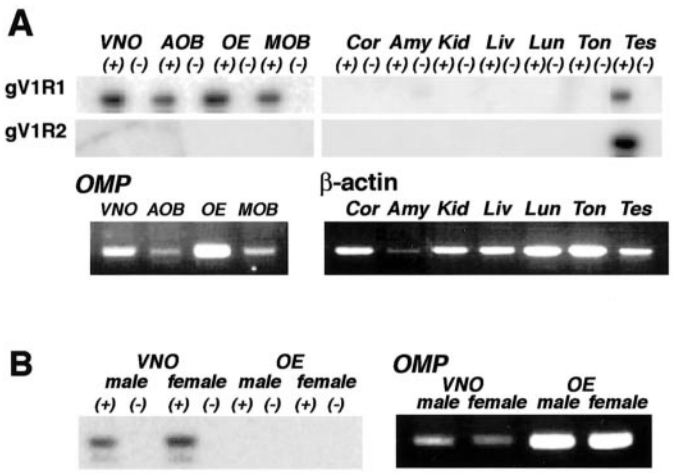

Figure 3 **(A)** Expression of gV1Rs in various tissues. The expressions of gV1R1 and gV1R2 genes were analyzed by Southern hybridization of RT–PCR products. VNO, vomeronasal organ; AOB, accessory olfactory bulb; OE, main olfactory epithelium; MOB, main olfactory bulb; Cor, cortex; Amy, amygdala; Kid, kidney; Liv, liver; Lun, lung; Ton, tongue; Tes, testis. cDNA was synthesized with reverse transcriptase, RT(+), or without reverse transcriptase, RT(–). The expressions of OMP and β-actin were analyzed by RT–PCR as a positive control. **(B)** Expression pattern of mouse V1Rs in VNO and OE. The expression of V1R family genes in mouse VNO and OE was analyzed by Southern hybridization of RT–PCR products.

results were obtained from four independent experiments (one male and three female goats) (data not shown).

To examine whether the expression of putative pheromone receptors in OE is a common feature in mammals, we analyzed the expression of V1R genes in mouse OE by Southern hybridization analysis of RT–PCR products. The lateral side of OE was carefully excised from 10 male and 10 female mice, and mRNA was purified from these OE. Using this mRNA as a template, RT–PCR was performed using specific primers derived from the conserved region of V1R family genes. Note that we detected at least 13 V1R family genes in the mouse using these primers (data not shown). The PCR products were blotted and hybridized with the probe that encoded the entire coding region of mouse V1R clone 2–4 (Hagino-Yamagishi *et al.*, 2001) under relaxed conditions. As shown in Figure 3B, V1R family genes were not expressed in OE in both male and female mice, although they were clearly expressed in VNOs. These results strongly suggest that mouse V1R genes are expressed only in VNO and not in OE, and the expression of the putative pheromone receptor gene in OE is characteristic of the goat and not of the mouse.

Then, the type of gV1R1-expressing cells in OE and VNO was determined by *in situ* hybridization analysis (Figure 4). The gV1R1-expressing cells were detected in the neuroepithelial layer (indicated by 'n') in both VNO and OE (Figure 4A,C) using an anti-sense riboprobe, but no signal was detected using the sense probe (Figure 4B,D). These

