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A Novel Family of Divergent Seven-Transmembrane Proteins: Candidate Odorant Receptors in *Drosophila*

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Summary

Although insects have proven to be valuable models for exploring the function, organization, and development of the olfactory system, the receptor molecules that bind odors have not been identified in any insect. We have developed a novel search algorithm, used it to search the *Drosophila* genomic sequence database, and identified a large multigene family encoding seven transmembrane domain proteins that are expressed in olfactory organs. We show that expression is restricted to subsets of olfactory receptor neurons (ORNs) for a number of these genes. Different members of the family initiate expression at different times during antennal development. Some of the genes are not expressed in a mutant of the Acj6 POU-domain transcription factor, a mutant in which a subset of ORNs show abnormal odorant specificities.

Introduction

Animals can detect a vast array of odors with remarkable sensitivity and discrimination. Olfactory information is first received by olfactory receptor neurons (ORNs), which transmit signals into the CNS where they are processed, ultimately leading to behavioral responses. An enormous amount of investigation into olfactory function, organization, and development has been carried out in insect model systems for many years (Kaissling, 1987; Hildebrand, 1995). However, a number of central questions have been refractory to incisive analysis because the receptor molecules to which odor molecules bind have not been identified in any insect.

To investigate the molecular mechanisms of olfactory function and development, we are studying the olfactory system of *Drosophila melanogaster*, which is highly sensitive and capable of odor discrimination (Siddiqi, 1987; Carlson, 1996). There are two olfactory organs on the adult fly: the third segment of the antenna and the maxillary palp (Figure 1A). In both organs, ORNs are housed in sensory hairs called sensilla. The organization of the ∼1200 ORNs of the antenna is complex but ordered. On the antenna, there are different morphological categories of sensilla: s. trichodea, s. coeloconica, large s. basiconica, and small s. basiconica (Figure 1B). The different morphological categories of sensilla are distributed in overlapping patterns across the surface of the antenna (Figures 1C–1F) (Venkatesh and Singh, 1984; Stocker, 1994).

Electrophysiological studies show that each morphological category of sensilla can be divided into different functional types (denoted by different colors in Figures 1C–1F), defined by the characteristic response profiles of their ORNs (Siddiqi, 1991; Clyne et al., 1997; M. de Bruyne, P. J. C., and J. R. C., unpublished data). For s. trichodea, the different functional types are segregated into zones on the surface of the antenna (Figure 1C); segregation is also observed for the different functional types of s. coeloconica (Figure 1D). This zonal organization is less conspicuous for large and small s. basiconica, of which different functional types are intermingled (Figures 1E and 1F). Electrophysiological data suggest that there are on the order of 30 different classes of ORNs in the antenna, a rough estimate based upon the odor response profiles of individual ORNs (and, in a few cases, the assumption that the neurons of particular functional types of sensilla have unique response profiles).

In contrast to the antenna, the organization of the ∼120 ORNs of the maxillary palp is simpler. There are ∼60 s. basiconica on the maxillary palp, each housing two ORNs (Singh and Nayak, 1985). The 120 ORNs fall into six different classes based upon their odorant response profiles (M. de Bruyne, P. J. C., and J. R. C., submitted; Clyne et al., 1999 [this issue of *Neuron*]). Neurons of the six ORN classes are always found in characteristic pairs in three functional types of s. basiconica, with the total number of neurons in each class being equal. Each class is distributed broadly over all, or almost all, of the olfactory surface of the maxillary palp.

Thus, electrophysiological and anatomical studies suggest that there are on the order of 35 classes of ORNs in the adult fly (∼30 on the antenna and 6 on the palp), each class with a distinct odor sensitivity. Classes of ORNs found in the antenna are arrayed in zones, while the classes of ORNs found in the maxillary palp are distributed in a less ordered fashion. ORNs in both the maxillary palp and the antenna extend their axons to the antennal lobe of the brain, where first-order processing of olfactory information occurs. The lobe contains ∼40 olfactory glomeruli, spheroidal modules where ORN axons converge and where their terminal branches form synapses with the dendrites of their target interneurons (Stocker, 1994; Hildebrand and Shepherd, 1997; Laissue et al., 1999).

What is the molecular basis for the distinct odor sensitivities of the different classes of ORNs? One possibility is that each class of ORN expresses a unique odorant receptor, as has been proposed for vertebrate olfactory systems (Ngai et al., 1993a; Ressler et al., 1993; Vassar et al., 1993; Buck, 1996; Hildebrand and Shepherd, 1997). Alternatively, each class of ORN might express a unique combination of a large set of receptors, as found in chemosensory cells of the nematode, *C. elegans* (Troemel et al., 1995). Both models call for a family of receptor

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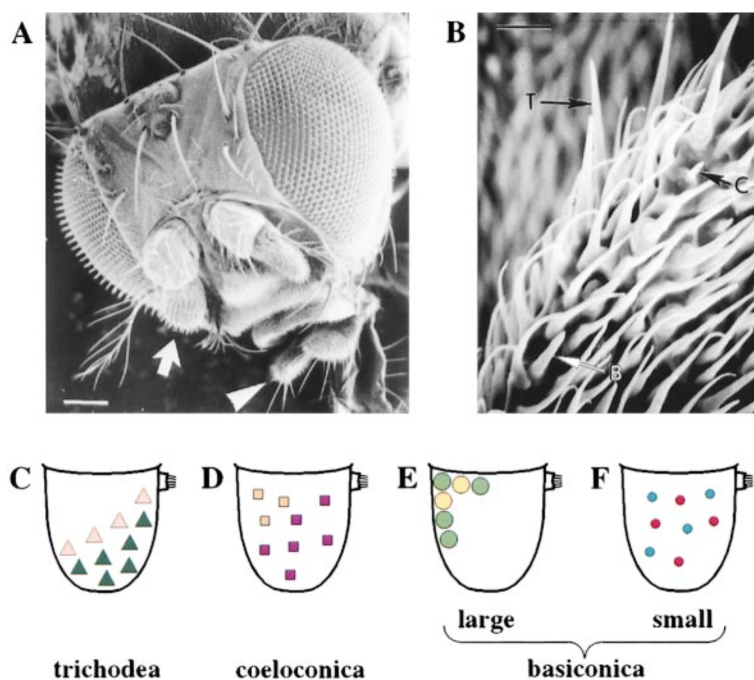


Figure 1. An Overview of the Olfactory System of the *Drosophila* Adult

(A) The two olfactory organs of the adult fly, the third antennal segment (arrow) and the maxillary palp (arrowhead). Scale bar, 100 μm. Taken from Riesgo-Escovar et al. (1995).

(B) Higher magnification of part of a third antennal segment showing the morphological categories of olfactory sensilla: s. basiconica ("B"), s. trichodea ("T") and s. coeloconica ("C"). There are ~150 trichoid, 200 basiconic, and 60 coeloconic sensilla on the antennal surface (Stocker, 1994). Scale bar, 5 μm. Taken from Riesgo-Escovar et al. (1997).

(C–F) Diagram of the olfactory sensilla on the anterior face of the third antennal segment. The different morphological categories of sensilla are indicated by different shapes, and the colors indicate different functional types of sensilla within each morphological category (see text). Dorsal is at the top and medial is to the left.

(C) Distribution of different functional types of s. trichodea. Note the zonal distribution of the two types.

(D) Distribution of different functional types of s. coeloconica. As for s. trichodea, each functional type occupies a distinct zone.

(E) The large s. basiconica are densely clustered in a small dorsomedial region, where the different functional types are intermingled. For simplicity, only two types are shown.

(F) The small s. basiconica are widely dispersed, and the different functional types are intermingled.

genes, and several lines of evidence suggest that for insects such a family would belong to the superfamily of seven-transmembrane G protein–coupled receptors (GPCRs). First, there is evidence that insects generate responses to odorants via GPCR-activated second-messenger systems. For example, a rapid and transient increase in inositol 1,4,5-trisphosphate (IP_3) has been observed in response to stimulation with pheromone and other odors using antennal preparations from various insect species (Breer et al., 1990; Boekhoff et al., 1993; Wegener et al., 1993). This increase in IP_3 can be blocked by pertussis toxin, implicating a G protein signaling cascade (Boekhoff et al., 1990). In *Drosophila*, *norpA* mutants, which lack the phospholipase C that is an essential component of phototransduction, also exhibit reduced olfactory responses of the maxillary palp (Riesgo-Escovar et al., 1995). A second reason to suspect that odorant receptors in *Drosophila* are GPCRs is that GPCRs have been shown to be odorant receptors in both vertebrates and *C. elegans*; moreover, abundant evidence indicates that olfactory information in these other organisms is transduced by GPCR-activated second-messenger systems (Buck, 1996; Bargmann and Kaplan, 1998). It would thus seem unlikely that a family of receptors that have a completely novel structure and that use a completely different transduction mechanism would have arisen in insects.

