Towards an Assay for Allelic Inactivation of Vomeronasal Receptor Genes in Mouse

by

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Abstract

Olfactory sensory perception is mediated by two functionally and anatomically distinct systems in mammals: the main olfactory system, which responds to tens of thousands of discrete environmental odorants by expressing as many as 1000 receptor genes, and the vomeronasal system, which discriminates between intraspecific chemical signals (pheromones) in order to elicit innate reproductive and social behaviors. The task in gene regulation for the two systems is similar - i.e. to choose one out of many receptor genes for each neuron to express. In a step towards understanding how such regulation may be facilitated at a neuronal level, olfactory receptor genes have been shown to be allelically inactivated (Chess et al., 1994). Here, we wish ask the question of whether VNO genes, like olfactory genes, are also allelically inactivated. Progress has been made towards development of an assay to answer this question. Preliminary data also suggests the possibility that more than one member of closely related gene subfamilies may be expressed per cell.

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Introduction

Sensory sytems translate external stimuli into internal representations of the world. In mammals, two distinct systems have evolved to accomplish this task. The main olfactory epithelium (MOE) resides in the posterior recess of the nasal cavity. Upon stimulation, its neurons transmit information to the main olfactory bulb, which in turn projects to the olfactory cortex, and then to higher sensory centers in the brain where the signals are processed to generate behavioral responses. Via this system, animals are able to distinguish between large numbers of volatile odorants. In mammals, it is estimated that there are as many as 1000 different olfactory receptor (OR) genes, each encoded by a distinct seven transmembrane domain protein (Buck and Axel, 1991). These genes are thought to couple with the heterotrimeric G-protein α subunit $G_{\alpha olf}$, thereby initiating a cascade of events leading to sensory neuron action potentials (Jones and Reed, 1989).

Olfactory receptor genes are found in numerous clusters dispersed throughout the mammalian genome. In humans, it appears that members of the OR gene family are present on almost all chromosomes, though a large percentage of these may be pseudogenes (Rouquier et al., 1998). Expression of a given gene is restricted to one of four discrete (but overlapping) topographic zones in the epithelium, yet members of the same gene cluster are expressed in neurons of various zones (Sullivan et al., 1996). Clusters appear to have originated by duplications of an ancestral repertoire, followed by subsequent

divergence within the many subfamilies (Ben-Arie et al., 1994). RNA in situ hybridization experiments have also permitted analysis of expression patterns of odorant receptors. In rodents, a given receptor gene identifies about 0.1% of the sensory neurons out of the reportoire of ~1000 (Vassar et al., 1993; Ressler et al., 1993). Different receptor probes appear to hybridize to distinct subpopulations of neurons, suggesting that each cell only expresses one or a small number of receptor genes. This is observed both in catfish (Ngai et al., 1993b) and in rat (Vassar et al., 1993; Ressler et al., 1993).

How does a neuron choose to express one specific olfactory receptor out of the ~1000 genes in the genome? One proposed model for regulation of these genes involves a cis-regulatory element that assures the stochastic expression of only one gene from a large array of linked receptor genes. This model is supported by evidence demonstrating that allelic inactivation - whereby each olfactory receptor neuron expresses only either its maternal or paternal allele - plays a role in OR gene expression. Thus, allelic inactivation might serve to silence one entire allelic array of genes, allowing a cis regulatory element on the active array to then stochastically select one olfactory gene to be expressed.

The vomeronasal system is functionally and anatomically distinct from the main olfactory system. The vomeronasal organ (VNO) resides more anteriorly in the nose, in a cigar shaped pouch within the nasal septum (Halpern, 1987) (Figure 1). VNO responses are elicited by pheromones - non-volatile odorants present in urine, sweat, and other bodily secretions, that trigger innate, stereotyped behaviors required for intraspecific communication. Pheromones may provide information about gender, dominance, reproductive status, overcrowding, or impending danger

(Halpern, 1987). In male rodents, for example, ablation of the VNO in virgin animal impairs sexual responses (Clancy et al. 1984, Meredith, 1986). Lesions can also diminish male specific aggressive behaviors (Bean, 1982; Clancy et al., 1984). In humans, there is debate as to the functionality of the VNO, which was long considered to be atrophied in adults. However, even if the human organ is not functional, it is possible that pheromonal responses may be mediated through the main olfactory system. Recent evidence shows that pheromones (specifically, axillary compounds) can regulate synchronization of menstrual cycles in women (Stern & McClintock, 1998). Steroid hormones, such as the 16-androstenes and estrogens, and fatty acids have been implicated as the chemical signals responsible for eliciting such behaviors, but the specific nature of the odorants involved in pheromonal responses has not yet been elucidated.