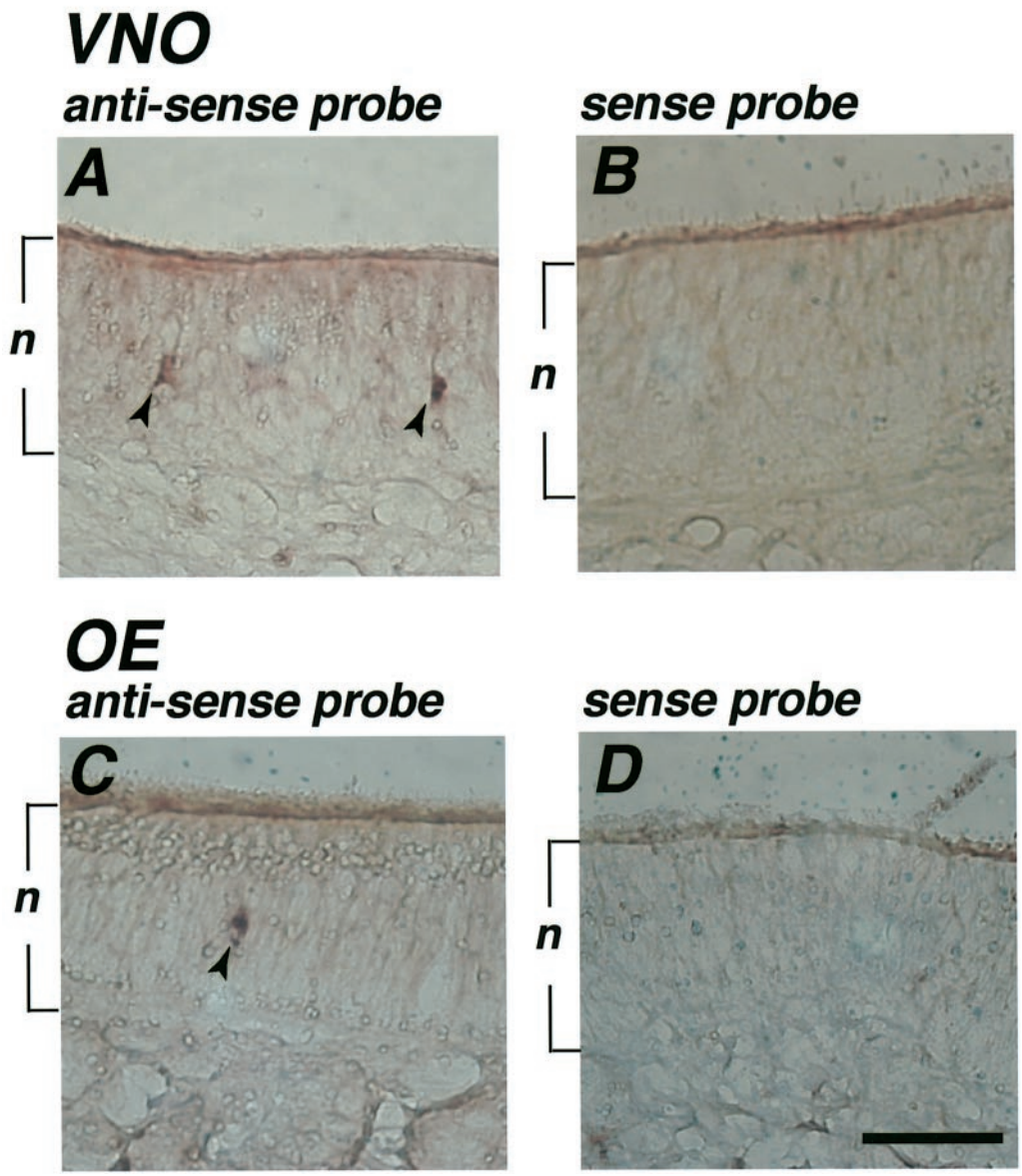

Figure 4 *In situ* hybridization analysis using DIG-labeled probe for gV1R1 gene in VNO and OE gV1R1-expressing cells were located specifically in vomeronasal (arrowheads in **A**) and olfactory neuroepithelial layers (arrowhead in **C**). No signal was detected in VNO and OE using sense probe (**B** and **D**). n, neuroepithelium. Bar: 50 μm.

results strongly suggest that the gV1R1-expressing cells in the VNO and OE are vomeronasal and olfactory sensory neurons, respectively.

In addition, the distribution of the gV1R1-expressing cells throughout the neuroepithelial layer of VNO, and a distinct spatial segregated pattern of the gV1R1-expressing cells, which was clearly detected in rodents, were not observed (data not shown).

The gV1R2 gene was not expressed in any tissues except the testis, suggesting that it is not expressed and does not function in the vomeronasal system (Figure 3A, bottom panel).

Discussion

We identified homologue genes of the rodent putative pheromone receptor in goat (gV1Rs), and found that the gV1R1 transcripts were expressed not only in sensory neurons of goat VNO but also in those of OE (Figure 3A, top panel; Figure 4A,C). The expression of the same receptor in the VNO and OE implied that the goat recognizes and processes ligand information through the two distinct olfactory systems.

The expression of the V1R homologue gene in OE was recently reported in human (Rodriguez *et al.*, 2000), despite

the fact that adult human does not have a functional vomeronasal system similar to that in rodents and even-toed ungulates (Stensaas *et al.*, 1991). Thus, the expression of V1R homologue genes in OE is not restricted in even-toed ungulates. In addition, previous reports showed that the rabbit and the pig with surgically removed or impaired VNOs still exhibited pheromone-induced stereotype behavior such as nipple-search behavior or reproductive behavior (Hudson and Distel, 1986; Dorries *et al.*, 1997), implying that pheromones could be detected not only by VNO but also by OE in these animals. These data support the notion that pheromone detection by the main olfactory system may play an important part in many mammalian species. The gene expression of the putative pheromone receptor in sensory neurons of goat VNO and OE shows the possible involvement of the two distinct sensory organs in mammalian pheromone detection.

Morphological observation of goat VNO revealed that the ratio of the volume of the neuroepithelial layer to that of the whole VNO in the goat is only 8% of that in the mouse (Figure 1), and the size of goat V1R gene family seems to be smaller than that of rat and mouse V1R gene families (Figure 2B). In addition, we have cloned eight genes of goat V2R homologues thus far, and found that these genes have multiple frameshifts and/or termination codons within their coding sequences (data not shown). Thus, the majority of goat V2R gene products may not function as receptors. Taken together, it seems that the ability of pheromone detection by VNO is more inefficient in goats than in rodents. The expression of the putative pheromone receptor gene in goat OE may compensate for the pheromone sensing function by VNO.

In rodents, olfactory neurons project their axons to MOB (Kosel *et al.*, 1981), whereas vomeronasal neurons project their axons to AOB (Halpern, 1987). These two olfactory systems seem to be clearly separated from each other. However in the goat, the gV1R1 transcripts are expressed not only in AOB but also in MOB (Figure 3A, top panel). Although we cannot completely rule out the possibility of trace amounts of contamination of AOB in the MOB sample, our results showed the possibility that the axons of gV1R1-expressing sensory neurons of VNO and/or OE project to MOB. Another possibility is that gV1R1 transcripts were expressed in cells of AOB/MOB themselves. To examine these possibilities, determination of the cell type that expresses the gV1R1 transcripts in the olfactory bulb is required.

Both gV1R1 and gV1R2 transcripts are expressed in the testis (Figure 3A), although gV1R2 is not a functional receptor (Figure 2A). Mouse V1Rs are also expressed in both VNO and the testis, and it has been argued that they are involved in sperm chemotaxis (Tatsura *et al.*, 2001). However, the biological significance of the expression remains unclear.

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