There have been extensive efforts to identify odorant and pheromone receptors in a variety of insects using a wide range of strategies. These efforts have been driven in part by interest in analyzing receptor genes in the context of highly tractable experimental systems in which there is a wealth of knowledge about olfactory function and organization. For example, *Drosophila* offers the advantages of a model genetic organism together with the ability to measure olfactory function conveniently in vivo, through either physiological or behavioral means. Interest in insect odorant receptors has also arisen because of the critical role of olfaction in the attraction of many insect pests to their plant hosts, of insect vectors of disease to their human hosts, and of insects to their mates. Nevertheless, efforts to identify odorant receptors in insects, based upon searches for genes bearing sequence similarities to odorant receptor genes from other organisms, or on other strategies, have been unsuccessful.

Here, we describe a novel multigene family encoding candidate odorant receptors that we identified from the *Drosophila* genomic sequence database. The 16 genes described here were discovered using novel computer programs that identify diagnostic features of the protein structure of the seven-transmembrane GPCR superfamily. Members of this new family are highly divergent from previously defined genes. Nearly all of the genes are found to be expressed in one or both of the olfactory organs, and for a number of genes we show that this expression is restricted to a subset of ORNs. We show that expression of different genes is initiated at different times during the development of the adult antenna, and that expression of a subset of these candidate receptor genes depends on the POU-domain transcription factor, Acj6.

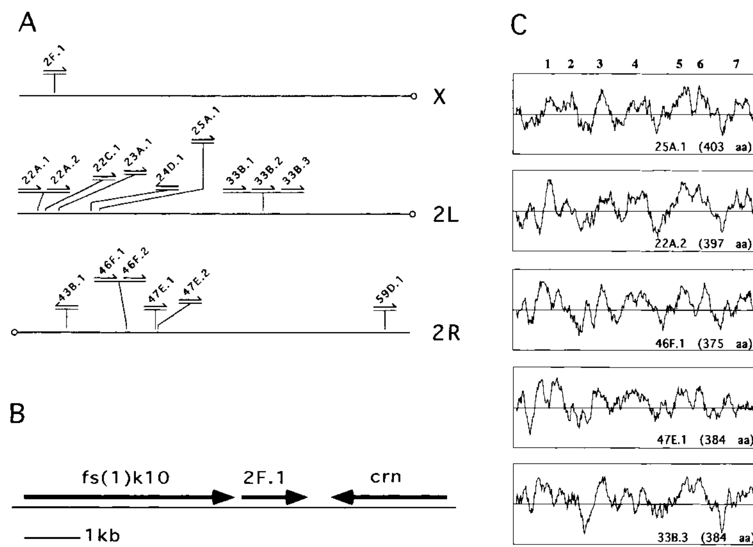


Figure 2. Genomic Organization and Hydropathy Plots of DOR Genes

(A) Genomic organization of DOR genes (not to scale). The genes shown are those identified from 16% of the total genomic sequence; most of the available sequence is from chromosome 2. The approximate chromosomal location of each gene is indicated. Genes separated by less than 1 kb are jointly underlined. Within each cluster, all genes are oriented in the same direction. The transcriptional orientation of the DOR genes with respect to the chromosome is unknown for 2F.1, 25A.1, 47E.2, 59D.1, and the cluster at 33B.

(B) The 2F.1 gene is flanked by two closely linked genes, *fs(1)k10* and *crn*. The arrowheads indicate the 3′ ends of the genes; for 2F.1, the end of the arrow indicates the position of the poly(A) addition signal sequence.

(C) Hydropathy plots of the genes whose expression patterns are shown in Figures 4–6. Hydrophobic peaks predicted by Kyte–Doolittle analysis appear above the center line. The approximate positions of the seven putative transmembrane domains are indicated above the first hydropathy plot. Similar plots were obtained for all the DOR genes.

Results

Identification of a Family of Putative G Protein–Coupled Receptors

In vertebrates and nematodes, it is estimated that there are hundreds of olfactory receptor genes, widely distributed in the genome (Buck and Axel, 1991; Troemel et al., 1995). With ~10% of the *Drosophila* genome sequenced, we thought it likely that some of the *Drosophila* odorant receptor genes had been sequenced. We adopted a two-part strategy to identify odorant receptor genes from the genomic database. First, we designed a computer algorithm to search the *Drosophila* genomic sequence for open reading frames (ORFs) from candidate odorant receptor genes. Second, we used RT–PCR to see if transcripts from any of these ORFs were expressed in olfactory organs.

For our computational screens, we used the genomic sequence data obtained by FTP from the Berkeley *Drosophila* Genome Project (BDGP) (http://www.fruitfly.org; version available in June 1998). We first identified ORFs of 300 bases or longer in all six frames. Next, a program written to identify GPCRs statistically by their physicochemical profile was used to screen for candidate ORFs (see Experimental Procedures). We then reduced the number of possible candidates by comparing them to *Drosophila* codon usage tables (http://flybase.bio.indiana.edu; version 10). Candidate ORFs whose codon usage differed at a significance level of 0.0005 by the chi-square statistic were discarded from the candidate set. Using these screening steps, we obtained 34 candidate ORFs.

Further analysis revealed that 8 of the 34 candidate ORFs corresponded to genes of known function, for example a cyclic nucleotide–gated channel (Baumann et al., 1994), and we did not analyze these ORFs further. Most of the remaining ORFs encoded fewer than seven predicted transmembrane domains. We therefore examined the genomic DNA surrounding each of the computer-identified ORFs for the presence of neighboring ORFs encoding additional transmembrane domains to which the original ORFs might be spliced. We used the *Drosophila* 5′ and 3′ intron–exon consensus splice sequences in this analysis to help identify linked exons (Mount et al., 1992). This analysis yielded several genes that could encode seven transmembrane domain proteins.

RT–PCR with primers designed from two of these final candidates yielded amplification products from antennal cDNA (data not shown). From RT–PCR experiments, the two genes did not appear to be expressed in the maxillary palp, abdomen, thorax, or head from which olfactory organs had been removed, suggesting that these genes were expressed specifically in the antenna. These two genes are located within 500 bp of each other at cytological position 22A (Figure 2A), and their predicted proteins are 75% identical at the amino acid level (see below).

To determine if these two candidates were part of a larger family of genes encoding seven transmembrane domain proteins, we used their sequences in BLAST searches of the *Drosophila* genome database to identify related genes (Altschul et al., 1990). Homologs of the two candidates were found, and their sequences were used in turn for further database searches. In total, 16 genes have been identified from the ~16% genomic sequence currently available. We have tentatively named this family of genes DOR (for *Drosophila* olfactory receptor), and each individual gene was named based upon its cytogenetic location in the genome. Thus, the two genes identified initially are DOR22A.1 and DOR22A.2, which we abbreviate here as 22A.1 and 22A.2. (The final digit in this nomenclature is used to distinguish the genes at a site and does not refer to the cytogenetic