Consistent with the differences in types of elicited behavior, the pathway utilized by VNO responses operates separately from the MOE. VNO information is conveyed through the accessory olfactory bulb, amygdala and hypothalamus. This pathway does not involve higher cognitive centers in the brain and reflects the reflexive, pre-programmed nature of VNO responses as compared to those generated by the MO system.

VNO neurons lack olfactory sensory transduction molecules, including $G_{\alpha olf}$ (Berghard et al., 1996) , but express high levels of $G_{\alpha 0}$ and $G_{\alpha i2}$ (Jia et al, 1996). These two G protein α subunits are expressed in topologically distinct zones in the VNO and their expression patterns correspond to those of the two distinct families of putative VNO genes cloned to date. The adenylyl cyclase expressed in the VNO is also a different isoform than that found in the MOE (Berghard et al, 1996). Absence of common signal transduction

components hints that VNO genes may in fact have evolved from a separate route from the main olfactory system.

Indeed, attempts to clone VNO receptors by homology to MOE receptors failed. The first family of VNO genes, colocalizing with $G_{\alpha i2}$ expression in the basal half of the VNO neuroepithelium, was cloned by differential screening of cDNA libraries constructed from single sensory neurons from rat VNO (Dulac and Axel, 1995). This process yielded a family of ~100 seven transmembrane domain receptors unrelated to those expressed in the MOE and to other known seven transmembrane domain receptors. A second multigene family of ~140 members was cloned that expresses in the apical $G_{\alpha 0}$ zone of the VNO neuroepithelium (Herrada and Dulac, 1997; Matsunami and Buck, 1997). The Gα0-VN genes are members of the large G protein-coupled receptor (GPCR) superfamily, but they have an unusually long N-terminal extracellular domain, suggesting a different mode of ligand binding, as well as an evolutionarily independent origin from the MOE and Gαi2-VN receptors. Numerous variant forms of Gα0-VN mRNAs have also been identified. In situ hybridization experiments using individual VNO candidate genes from either family have hinted that each receptor is expressed in a small percentage of VN neurons, and are randomly distributed within its Gα zone. This suggests that VN neurons express only one or a small number of receptor genes, similar to the MO system.

Here, we have made progress towards developing an assay for monoallelic expression. To examine the number of receptor genes expressed per cell, we have also analyzed single cells by RT-PCR. Preliminary data indicates more than one member of a closely related gene subfamily may be expressed.

Results

An Assay for Allelic Inactivation

Progress towards understanding sensory coding in the vomeronasal system shadows that of the main olfactory system. By in situ hybridization, it appears that each VN receptor expresses in a small percentage of VN neurons in a random and possibly non-overlapping manner, suggesting that each neuron expresses only one or a small number of genes. The overall size of the VN system, with ~240 total members in the two multigene families, is smaller than that of the main system, but it remains possible that the underlying mechanism of regulation is the same.

In earlier work done on the main olfactory system (Chess et al., 1994), an assay was set up to determine whether cells expressing a given receptor transcribe this receptor gene from both maternal and paternal alleles, or only from one or the other. This assay distinguishes the transcripts from the two alleles of I7, an olfactory receptor gene, by taking advantage of the numerous polymorphisms that exist between Mus musculus domesticus and Mus spretus, two distantly related strains of mice still capable of interbreeding. By examining diluted pools of cells from dissected olfactory turbinates of F1 progeny (where dilution is performed such that the majority of pools are negative, indicating that a positive signal is statistically likely to come from only one receptor-expressing cell in the pool), it is possible to use RT-PCR to determine which parental allele I7 expression derives from. In the MO system, it has been found that OR genes are monoallelically expressed, and parental origin of the chosen allele appears random. Additionally, two-color FISH analysis also shows that receptor alleles are asynchronously replicated (a

phenomenon consistently present in monoallelically expressed genes (Knoll et al., 1994; Kitsberg et al., 1993)) (Chess et al., 1994;). Together, these two results suggest that allelic inactivation is involved in the regulation of receptor choice, providing support for a model where a hierarchy of controls acts to narrow down to one (or a small number of) active gene per neuron.