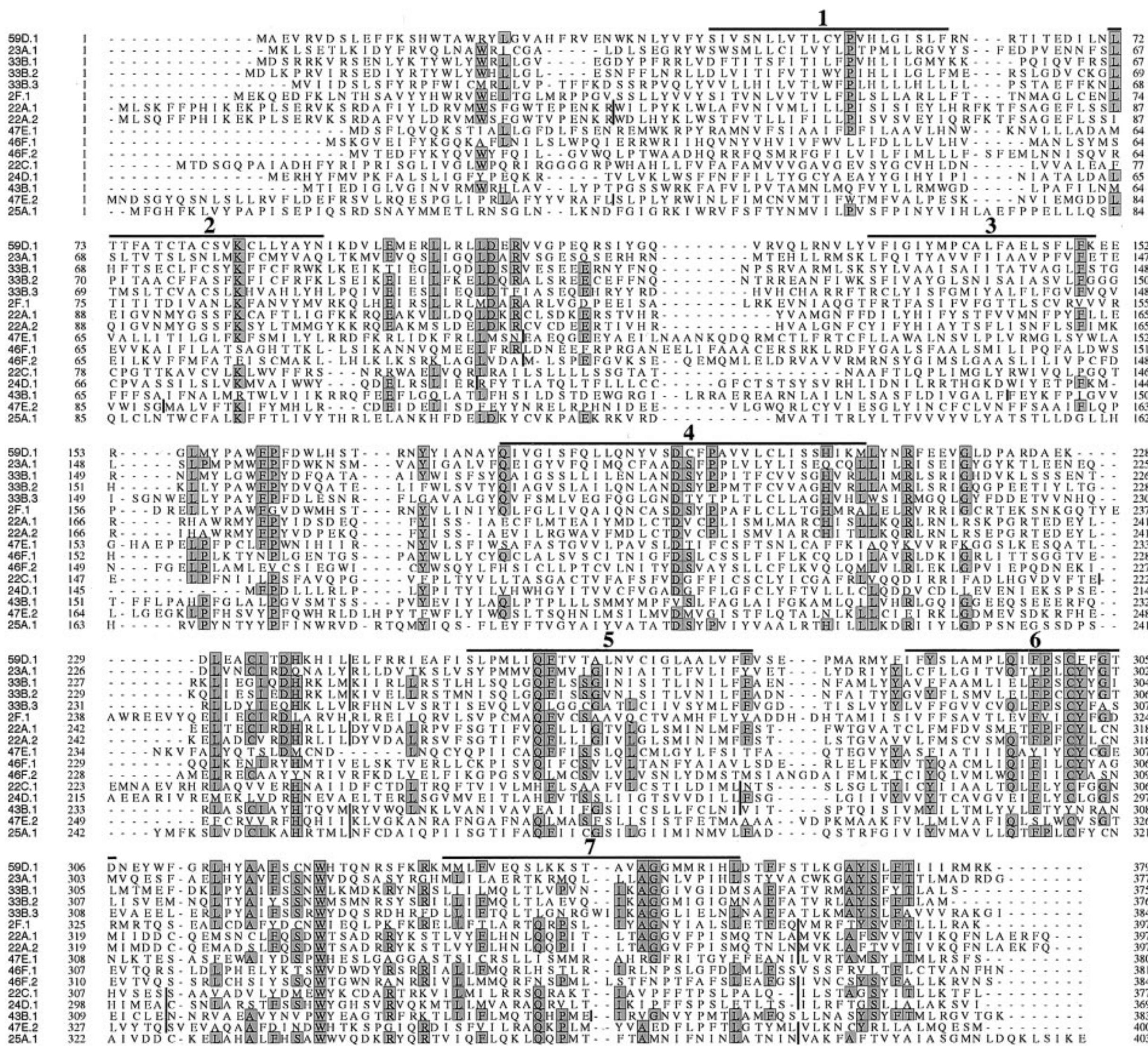


Figure 3. Amino Acid Sequence Alignment of DOR Genes

All DNA sequences were obtained from the BDGP database, and the determination of predicted amino acid sequences is described in Experimental Procedures. Residues conserved in >50% of the predicted proteins are shaded. The approximate locations of predicted transmembrane domains 1–7 are indicated. Exon–intron boundaries are shown with vertical lines.

band number.) The genomic locations of all of the DOR genes identified so far are indicated in Figure 2A, and an alignment of their amino acid sequences is presented in Figure 3. Of the 16 family members, 13 have been found to be expressed in either the antenna or the maxillary palp, or in both, based upon RT–PCR analysis (Table 1) and in situ hybridizations to RNA in tissue sections (described below).

The DOR genes have no significant similarities to any known genes, and do not appear in any of the *Drosophila* EST databases. However, Kyte–Doolittle hydropathy plots of the predicted proteins show that each has approximately seven peaks that could represent transmembrane domains (Figure 2C) (Kyte and Doolittle, 1982). The lengths of the 16 proteins are between 369 and 403 amino acids, similar to the lengths of most previously described families of GPCRs (Probst et al., 1992). In addition, the spacing of the putative transmembrane domains gives rise to predicted intracellular and extracellular loops similar in size to those in many families of GPCRs (Probst et al., 1992).

Amino acid sequence identity among the DOR genes ranges from ~10%–75%, with many genes showing a relatively low level of identity to each other (~20%). Two pairs of clustered genes, 22A.1/22A.2 and 33B.1/33B.2 show the highest identity, with 75% and 57% identities, respectively. However, not all clustered genes show high degrees of similarity. 33B.3, for example, is only 28% identical to both 33B.1 and 33B.2, and 46F.1 and 46F.2 are only 29% identical. In addition to exhibiting sequence identity, many of the genes contain introns in corresponding locations (Figure 3), consistent with their constituting a family derived from a common ancestral gene.

Table 1. Tissue-Specific Expression of DOR Genes Determined by RT–PCR

Gene	Antenna	Maxillary Palp	*acj6*[6]
2F.1	+	ND	ND
22A.1	+	−	+
22A.2	+	−	+
22C.1	−	−	ND
23A.1	+	−	+
24D.1	−	−	ND
25A.1	+	+	+
33B.1	+	−	−
33B.2	+	−	−
33B.3	+	+	−
43B.1	+	−	+
46F.1	−	+	−
46F.2	+	−	−
47E.1	+	−	+
47E.2	+	−	+
59D.1	−	−	ND

For each gene, expression was examined in the null mutant *acj6*[6] in those organs that showed expression in wild type, except that for DOR 25A.1, expression in *acj6*[6] has been examined only in the antenna. ND, not determined.

Table 2. Summary of RNA In Situ Hybridization Data

Gene	Antenna	Maxillary palp	*acj6*[6]
33B.3	−[a]	17 ± 1 (n = 5)	−
46F.1	−	18 ± 1 (n = 5)	−
47E.1	40 ± 1 (n = 6)	−	+
22A.2	22 ± 1 (n = 11)	−	+
25A.1	16 ± 1 (n = 5)	−[a]	+
22A.1	17 ± 1 (n = 9)	−	ND[a]
2F.1	+	−	ND
23A.1	−[a]	−	ND[a]
33B.1	−[a]	−	ND
33B.2	−[a]	−	ND
46F.2	−[a]	−	ND

The number of cells expressing each DOR gene ± SEM is shown. "n" indicates the number of olfactory organs for which a complete set of cross sections was counted. "+" indicates that stained cells were clearly observed but not quantitated; "−" indicates no labeled cells were observed.
ND, not determined.
[a] Expression detected by RT–PCR (Table 1).

There are 67 residues that are conserved among at least 50% of the genes, and most of these (49) are in the C-terminal halves of the proteins (Figure 3). Among the conserved residues are a serine and a threonine in the intracellular C-terminal tail, residues frequently conserved in this region of GPCRs (Probst et al., 1992). The most divergent region in the sequences is a stretch of 30 amino acids representing part of the first extracellular loop and nearly all of transmembrane domain 3. The divergence in this region also occurs in the most conserved pairs of genes: 22A.1 and 22A.2 are 75% identical overall but only 50% identical in this region, and 33B.1 and 33B.2 are 57% identical overall but only 33% identical in this region. We note that transmembrane domains 3, 4, and 5 are exceptionally divergent in rat odorant receptors and have been proposed to play a role in odorant binding (Buck and Axel, 1991).

Some of the genes are clustered in the genome (Figure 2A), while others are apparently isolated. Within a cluster, the average intergenic distance is on the order of 500 bp. Clustered DOR genes do not necessarily have introns in corresponding locations (e.g., 46F.1 and 46F.2), but all clustered genes have their transcriptional orientations in the same direction (Figure 2A). At least one of the DOR genes (2F.1) is flanked closely on both sides by two apparently unrelated genes (Figure 2B) (Haenlin et al., 1987).

To determine whether any of the DOR genes have closely related homologs, we used coding regions from nine of the genes to probe Southern blots of *Drosophila* genomic DNA at high or reduced stringency. For the closely related genes, such as 22A.1 and 22A.2, we used a combined probe. Each probe appeared to detect only its own sequence at high stringency, while at lower stringency most genes detected one or two novel bands (data not shown). As expected, because of the overall low level of similarity, none of these extra bands corresponded to any of the other known DOR genes. These data indicate that some of these genes have one or two closely related homologs but that none belongs to a large subfamily of highly related genes.

DOR Genes Are Expressed in Subsets of Olfactory Receptor Neurons

Olfactory receptor neurons of the adult fly are located in both the antenna and the maxillary palp. To ask whether any of the DOR genes are expressed in these neurons, we carried out in situ hybridization to RNA in adult tissue sections. Of 11 genes examined, 7 showed detectable expression, which in every case was observed only in the olfactory organs (Table 2). The 46F.1 probe hybridized to a subset of ORNs in the maxillary palp (Figure 4A). Counting of labeled ORNs in serial sections revealed that the total number of 46F.1-staining ORNs per maxillary palp was 18 ± 1 (Table 2), or 15% of the 120 olfactory neurons in the maxillary palp. A similar number of neurons, 17 ± 1, was labeled by another probe, 33B.3 (Figure 4B). The neuronal identity of the labeled cells was apparent from the presence in many cases of a well-defined axon projecting from the labeled cell body and joining the maxillary nerve (Figures 4B and 4C). For both probes, the labeled neurons were distributed broadly over the olfactory surface of the organ, and were interspersed among unlabeled neurons (Figures 4A–4C). We note that staining in many cells appeared annular, which we interpret to reflect a perinuclear distribution of mRNA, as expected of an mRNA present at highest concentrations in the cell bodies of these ORNs (Figure 4B). The 33B.3 and 46F.1 genes are evidently expressed in different subsets of ORNs, because the number of neurons hybridizing with a mixed probe was greater than the number of neurons that hybridized when either probe was used individually (data not shown). For neither probe was hybridization detected in the antenna, head, or thorax.

Many of the DOR genes are expressed in the antenna and not in the maxillary palp, as determined by RT–PCR (Table 1). For several genes, we confirmed this localization by in situ hybridization. The 47E.1 probe hybridized to 40 ± 1 cells in a broad area across the antenna

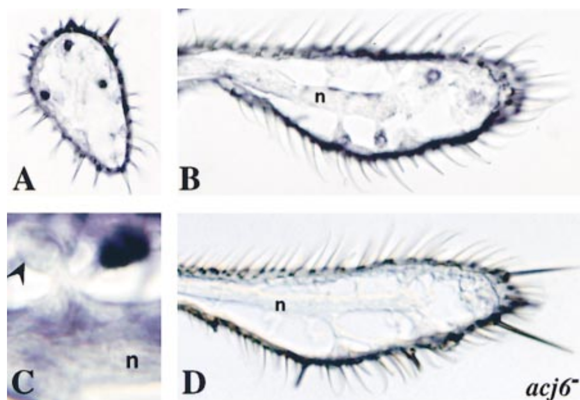


Figure 4. DOR Genes Are Expressed in Subsets of Olfactory Receptor Neurons in the Maxillary Palp

In situ hybridizations to tissue sections of maxillary palps. (A) shows a frontal section; all other sections are sagittal.

(A) A 46F.1 probe reveals expression in a subset of ORNs that are broadly distributed. The background staining at the periphery of the organ represents nonspecific labeling of the cuticle, observed equally for sense and antisense probes.

(B) A 33B.3 probe also hybridizes to a subset of cells. Note the perinuclear distribution of the staining: the annular staining pattern of the neuron at the top, right of center, is particularly conspicuous. Unlabeled ORNs are visible under the cuticular surface (top center).

(C) At higher magnification, it can be seen that the cells expressing 46F.1 are neurons. Note the axons projecting from the cells into the nerve ("n") that runs through the middle of the maxillary palp. The arrowhead indicates an ORN that is not expressing 46F.1, adjacent to an ORN that is strongly stained. The light staining of the nerve is background staining, observed equally for sense and antisense probes.

(D) 33B.3 is not expressed in the *acj6* null mutant, *acj6*^{6}.

(Figures 5A and 5B), including both anterior and posterior faces, similar to the distribution pattern of small s. basiconica (Figure 1F). A probe from the 25A.1 gene hybridized to fewer cells, 16 $\pm$ 1, but in a region of the antenna similar to that of 47E.1 staining, as judged by reconstruction of serial sections (Figures 5C and 5D). The 22A.2 probe hybridized to 22 $\pm$ 1 cells in a different distribution, clustered in the dorsomedial region of the antenna (Figure 5E). This pattern matches the distribution of the large s. basiconica (Figure 1E). The expression patterns of the three genes in the antenna are illustrated schematically in Figure 5G. None of these three probes revealed expression in the maxillary palp, head, or thorax. In summary, we have demonstrated that the DOR family is expressed in ORNs and that the expression of individual members is restricted to distinct subsets of cells in the olfactory organs.

Expression Patterns of Candidate Olfactory Receptor Genes during Development

Recent evidence supports a dual role for the vertebrate olfactory receptor genes: first, an instructive role in guiding the axons of ORNs to the correct glomeruli during development (Mombaerts et al., 1996; Wang et al., 1998) and, second, as odorant receptors in the adult (Zhao et al., 1998). To address the possibility that the DOR genes might also play a role in development, we hybridized three DOR probes to antennal sections from different times during pupal development. In *Drosophila*, ORN axons first leave the developing antenna at ~16 hr after puparium formation (APF) (Lienhard and Stocker, 1991; Ray and Rodrigues, 1995; Reddy et al., 1997), and the diameter of the antennal nerve continues to increase until 72 hr APF (Stocker et al., 1995). Glomeruli first become visible in the antennal lobe at ~48 hr APF. We examined developing antennae at 16 hr, 24 hr, 36 hr, 48 hr, 54 hr, 60 hr, 72 hr, and 93 hr APF (adults eclosed from the pupal case at ~100 hr).

Cells positive for 22A.2 were first seen at 60 hr APF, indicating that detectable expression begins between 54 hr and 60 hr, well within the period in which the antennal nerve is still increasing in diameter (Figures 6A and 6B). A subset of cells was labeled at this time, and they were restricted to a subregion of the developing antenna; the pattern appears comparable to that of the mature antenna, although we have not characterized it in as much detail as that of the adult. Labeling with 22A.2 was also observed in antennae at all subsequent time points. Interestingly, cells positive for 47E.1 and 25A.1 were not observed until much later, at the 93 hr time point; they were not observed at any of the earlier times (Figures 6C and 6D and data not shown). For comparison, we also performed in situ hybridization with a probe representing the odorant-binding protein OS-E (McKenna et al., 1994), which is believed to play a role in olfactory function, but which has not been implicated in a developmental process. OS-E was also first observed at 93 hr, at which time it shows very abundant expression (Figures 6E and 6F).

Expression of a Subset of Candidate Receptor Genes Depends on the POU-Domain Transcription Factor Acj6

Little is known about the regulation of odor receptor genes, a process critical to the establishment of olfactory neuron identity and ultimately to the process of olfactory coding. In *C. elegans*, the *odr-7* gene, a member of the nuclear receptor superfamily, has been shown to regulate the odorant receptor gene *odr-10* (Sengupta et al., 1994, 1996). In *Drosophila*, null mutations of the *acj6* gene, which encodes a POU-domain transcription factor, eliminate the odor response of three of the six classes of maxillary palp ORNs (Clyne et al., 1999). A fourth ORN class on the maxillary palp is altered to a new class of ORN with a novel odor sensitivity. These data suggest that Acj6 plays a role in the differentiation of certain maxillary palp ORNs, perhaps by determining which olfactory receptor gene(s) are expressed. To address the possibility that Acj6 regulates odorant receptor genes, we hybridized probes from the 33B.3 and 46F.1 genes to sections of maxillary palps from the null mutant *acj6*^{6}. No hybridization was detected in either case (Figure 4D and data not shown), nor was expression of either gene detected by RT–PCR from *acj6*^{6} maxillary palps (Table 1).

acj6 mutations also affect the physiological response of antennal neurons to odors (Ayer and Carlson, 1991, 1992). We therefore hybridized the 22A.2, 25A.1, and 47E.1 probes to sections of *acj6*^{6} antennae. All three probes hybridized to groups of cells in the same locations as in the wild-type antenna (Figure 5F, Table 2,