In order to assay for monoallelic expression in the vomeronasal system, we wished to find a VNO gene that 1) expresses both in musculus and spretus, 2) is single copy, 3) is polymorphic between musculus and spretus, 4) contains an intron to facilitate RT-PCR analysis (eliminating the concern of contamination from DNA templates).

Finding a Gene for the Monoallelic Expression Assay

Murine homologs of the rat Gα0-VN family of VNO receptors were identified in a genomic library screen (Davison, unpublished data, 1997). Five distinct genes were cloned, and sequencing found one gene, VN22, to have an intron in the 5′ UTR (not enough sequence was available for the other clones to determine sites of introns). RT-PCR experiments on VN22 ran into complications, when it became evident that this gene was not single copy. Southern blot analysis using sequences from the 5'UTR on VN 10, 22, 25, and 61 also revealed that all of these sequences had closely related family members (Figure 2). Nevertheless, we hoped that these closely related family members would be distinguishable at the sequence level. To elucidate the exon-intron structure of these genes, a VNO cDNA library (courtesy of Linda Buck) was screened for both $G_{\alpha 0}$ and $G_{\alpha i2}$ -VN genes using probes from conserved areas of coding regions. In the primary screen 100 positive plaques were identified, leading eventually to the cloning of ten $G_{\alpha 0}$ and nine $G_{\alpha i2}$-VN genes.

We wished to pursue the $G_{\alpha i2}$-VN gene family in mouse first, as the $G_{\alpha 0}$-VN family had already been shown to contain many highly similar sequences and splice variants (Matsunami and Buck 1997; Herrada and Dulac 1997) which might confuse RT-PCR analysis. However, sequencing of the $G_{\alpha i2}$-VN cDNAs revealed that none were full length (usually falling ~100 bp short of transcriptional start), perhaps due to secondary structure in the mRNA. PCR using a 5′ primer and the T7 primer on the lambda library will hopefully recover any 5′ ends (where introns likely exist). Another option is to try 5′ RACE.

Careful analysis of the mouse Gα0-VN sequences revealed that it would be extremely difficult to find a single copy sequence to use for our RT-PCR assay. In addition to the many variant mRNAs, closely related family members made it almost impossible to design primers that would only see one sequence. As stated earlier, in situ hybridization experiments in both the MO system and both families of VNO genes have found that each individual receptor probe hybridizes to a small percentage of neurons, distributed randomly within its acceptable zone (Vassar et al., 1993; Ressler et al., 1993, Herrada and Dulac, 1997; Matsunami and Buck, 1997). When a mix of probes is used, a larger percentage of neurons is labelled, roughly equal to the sum of the individual labeled components. This leads to the conclusion that different neurons express different receptor genes, but it does not rule out the possibility that a neuron may express a small number of receptors, perhaps closely related receptors, as opposed to just one.

When this is taken into account, it necessitates the sequencing of all possible RT-PCR products from both musculus and spretus in order to give an unequivocal answer to the monoallelic expression question. Simultaneous amplification of more than one receptor gene could still

permit an assay if enough sequences were gathered such that it would be clear which alleles of which genes originated from which parent. If this were not the case, however, complications could arise in analysis. If only one product were found in a single cell from an F1 animal, it would be reasonable to conclude that the gene is monoallelically expressed. However, if two products were found, it might be unclear as to whether this reflects biallelic expression, or whether both products might in fact be coming from two closely related genes from one parental genome, i.e. it would be difficult to ascertain whether any given transcript derives from musculus or spretus, to determine which are homologous genes in the two strains of mice, or to ascribe sequence differences to species-specific polymorphisms, since two closely related genes even within the same strain can be well over 90% identical at the nucleotide level. Expressed pseudogenes would also confound this issue further.

Some of these problems could be alleviated if we could be sure that only one gene were expressed per cell. Analysis of closely related receptor gene subfamily members might permit an investigation of how likely it is that a given neuron expresses "one", as opposed to, "a small number" of receptor genes - a question never directly examined before.