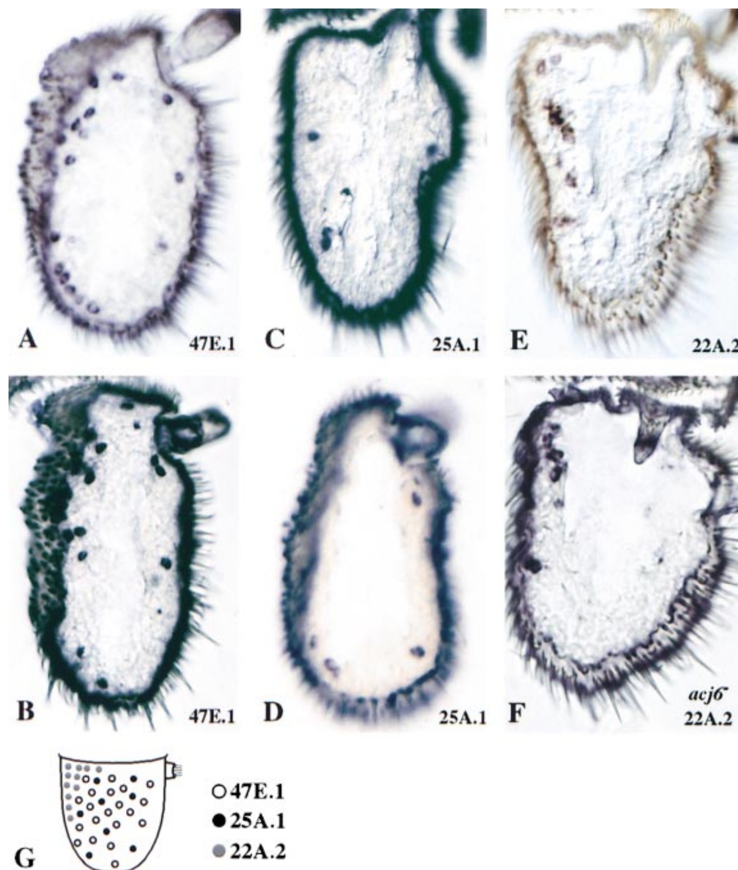


Figure 5. DOR Genes Are Expressed in Subsets of Antennal Cells

Shown are in situ hybridizations to tissue sections of third antennal segments. In (A), (B), (D), and (F), the plane of section passes through the fluid-filled interior of the antenna.

(A and B) A 47E.1 probe hybridizes to a subset of cells that are broadly distributed.

(C and D) A 25A.1 probe hybridizes to a smaller subset of cells. The angle of section in (C) differs somewhat from the other panels.

(E) A 22A.2 probe hybridizes to a subset of cells in the dorsomedial region where the large s. basiconica are located.

(F) 22A.2 is expressed in the *acj6*^{6} mutant, in contrast to 33B.3 (Figure 4D).

(G) Summary of distributions of labeled cells for 47E.1 (open circles), 25A.1 (closed circles), and 22A.2 (shaded circles) on the anterior face of the antenna, based on analysis of expression in 30–50 antennae for each gene.

and data not shown). RT–PCR amplification showed that expression of certain other DOR genes, 33B.1, 33B.2, 33B.3, and 46F.2, was eliminated in the antenna of *acj6*^{6} (Table 1). Thus, in the *acj6*^{6} mutant, one subset of candidate odorant receptor genes was not expressed while a different subset remained unaffected. Interestingly, genes within a cluster all showed similar dependency on Acj6: 33B.1, 33B.2, and 33B.3, for example, all depended on Acj6, whereas 22A.1 and 22A.2 did not. In summary, these data support a role for *acj6* in the regulation of a subset of olfactory receptor genes.

Discussion

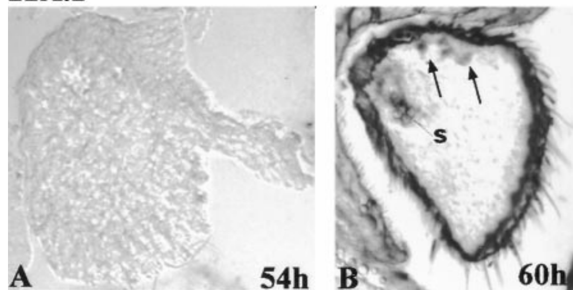
Candidate *Drosophila* Odorant Receptor Genes

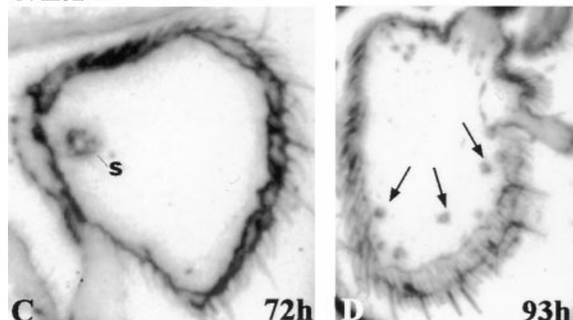
We used a novel strategy to search the *Drosophila* genomic sequence database for genes encoding potential GPCRs, leading to the identification of a multigene family with properties expected of odorant receptors. In addition to these genes, we also identified by this strategy a wide variety of other transmembrane proteins, a few previously identified by other means and many representing novel proteins with similarity to known transmembrane proteins. These results suggest that the algorithm may be of widespread use in identifying new receptors, channels, and other transmembrane proteins.

The family of candidate odorant receptor genes currently contains 16 members, identified from the 16% of the *Drosophila* genomic sequence that is available. By extrapolation, the size of this family may be on the order of 100 genes, making it the largest gene family identified in *Drosophila*.

There are several lines of evidence indicating that these genes are likely to encode *Drosophila* odorant receptors. First, the predicted proteins encoded by the genes each contain approximately seven potential transmembrane domains, as expected of GPCRs. Second, we have found that the genes are expressed in one or both of the two olfactory organs, and for a number of genes have shown that this expression is restricted to a subset of ORNs, as expected for odorant receptors. Third, the large number of family members, and the clustered location of some in the genome, are reminiscent of odorant receptors in other organisms.

Comparison of the sequences of these candidate odorant receptors to those from other organisms shows that they are extremely divergent from known odorant receptors and other GPCR families. This is not surprising, as searches for these genes based on sequence similarity to odorant receptors from other organisms had not succeeded, and the odorant receptor families in vertebrates and *C. elegans* are essentially unrelated. There is a great deal of sequence divergence among the DOR genes, much more than among the rat sequences reported by Buck and Axel (1991), for example. Moreover, genomic Southern blots have shown that none of nine DOR genes tested defines a subfamily of more than two or so well-conserved genes. The DOR family therefore differs in this respect from the mouse family,





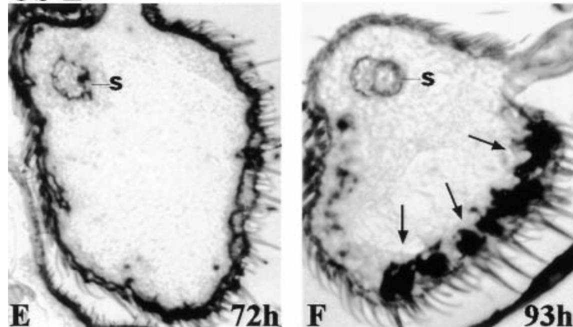


Figure 6. Expression of DOR Genes during Antennal Development

In situ hybridizations to tissue sections of third antennal segments at different times during pupal development. The times indicated refer to hours APF (after puparium formation). Arrows indicate labeled cells.

(A) Expression of 22A.2 is not observed at 54 hr APF. Note that background staining is absent in sections taken at 54 hr (or at earlier times), presumably due to the immaturity of the cuticle.

(B) Expression of 22A.2 is observed at 60 hr APF.

(C) 47E.1 expression is not observed at 72 hr APF. Background staining is seen with both sense and antisense probes on the cuticular surface of the sacculus (s), a multichambered sensory pit, and the dot at the bottom of the third antennal segment is nonspecific staining of a section of tracheal tissue.

(D) Expression of 47E.1 is detected at 93 hr APF.

(E) The odor binding protein OS-E is not expressed at 72 hr APF. The small dots at the bottom of the antenna are nonspecific staining of a section of tracheal tissue, observed with both sense and antisense probes.

(F) Abundant expression of OS-E is seen at 93 hr APF.

for example, where most odorant receptor genes belong to subfamilies of approximately seven to ten genes (Ressler et al., 1993).

Although, at present, the clusters of DOR genes we have identified contain smaller numbers of genes (three or fewer) than in other organisms (Troemel et al., 1995; Sullivan et al., 1996; Barth et al., 1997), a number of interesting features of the clustered genes are already apparent. As found in other organisms (Barth et al., 1997), *Drosophila* odorant receptor genes within a cluster are not necessarily coordinately regulated, such that genes within a cluster are expressed in different subsets of cells, and even in different olfactory organs (e.g., 46F.1 is expressed in the maxillary palp, whereas 46F.2 is expressed in the antenna). So far, all genes identified within a cluster, however, are transcribed in the same orientation. Genes within a cluster sometimes do, but sometimes do not, share intron positions, suggesting that introns may have become lost following gene duplication; a phylogenetic study revealed extensive gene duplication and intron loss among the chemoreceptor genes of *C. elegans* (Robertson, 1998).

Organization of the *Drosophila* Olfactory System

The number and broad distribution of maxillary palp neurons expressing 46F.1 and 33B.3 are intriguing in light of electrophysiological studies. There are ~120 ORNs on the palp, which fall into six different classes based upon their odorant response profiles. Each class contains roughly equal numbers of neurons, distributed broadly over the olfactory surface of the palp. Thus, if an individual receptor gene is expressed in all ORNs of a functional class, one might expect a gene to be expressed in a broad distribution, in ~20 neurons, in good agreement with the distribution and numbers observed for both 46F.1 and 33B.3 (18 ± 1 and 17 ± 1, respectively).

The two DOR genes whose expression was detected by in situ hybridization in the maxillary palp are expressed in ORNs housed within s. basiconica, the only morphological class of sensilla on the palp. In the antenna, the 22A.2 probe consistently hybridized to a subset of cells in a portion of the dorsomedial region of the antenna that contains almost exclusively large s. basiconica (Figure 1E). The 47E.1 and 25A.1 probes hybridize to subsets of cells in a distinctly different region of the antenna, which may correlate with the distribution of small s. basiconica, of which at least two functional types are intermingled (Figure 1F). We note that the numbers of cells to which 47E.1 and 25A.1 hybridize are different: 40 ± 1 and 16 ± 1; one possible interpretation is that they are expressed in distinct functional types of small s. basiconica. We note that this region also contains s. trichodea and s. coeloconica, however, and although the labeling patterns do not correlate with the distribution of either of two functional classes of s. trichodea (Clyne et al., 1997), a definitive identification of the sensillar type will require further investigation. If, in fact, all of the DOR genes are expressed in only one of the morphological categories of sensilla, the s. basiconica, we would predict that there are other, as yet unidentified, families of receptors that are expressed in the other morphological categories of sensilla. This would mean that the number of odorant receptors in *Drosophila* might be substantially larger than 100.

We have identified three DOR genes that are expressed in the maxillary palp (Table 1), from the 16% of

the genome analyzed. As these three genes, like most DOR genes, are not clustered in the genome, linear extrapolation suggests that the entire genome contains on the order of 18 DOR genes expressed in the maxillary palp, an organ that has six functional classes of neurons (M. de Bruyne, P. J. C., and J. R. C., submitted; Clyne et al., 1999). If all neurons within a functional class, i.e., with the same odor specificity, are identical in terms of their receptor expression, then the ratio of expressed genes to neuronal classes in this organ would be consistent with a model in which an individual ORN expresses a small number of odorant receptors; however, further data are needed to establish conclusively the number of receptor genes expressed per cell. Olfactory neurons in other organisms appear to lie at either of two extremes: in the vertebrates, it is believed only one receptor is expressed per ORN (Ngai et al., 1993b; Ressler et al., 1993; Vassar et al., 1993); in *C. elegans*, ~550 chemoreceptors are likely to be distributed among 14 classes of chemosensory neurons (Troemel et al., 1995).

ORNs in *Drosophila* and other insects project to an olfactory processing center, the antennal lobe, which is much like the olfactory bulb of vertebrates. Like its vertebrate counterpart, the antennal lobe contains olfactory glomeruli, of which the antennal lobe of *Drosophila* has ~40 (Stocker et al., 1995; Laissue et al., 1999). In vertebrates, there is an approximate equivalence between the estimated number of odorant receptor genes and the number of glomeruli (Barth et al., 1996; Buck, 1996); since *C. elegans* does not contain glomeruli, it has not been possible until now to consider whether the evolutionary conservation of this equivalence extends to invertebrates. If, in fact, the number of DOR genes is 100, then the ratio of odorant receptor genes to glomeruli would exceed two and would rise if additional families of odorant receptor genes were discovered. We note that the number of glomeruli receiving input from the maxillary palp has been variously estimated as three and five (Venkatesh and Singh, 1984; Stocker et al., 1995); if our estimate of 18 genes expressed in the maxillary palp is correct, then the ratio of these receptor genes to their corresponding glomeruli would fall in the range of three to six.

Regulation of DOR Genes

The DOR family is subject to complex regulation. First, the expression of individual DOR genes exhibits highly specific tissue and spatial localization. Some genes are expressed in the antenna but not the maxillary palp; others show expression in the maxillary palp but not the antenna. Within an organ, expression of a particular DOR gene is restricted to a subset of cells. In the antenna, the patterns of expression are spatially regulated, exhibiting regional specificity of expression as detailed above. In the maxillary palp, expression is limited to a population of neurons approximately equal in number to the neurons of a functional class.

DOR genes are also subject to interesting temporal regulation. One gene, 22A.2, is expressed in the developing antenna during a time when the antennal nerve is still increasing in diameter (Stocker et al., 1995). These data leave open a possible role for *Drosophila* olfactory receptors in axon guidance and glomerulus formation, a role for which evidence has been found in vertebrates (Mombaerts et al., 1996; Wang et al., 1998) but not *C. elegans*. In zebrafish, odorant receptors show asynchronous onset of expression during development of the olfactory placode (Barth et al., 1996). The DOR genes also show heterogeneity in their temporal regulation: expression of two other DOR genes begins much later than for the 22A.2 gene. If, in fact, individual ORNs express more than one DOR gene, perhaps some have acquired a specialized role in development.

We have also found evidence that different DOR genes are expressed at different levels of abundance within cells. Although RT–PCR experiments showed expression of 25A.1 in both antenna and maxillary palp, in situ hybridization revealed expression of 25A.1 only in the antenna of each animal examined; conversely, although RT–PCR experiments showed expression of 33B.3 in both olfactory organs, in situ hybridization detected label only in the maxillary palp of each animal examined (Tables 1 and 2). These results suggest that a receptor gene may be expressed at different cellular levels in the two organs and that different genes may be expressed at different cellular levels in the same organ. Such an explanation would suggest that there are mechanisms governing not only the spatial and temporal control of DOR genes but also their levels of expression.

If DOR genes are, in fact, expressed at different cellular levels in particular ORNs, then perhaps the five DOR genes that we were unable to detect in the antenna by in situ hybridization—despite clear evidence for their antennal expression from RT–PCR, a more sensitive technique—are among those expressed at low levels. We note that in *C. elegans*, expression of a number of candidate odorant receptors was undetectable using GFP fusion genes (Troemel et al., 1995).

As a first step in investigating the mechanisms through which the complex regulation of DOR genes is achieved, we tested the role of the POU-domain transcription factor Acj6, which we have previously found to act in governing olfactory neuron identity. We found that Acj6 is, in fact, required for expression of the DOR family. Two lines of evidence, RT–PCR and in situ hybridization analysis, both indicate that proper expression of a specific subset of DOR genes depends on Acj6. The results suggest that the odor specificity of a subset of ORNs is governed at least in part by the action of the Acj6 POU-domain transcription factor on DOR genes, and they are fully consistent with the notion that DOR genes may encode odorant receptors.

The isolation of genes likely to encode odorant receptors in *Drosophila* opens a number of avenues for future investigation. *Drosophila* provides the ability to manipulate odor receptors genetically and test the functional consequences of such manipulations in vivo, either physiologically or behaviorally. Such analysis may be useful in examining potential roles of DOR proteins in olfactory response and in development. It may also be possible to isolate homologous genes in other insects, including some that provide excellent opportunities for research and some of agricultural or medical importance that rely on olfactory cues to locate their hosts.

Experimental Procedures

Computer Algorithm to Recognize G Protein–Coupled Receptors (GPCRs)

Briefly, the algorithm uses statistical characterization of amino acid physicochemical profiles in combination with a nonparametric discriminant function. The key approach is to use the information in the interplay between the local structure (transmembrane α helix) and the global structure (repeated multiple domains) and characterize this information with concise statistical variables. The algorithm was trained on a set of 100 putative GPCR sequences from the GPCRDB (http://swift.embl-heidelberg.de/7tm) and a set of 100 random proteins selected from the SWISSPROT database. (We later expanded this training set greatly, but that version was not used for the genes reported in this paper.) In the first step, we used three sets of descriptors to summarize the physicochemical profiles of the sequences. These are: GES scale of hydropathy (Engelman et al., 1986), polarity (Brown, 1991), and amino acid usage frequency. For the first two of these measurements, we computed a sliding window profile (White, 1994) using a kernel of a 15 amino acid constant function convoluted with a 16 amino acid Gaussian function. These profiles were then summarized with three statistics: the periodicity (characterizing the quasiperiodic presence of the transmembrane domain), average derivative (characterizing the abrupt change between the transmembrane domain and the nontransmembrane domain), and the variance of the derivative (also characterizing the abrupt change). Therefore, each sequence was characterized by seven variables. These seven variables were used in a nonparametric linear discriminant function that was then optimized to separate the known GPCRs from random proteins in the training set. The same linear discriminant function with the scores derived from the training set was then used to screen the genomic database for candidate genes. The candidate sequences were given significance values by an odds ratio of the GPCRs and non-GPCRs computed using the observed empirical distribution of the training set. Those sequences with better than 95% odds ratio were considered for further analyses, described in the Results. More detailed information about the algorithm is available as supplemental data in the online version of this paper (http://www.neuron.org/cgi/content/full/22/2/327/DC1).