Single Cell Analysis

To look at the question of whether closely related receptor genes may be expressed in the same neuron, we sorted single cells using a fluorescence activated cell sorter (FACS) and performed RT-PCR using intron-spanning primers chosen from conserved regions that would amplify AF011413 (a $G_{\alpha 0}$-VN gene) and its very closely related family members. Using single cells instead of diluted pools of cells simplifies analysis, eliminating the possibility

that more than one RT-PCR product could be the result of more than one cell expressing the receptor gene. One positive cell, H12, gave highly unexpected results. After obtaining the initial positive signal, ten separate aliquots of the RT reaction products were amplified. Each gave a strong positive signal, indicating a high efficiency of reverse transcription (Figure 3). Sequencing of eight cloned PCR products (taken randomly from five of the ten aliquots) revealed eight different products.

The eight known VN-$G\alpha 0$ sequences to which our primers were designed (AF011411, AF011412, AF011413, AF011418, AF011419, AF011420, AF011421, AF011422, AF011423) are all >90% identical. Our primers were chosen to amplify all eight known sequences. The exon-intron structure of these sequences bear similarity to the human calcium sensing receptor (CSR), a GPCR (Brown et al., 1993). Splice variants of these sequences often lack segments of the N-terminal domain, usually corresponding to a human CSR encoded by a single exon or pair of exons (Matsunami and Buck, 1997) (Figure 4). For example, AF0011413 and AF011423 are identical except for a stretch of 360 bp which is missing from the latter gene, corresponding to exons 4 and 5, which then results in a frameshift for exon 6. Likewise, pairs AF011412/AF011422 and AF011421/AF011420 are also identical - the former in each pair appears to lack exon 5, while the latter lacks both exons 4 and 5. However, AF011422, AF011420 and AF011421 all appear to have frameshifts that affect downstream exons. AF011418 and AF011419 also appear to be distinct sequences with missing exons and downstream frameshifts. It is possible that there are full length sequences corresponding to these sequences which have not yet been cloned. Among the sequences, similarities range from 91 to 98% identical, suggesting that these are indeed distinct genes, though closely related.

Our positive single cell H12 PCR products confirm the existence of many closely related expression products. Furthermore, they suggest that more than one gene may be expressed per cell. Among our eight PCR sequences, products 2.1, 3.1, 3.2 and 4.2 are all between 98-99% identical, and are >99% identical to AF011421 (Figure 5). Product 1.3 is most closely related to AF011411, with 97% similarity. Product 5.1 is identical to AF011412, though it seems to lack some of exon 4 as well as exon 5. Product 2.2 is 96% similar to AF011413, though it lacks some of exon 4 and 5. Product 4.1, the only "full-length" product that does not appear to lack any exons, appears to be unique, with only 89-93% homology to any previously sequenced gene.

Two other single cells giving positive signals, F3 and A12, are in the process of being examined. Unlike H12, which gave strong positive signals from all aliquots of its RT reaction, only two aliquots each from F3 and A12 resulted in visible PCR bands. However, the separate aliquots from each cell may still give us important information regarding the status of gene expression of this closely related family in a single cell.

Discussion

Because of the many functional similarities between the main olfactory system and the vomeronasal system, a study of mechanisms of receptor choice promises to provide interesting angles from which to view gene regulation in mammals as a whole, as well as to reveal clues about the evolution of these systems. In the MO system, allelic inactivation may facilitate the integration of information by simplifying the task of receptor choice (allowing a neuron to choose only from one set of active genes). In the VN system, we have not yet determined whether expression is monoallelic.

Signal transduction pathways utilized by the two systems differ, as do details of sensory integration. The VN system also handles a smaller repertoire of receptors. Thus, the separate evolution of a distinct system of regulation of receptor choice is quite possible, though the consistency of expression patterns in terms of randomness and numbers of genes expressed per neuron may be similar.

How many receptor genes are expressed per cell?

Our study shows that the model of one-receptor-gene-per-cell may not be quite so straightforward. Previous in situ hybridization experiments on both the main olfactory system and the vomeronasal system seemed to indicate that expression of individual receptor genes is restricted and additive, suggesting that each neuron probably expresses a single receptor. Based on this assumption, the model next focused on how this gene choice might be regulated. In order to facilitate our assay for monoallelic expression, we wished to confirm that an individual neuron only expresses one receptor by examining single cells by RT-PCR. Here we have presented evidence that more than one gene product is expressed per cell. It remains unclear how many of these products are actually functional genes as opposed to expressed pseudogenes - however, it is clear that they are not merely splice variants of one gene, as the sequences themselves differ. It is possible that recent duplications of these genes may have included duplication of functional regulatory elements which allow for expression, even though the gene itself may no longer be viable. A careful single-cell examination of expression of closely related genes in the main olfactory system may prove interesting.