Sequence Analysis

Our computer algorithms identified the ORFs for the second exons of 22A.1 and 22A.2, which encode transmembrane domains 1–4. These ORFs are on the BDGP P1 clone called DS005342. We examined the DS005342 sequence around the initial ORFs for neighboring ORFs that encoded additional potential transmembrane domains. Key to the identification of these neighboring ORFs was the presence of intron–exon consensus splice sequences: GTRAGT for the 5′ end and HAG for the 3′ end (Mount et al., 1992). 22A.1 and 22A.2 were found to have two other introns in corresponding locations, all of which had conserved splice sequences.

The amino acid sequences of 22A.1 and 22A.2 were used in searches of the *Drosophila* genome database using the tBLASTn program of the BDGP. These searches yielded partial sequences of most other members of the DOR family. To complete the sequences of these genes, we performed an analysis of the genomic DNA around each identified ORF as we did for 22A.1 and 22A.2, using the locations of conserved introns in the genes, the intron consensus splice sequences, and the tBLASTn alignments as guides. Use of the genes identified in the second round as query sequences in tBLASTn searches and subsequent similar analysis of genomic DNA yielded the remaining genes. Additional searches of GenBank and SwissProt databases were performed with the NCBI (National Center for Biotechnology Information) BLAST network.

The sequence alignment in Figure 3 is based on the alignments predicted by the tBLASTn program of the BDGP and then edited extensively by hand. We note that the 5′ splice sequences for the most 3′ introns of both 2F.1 and 47E.1 are unfavorable. We assumed that these introns were spliced nonetheless, as the resulting amino acid sequence showed greater sequence identity to other DOR family members. However, if these introns are not spliced out, then the lengths of 2F.1 and 47E.1 would not be significantly altered from the lengths indicated in Figure 3. The 2F.1 gene was independently predicted to be a gene (GenBank accession number 2661571) by the EMBL genefinder program.

Reverse Transcriptase PCR

Individual flies were frozen in liquid nitrogen, and antennae and maxillary palps were dissected by hand. On average, 150 antennae or 200 maxillary palps were used for RNA preparation. Total RNA was prepared as described elsewhere (McKenna et al., 1994). The RNA was treated with DNaseI (GIBCO BRL) for 30 min at 37°C, phenol/–chloroform extracted, and reprecipitated. The entire RNA preparation was used for oligo dT–primed cDNA synthesis using Superscript II Reverse Transcriptase (GIBCO BRL) according to the manufacturer's directions. PCR was performed using Sigma Taq polymerase under standard cycling conditions, with an annealing temperature of 60°C, gene-specific primer concentration of 1 pM, and magnesium concentration of 2.5 mM. For all genes except 2F.1, primer pairs that span introns were used in order to distinguish PCR bands amplified from cDNA from those amplified from any remaining genomic DNA.

Nucleic Acid Hybridization

For in situ hybridization, coding regions of the DOR genes were subcloned into the pGEM-T Easy vector (Promega). Digoxygenin-labeled RNA probes were generated and hydrolyzed according to the manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN). In situ hybridizations to RNA in tissue sections were performed using a modified version of procedures described elsewhere (Hafen and Levine, 1986; Chadwick and McGinnis, 1987). Briefly, heads were dissected from animals and fixed in 4% paraformaldehyde/PBS for 15 min. Tween-20 was then added to 0.1% and heads were fixed for an additional 30 min. Samples were washed twice for 5 min in 0.1% Tween 20/PBS (PBST), cut into 8 μm frozen sections, and mounted on poly-L-lysine-treated slides (Sigma). Sections were dried onto slides for 30 min at room temperature and then fixed for an additional 30 min in 4% paraformaldehyde/PBST. Samples were washed for a total of 2 hr in PBST with five changes of buffer, followed by an incubation for 5 min in 1:1 PBST:hybridization buffer (50% formamide, 5X SSC, 50 mg/ml heparin, 0.1% Tween 20), and then prehybridized for 2 hr at 55°C in hybridization buffer. Hybridizations were carried out for 12–18 hr at 55°C at probe concentrations of 5 ng/ml. Posthybridization washes consisted of three washes of 1 hr each in fresh hybridization buffer at 55°C followed by an overnight wash. The next day, specimens were washed for 1 hr in fresh hybridization buffer and then 5 min in 1:1 PBST:hybridization buffer, followed by five washes in PBST for 5 min each. Digoxygenin-labeled RNA probes were detected according to the manufacturer's instructions using alkaline phosphatase–conjugated anti-digoxygenin antibody (Boehringer Mannheim, Indianapolis, IN).

For the developmental studies, *Drosophila* were collected as white prepupae and kept at 25°C on moist filter paper for the indicated number of hours, at which time they were fixed. At 25°C, the approximate time from the white prepupal stage to eclosion is 100 hr (Ashburner, 1989).

For genomic Southern blots, hybridizations were at 65°C (high stringency) or 55°C (reduced stringency) in 7% SDS, 0.5 M sodium phosphate buffer [pH 7.2], and 1 mM EDTA [pH 8.0].

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References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. J. Mol. Biol. *215*, 403–410.

Ashburner, M. (1989). Drosophila: A Laboratory Handbook (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Ayer, R.K., and Carlson, J. (1991). *acj6*: a gene affecting olfactory physiology and behavior in *Drosophila*. Proc. Natl. Acad. Sci. USA *88*, 5467–5471.

Ayer, R.K., and Carlson, J. (1992). Olfactory physiology in the *Drosophila* antenna and maxillary palp: *acj6* distinguishes two classes of odorant pathways. J. Neurobiol. *23*, 965–982.

Bargmann, C.I., and Kaplan, J.M. (1998). Signal transduction in the Caenorhabditis elegans nervous system. Annu. Rev. Neurosci. *21*, 279–308.

Barth, A.L., Justice, N.J., and Ngai, J. (1996). Asynchronous onset of odorant receptor expression in the developing zebrafish olfactory system. Neuron *16*, 23–34.

Barth, A.L., Dugas, J.C., and Ngai, J. (1997). Noncoordinate expression of odorant receptor genes tightly linked in the zebrafish genome. Neuron *19*, 359–369.

Baumann, A., Frings, S., Godde, M., Seifert, R., and Kaupp, U.B. (1994). Primary structure and function of a Drosophila cyclic nucleotide–gated channel present in eyes and antennae. EMBO J. *13*, 5040–5050.

Boekhoff, I., Strotmann, J., Raming, K., Tareilus, E., and Breer, H. (1990). Odorant-sensitive phospholipase C in insect antennae. Cell. Signal. *2*, 49–56.

Boekhoff, I., Seifert, E., Goggerle, S., Lindemann, M., Kruger, B., and Breer, H. (1993). Pheromone-induced second-messenger signaling in insect antennae. Insect Biochem. Mol. Biol. *23*, 757–762.

Breer, H., Boekhoff, I., and Tareilus, E. (1990). Rapid kinetics of second messenger formation in olfactory transduction. Nature *345*, 65–68.

Brown, T.A. (1991). Molecular Biology Labfax (San Diego, CA: Academic Press).

Buck, L. (1996). Information coding in the vertebrate olfactory system. Annu. Rev. Neurosci. *19*, 517–544.

Buck, L., and Axel, R. (1991). A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. Cell *65*, 175–187.

Carlson, J. (1996). Olfaction in *Drosophila*: from odor to behavior. Trends Genet. *12*, 175–180.

Chadwick, R., and McGinnis, W. (1987). Temporal and spatial distribution of transcripts from the *Deformed* gene of *Drosophila*. EMBO J. *6*, 779–789.

Clyne, P., Grant, A., O'Connell, R., and Carlson, J. (1997). Odorant response of individual sensilla on the *Drosophila* antenna. Invert. Neurosci. *3*, 127–135.

Clyne, P.J., Certel, S.J., de Bruyne, M., Zaslavsky, L., Johnson, W.A., and Carlson, J.R. (1999). The odor specificities of a subset of olfactory receptor neurons are governed by Acj6, a POU-domain transcription factor. Neuron *22*, this issue, 339–347.

Engelman, D.M., Steitz, T.A., and Goldman, A. (1986). Identifying nonpolar transbilayer helices in amino acid sequences of membrane proteins. Annu. Rev. Biophys. Biophys. Chem. *15*, 321–353.

Haenlin, M., Roos, C., Cassab, A., and Mohier, E. (1987). Oocyte-specific transcription of fs(1)k10: a Drosophila gene affecting dorsal–ventral developmental polarity. EMBO J. *6*, 801–807.

Hafen, E., and Levine, M. (1986). In *Drosophila*: A Practical Approach, D.B. Roberts, ed. (Oxford: IRL Press Limited), pp. 139–157.

Hildebrand, J. (1995). Analysis of chemical signals by nervous systems. Proc. Natl. Acad. Sci. USA *92*, 67–74.

Hildebrand, J.G., and Shepherd, G.M. (1997). Mechanisms of olfactory discrimination: converging evidence for common principles across phyla. Annu. Rev. Neurosci. *20*, 595–631.

Kaissling, K.-E. (1987). R.H. White Lectures on Insect Olfaction, K. Colbow, ed. (Burnaby, B.C. Canada: Simon Fraser University Press).

Kyte, J., and Doolittle, R.F. (1982). A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. *157*, 105–132.

Laissue, P., Reiter, C., Hiesinger, P., Halter, S., Fischbach, K., and Stocker, R. (1999). 3-D reconstruction of the antennal lobe of Drosophila melanogaster. J. Comp. Neurol. *405*, in press.

Lienhard, M., and Stocker, R. (1991). The development of the sensory neuron pattern in the antennal disc of wild-type and mutant (lz^3, ss^a) *Drosophila melanogaster*. Development *112*, 1063–1075.

McKenna, M., Hekmat-Scafe, D., Gaines, P., and Carlson, J. (1994). Putative *Drosophila* pheromone-binding proteins expressed in a subregion of the olfactory system. J. Biol. Chem. *269*, 16340–16347.

Mombaerts, P., Wang, F., Dulac, C., Chao, S., Nemes, A., Mendelsohn, M., Edmonson, J., and Axel, R. (1996). Visualizing an olfactory sensory map. Cell *87*, 675–686.

Mount, S.M., Burks, C., Hertz, G., Stormo, G.D., White, O., and Fields, C. (1992). Splicing signals in *Drosophila*: intron size, information content, and consensus sequences. Nucleic Acids Res. *20*, 4255–4262.

Ngai, J., Chess, A., Dowling, M., Necles, N., Macagno, E., and Axel, R. (1993a). Coding of olfactory information: topography of odorant receptor expression in the catfish olfactory epithelium. Cell *72*, 667–680.

Ngai, J., Dowling, M., Buck, L., Axel, R., and Chess, A. (1993b). The family of genes encoding odorant receptors in the channel catfish. Cell *72*, 657–666.

Probst, W.C., Snyder, L.A., Schuster, D.I., Brosius, J., and Sealfon, S.C. (1992). Sequence alignment of the G-protein coupled receptor superfamily. DNA Cell Biol. *11*, 1–20.

Ray, K., and Rodrigues, V. (1995). Cellular events during development of the olfactory sense organs in *Drosophila melanogaster*. Dev. Biol. *167*, 426–438.

Reddy, G., Gupta, B., Ray, K., and Rodrigues, V. (1997). Development of *Drosophila* olfactory sense organs utilizes cell–cell interactions as well as lineage. Development *124*, 703–712.

Ressler, K., Sullivan, S., and Buck, L. (1993). A zonal organization of odorant receptor gene expression in the olfactory epithelium. Cell *73*, 597–609.

Riesgo-Escovar, J., Raha, D., and Carlson, J. (1995). Requirement for a phospholipase C in odor response: overlap between olfaction and vision in *Drosophila*. Proc. Natl. Acad. Sci. USA *92*, 2864–2868.

Riesgo-Escovar, J., Piekos, W., and Carlson, J. (1997). The *Drosophila* antenna: ultrastructural and physiological studies in wild-type and lozenge mutants. J. Comp. Physiol. [A] *180*, 151–160.

Robertson, H.M. (1998). Two large families of chemoreceptor genes in the nematodes Caenorhabditis elegans and Caenorhabditis briggsae reveal extensive gene duplication, diversification, movement and intron loss. Genome Res. *8*, 449–463.

Sengupta, P., Colbert, H., and Bargmann, C. (1994). The *C. elegans* gene *odr-7* encodes an olfactory-specific member of the nuclear receptor superfamily. Cell *79*, 971–980.

Sengupta, P., Chou, J., and Bargmann, C. (1996). *odr-10* encodes a seven transmembrane domain olfactory receptor required for responses to the odorant diacetyl. Cell *84*, 899–909.

Siddiqi, O. (1987). Neurogenetics of olfaction in *Drosophila melanogaster*. Trends Genet. *3*, 137–142.

Siddiqi, O. (1991). Olfaction in Drosophila. In Chemical Senses, C. Wysocki and M. Kare, eds. (New York: Marcel Dekker), pp. 79–96.

Singh, R.N., and Nayak, S.V. (1985). Fine structure and primary sensory projections of sensilla on the maxillary palp of *Drosophila melanogaster meigen* (Diptera: Drosophilidae). Int. J. Insect Morphol. Embryol. *14*, 291–306.

Stocker, R. (1994). The organization of the chemosensory system in *Drosophila melanogaster*: a review. Cell Tissue Res. *275*, 3–26.

Stocker, R.F., Tissot, M., and Gendre, N. (1995). Morphogenesis and cellular proliferation pattern in the developing antennal lobe of Drosophila. Roux's Arch. Dev. Biol. *205*, 62–72.

Sullivan, S., Adamson, M., Ressler, K., Kozak, C., and Buck, L. (1996). The chromosomal distribution of mouse odorant receptor genes. Proc. Natl. Acad. Sci. USA *93*, 884–888.

Troemel, E., Chou, J., Dwyer, N., Colbert, H., and Bargmann, C. (1995). Divergent seven transmembrane receptors are candidate chemosensory receptors in *C. elegans*. Cell *83*, 207–218.

Vassar, R., Ngai, J., and Axel, R. (1993). Spatial segregation of odorant receptor expression in the mammalian olfactory epithelium. Cell *74*, 309–318.

Venkatesh, S., and Singh, R. (1984). Sensilla on the third antennal segment of *Drosophila melanogaster meigen*. Int. J. Insect Morphol. Embryol. *13*, 51–63.

Wang, F., Nemes, A., Mendelsohn, M., and Axel, R. (1998). Odorant receptors govern the formation of a precise topographic map. Cell *93*, 47–60.

Wegener, J.W., Boekhoff, L., Tareilus, E., and Breer, H. (1993). Olfactory signaling in antennal receptor cells of the locust (Locust migratoria). J. Insect Physiol. *39*, 153–163.

White, S.H. (1994). Hydropathy plots and the prediction of membrane protein topology. In Membrane Protein Structure, S.H. White, ed. (New York: Oxford University Press), pp. 97–124.

Zhao, H., Ivic, L., Otaki, J., Hashimoto, H., Mikoshiba, K., and Firestein, S. (1998). Functional expression of a mammalian odorant receptor. Science *279*, 237–242.

GenBank Accession Numbers

The genomic clone (P1 or cosmid), the sequence range in the genomic clone for the predicted coding region, and the GenBank Accession Number of the genomic clone (in parentheses) are listed below for each DOR gene. The sequence range is correct as of 26 January 1999; however, some may change as some P1 clones are in unordered pieces.

59D.1, DSO7462 1894-700 (AC005672); 23A.1, DSO6400 122346-121150 (AC005558); 33B.1, DSO6189 29614-28415 (AC006240); 33B.2, DSO6189 27938-26630 (AC006240); 33B.3, DSO6189 26150-24920 (AC006240); 2F.1, 30B8 22942-24202 (AL009195); 22A.1, DSO5342 13596-15031 (AC004121); 22A.2, DSO5342 15686-17139 (AC004121); 47E.1, DSO0724 63353-64694 (AC006066); 46F.1, DSO5033 73610-72317 (AC005974); 46F.2, DSO5033 72218-71006 (AC005974); 22C.1, DSO0164 26252-29627 (AC004716); 24D.1, DSO6482 45051-43643 (AC004371); 43B.1, DSO2358 29787-31457 (AC005425); 47E.2, DSO0971 24291-22692 (AC005638); 25A.1 DSO8170 61009-62575 (AC005463).

The predicted amino acid sequences of the DOR proteins have been included as supplemental data in the online version of this paper (http://www.neuron.org/cgi/content/full/22/2/327/DC1).