Location of VN Genes

Chromosomal location of the VN genes may also reveal clues as to possible mechanisms in regulation. Though we know that there are many OR gene clusters distributed throughout the mouse and human genomes (Sullivan et al., 1996; Rouquier et al., 1998) , less is known about the chromosomal location of the VN genes. Preliminary evidence suggests that many of the $G_{\alpha i2}$-VN genes reside at the same genomic locus. For example, probes from VN 10, 22, and 25 appear to pick up many of the same bands on our Southern blot (Figure 2). PCR also found VN 22 and 25 on a single YAC containing approximately 800kb of mouse genomic DNA (Kreitzer, Davison, and Chess, unpublished data). Confinement of $G_{\alpha i2}$-VN genes to one locus might be required by its system of receptor choice. Less is known about the chromosomal location of $G_{\alpha 0}$-VN genes, though some preliminary evidence suggests that one cluster may be linked to an OR cluster on mouse chromosome 7 (Matsunami and Buck, 1997). It also remains a possibility that the regulation of the two families of VN genes may be different. Since we know there are closely related subfamily members in the $G_{\alpha i2}$-VN family as well (which again contributed to difficulty in obtaining an assay for monoallelic expression), it would be useful to conduct similar single-cell experiments to assess the expression patterns of these genes as well. This may also be informative in formulating models of how regulation of MO, $G_{\alpha i2}$-VN and $G_{\alpha 0}$-VN receptor genes compare.

Experimental Procedures

Screening of VNO cDNA Library

A mouse VNO cDNA library prepared in Lambda ZAP II vector (courtesy of Linda Buck) was screened according to standard protocols (Stratagene). High stringency hybridization was carried out with a mixed probe composed of PCR products amplified from mouse genomic DNA using degenerate primers. For the $G_{\alpha i2}$-VN family: F22, 5'-CTYAGTTCIARAARCTCTGTTTA-3'; R23, 5'TCMTRAGYAICAKGATGGW-3' For the $G_{\alpha 0}$-VN family: F1, 5'ACIC CIAYIGTIAARGCIAAY-3'; F2, 5'-CCIAYIGTIAARGCIAAYAAY-3'; F3, 5'-ACIG TIYTIGCIAARACIATH-3'; R2, 5'-GGIARRAAIGTIAYCCAIAC-3'; Cloning and conversion of positive plaques to plasmids was carried out using Stratagene's protocol for the Lambda ZAP II vector. Forward and reverse sequencing was done using T3 and T7 primers.

Single Cell RT-PCR Assays

Cells were collected from vomeronasal organs dissected from adult C57/BL6 mice and FAC sorted into plates of 96 PCR tubes with one cell per tube. First strand synthesis was accomplished using avian myoblastosis virus reverse transcriptase (AMV-RT) and primer 13ALLRT6, 5'-CTTGAAAGCCATGACCA CAG-3'. Optimized conditions for PCR performed with Gibco Taq polymerase were: initial denaturation at 94C for 4', followed by 94C 25s, 52C 1', 72C 1'. First round PCR contained 45 cycles and used primers 13ALL3F1, 5'-ACAAGTAGA GTCTCAGAAAAAGG-3', and 13ALL6R1, 5'-AGAGATGAGCAGGATGTAGC-3'. Second round PCR contained 35 cycles and used nested primers 13ALL4F1, 5'-GTGAA CATGAAGCATAGGG-3' and 13ALL6R2, 5'-GCAGGATGTAGCTGAGAAT-3'. In controls done on total RNA isolated from adult VNO's, these primers gave

the expected 532 bp band as well as what appeared to be another smaller band, which could be a product of variant splicing. First round PCR primers crossed two introns while second round PCR primers crossed one, giving a DNA product of ~1.7kb. These primer pairs were chosen specifically to amplify AF011413 and its closely related family members to see if more than one receptor could be expressed per cell, and also to examine whether both expression of splice variants as well as full length receptors occurs in an individual VNO neuron. After primary positives were identified, PCR was performed on 1ul aliquots of the RT reaction of the positive cell. PCR products were subcloned into pGEM-T (Promega) or pCR2.1 (Invitrogen) vector and sequenced using T7 primer. Sequence analysis was performed using MacVector, Sequencher, and GCG sequence analysis programs.

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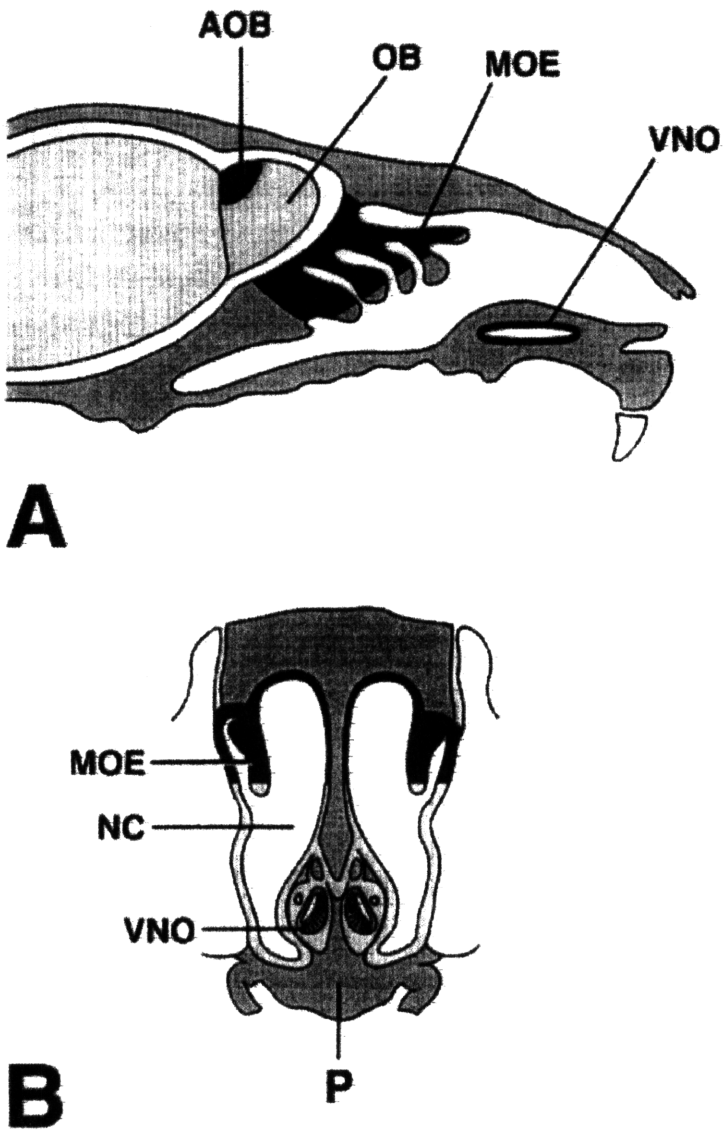

Figure 1. Anatomical Distinction of the Vomeronasal and Main Olfactory Systems

(A) Sketch of a parasagittal section through the skull of a rat (mouse skull morphology differs only slightly). The main olfactory epithelium (MOE) is found deep inside the nasal cavity (NC); the vomeronasal organ (VNO) lies more anteriorly, just above the palate (P) in a pouch against the septum. VNO neurons are segregated into two neuronal populations distinguished by differential expression of the G-protein α subunits $G_{\alpha i2}$ and $G_{\alpha o}$. Axons from MOE sensory neurons project to the main olfactory bulb (OB), whereas VNO neurons project to the accessory olfactory bulb (AOB).
(B) A coronal section of the above.

(Dulac and Axel, 1995)

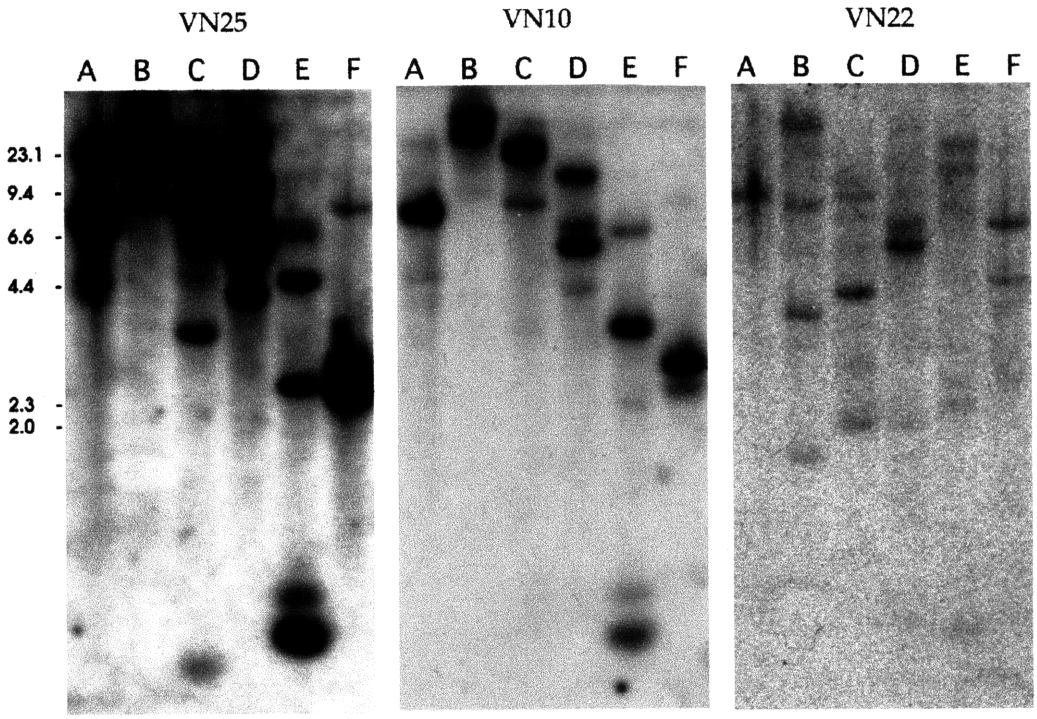

Figure 2. Genomic Southern Blot Analysis of VNO Genes

Five micrograms of mouse kidney DNA was digested with BamHI (A), BglI (B), BglII (C), HindIII (D), SacI (E), XbaI (F), size-fractionated, blotted, and the blot was hybridized with radiolabeled probes prepared from 5' untranslated regions of VN25, VN10, and VN22 genes. VN61 and VN109 gave similar results (data not shown).

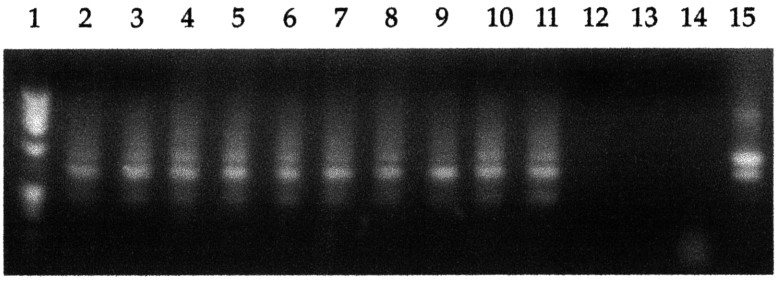

Figure 3. Single cell RT-PCR

Aliquots of the RT reaction from single cell H12 were amplified in two successive rounds of PCR, using primers that would see AF011413 and closely related subfamily members. Lane 1, φX-HaeIII marker; lanes 2-11, PCR products from 1ul aliquots of RT template; lane 12, negative control (for primary followed by secondary PCR); lane 13, negative control (secondary PCR); lane 14, negative control (primary PCR); lane 15, positive control.

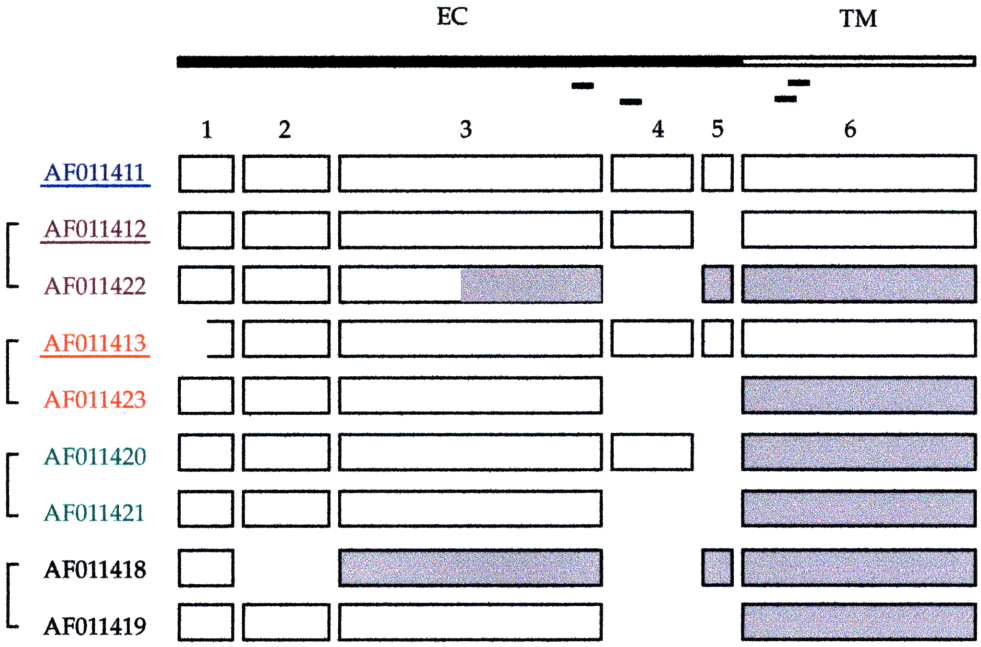

Figure 4. Closely related VN-αG0 sequences.

Primers were designed to conserved regions in this subfamily (▬).
Empty boxes denote open reading frames. Underlined sequences have no frame shifts. Shaded areas represent coding sequences that are untranslated due to a frame shift. EC denotes the proposed N-terminal extracellular domain. TM represents the predicted transmembrane domains.
AF011411 and AF011412 share 96% nucleotide sequence identity. AF011413 is 92% identical to AF011411 and 91% identical to AF011412. Unboxed sequences with frame shifts are variant mRNAs. These are shown paired with their most closely related subfamily member and are likely the same sequence only differing by variant splicing.
AF001420/AF001421 are 93% identical to both AF001411 and AF011412.
AF011418/AF011419 are 97% identical to AF011411.

(adapted from Matsunami and Buck, 1997)

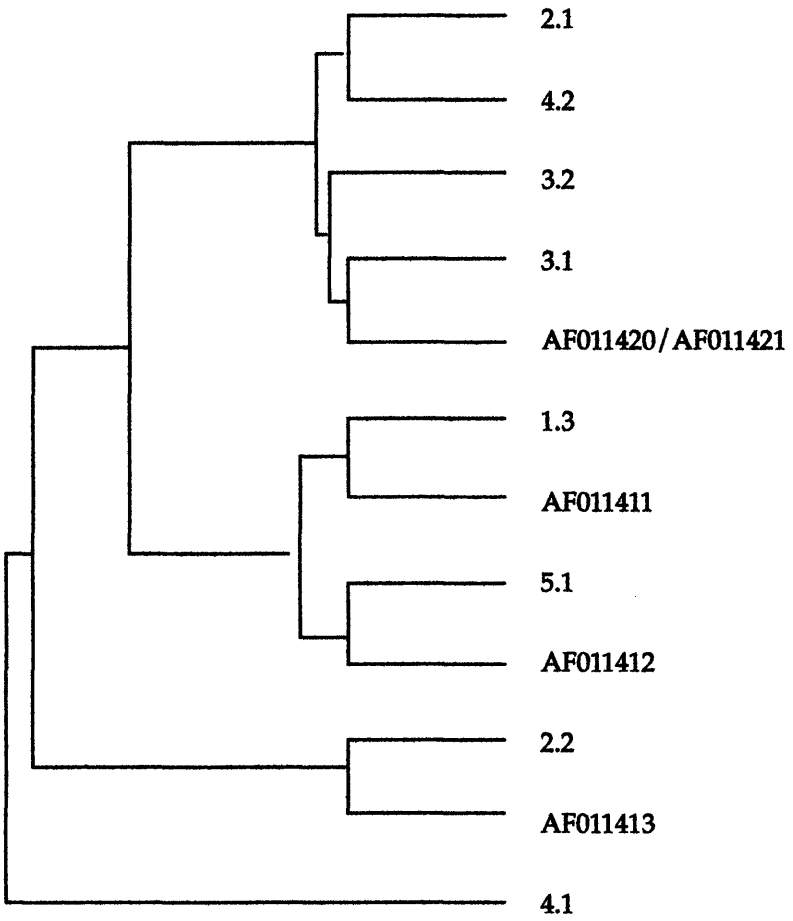

Figure 5. Homology derived relationships between single cell H12 PCR products and known subfamily member genes.