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Divergent Seven Transmembrane Receptors Are Candidate Chemosensory Receptors in C. elegans

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Summary

Using their senses of taste and smell, animals recognize a wide variety of chemicals. The nematode C. elegans has only fourteen types of chemosensory neurons, but it responds to dozens of chemicals, because each chemosensory neuron detects several stimuli. Here we describe over 40 highly divergent members of the G protein–coupled receptor family that could contribute to this functional diversity. Most of these candidate receptor genes are in clusters of two to nine similar genes. Eleven of fourteen tested genes appear to be expressed in small subsets of chemosensory neurons. A single type of chemosensory neuron can potentially express at least four different receptor genes. Some of these genes might encode receptors for water-soluble attractants, repellents, and pheromones.

Introduction

The olfactory and gustatory systems detect a variety of structurally unrelated molecules, from ions to complex organic compounds. These heterogeneous chemical signals are recognized by specialized sensory receptors and sensory neurons. Unlike the visual system, which detects many wavelengths of light but uses only a few types of receptor neurons, the olfactory system contains many types of sensory neurons that each detect particular chemical cues.

To analyze the mechanisms of sensory recognition and discrimination by the nervous system, we are studying chemosensation in the nematode Caenorhabditis elegans. C. elegans can detect touch, temperature, and light, but its responses to chemicals are the most diverse responses in its behavioral repertoire. C. elegans eats bacteria; chemicals produced by bacteria stimulate chemotaxis, egg laying, feeding, and defecation (Ward, 1973; Dusenbery, 1974; Horvitz et al., 1982; Avery and Horvitz, 1990; Thomas, 1990; Bargmann et al., 1993), while toxic or aversive compounds are avoided (Culotti and Russell, 1978). Pheromones contribute to mating between males and hermaphrodites (Liu and Sternberg, 1995). A pheromone also controls the development of an alternative larval stage called a dauer larva (Golden and Riddle, 1984).

The neurons involved in these chemosensory responses can be precisely defined within the nervous system of C. elegans. An adult hermaphrodite has exactly 302 neurons, whose positions, morphology, and synaptic connections are reproducible from animal to animal (White et al., 1986). Among these neurons are 32 neurons that appear to be chemosensory, since they have ciliated endings that are exposed to the environment through specialized sensory structures (Ward et al., 1975; Ware et al., 1975). These neurons can be divided into fourteen types, where one neuron type typically consists of two bilaterally symmetric neurons. For ten types of neurons, chemosensory function has been directly demonstrated by observing behavioral deficits after laser killing of defined cell types. For example, the two ASE chemosensory neurons respond to water-soluble attractants including salts, cAMP, and biotin; the two AWC olfactory neurons respond to volatile aldehydes, ketones, alcohols, and thiazoles; and the two ASH neurons respond to both chemical and mechanical stimuli (Bargmann and Horvitz, 1991a; Bargmann et al., 1993; Kaplan and Horvitz, 1993). The neurons that sense attractants (seven types) and repellents (two types) do not overlap, and they synapse onto distinct synaptic targets that mediate chemotaxis and avoidance behaviors (White et al., 1986).

Interestingly, different kinds of sensory information can be sorted out within a single type of sensory neuron. For example, three pairs of neurons regulate both chemotaxis and dauer larva formation, while two pairs of neurons regulate both chemotaxis and egg laying (Bargmann and Horvitz, 1991b; E. Sawin and H. R. Horvitz, personal communication). Thus, two distinct responses can be generated by a single sensory cell type. In addition, animals can adapt independently to two different chemicals that are detected by the same chemosensory neuron (Colbert and Bargmann, 1995), and the response to one chemical detected by a chemosensory neuron can be saturated without blocking the response to a second chemical detected by that neuron (Ward, 1973; Bargmann et al., 1993).

How can a small number of chemosensory neurons generate responses to a much larger number of chemicals? One possibility is that each chemosensory neuron possesses multiple receptor proteins or binding sites for different compounds. In that case, some aspects of discrimination between sensory stimuli could occur within a single sensory neuron. Alternatively, each sensory neuron might express only one type of receptor that binds to many chemicals; in this case, downstream integration of information from several types of sensory neurons could be used to generate diverse responses. Unfortunately, it has not been possible to examine chemosensory receptor expression directly. A family of G protein–coupled vertebrate olfactory receptors has been identified (Buck and Axel, 1991), but homologs of these receptors have not been identified in C. elegans. G protein–mediated second messengers have been implicated in insect chemoreception (Breer et al., 1990), but the receptors that mediate chemosensation in invertebrates are unknown.

*These authors made similar contributions to this work.

We describe here a family of seven transmembrane receptor genes whose products might mediate chemosensation in C. elegans. These genes were sequenced by the C. elegans genome sequencing consortium, which has sequenced about 15% of the genome (Sulston et al., 1992; J. Sulston, A. Coulson, R. Waterston, et al., personal communication). The genes are highly divergent from known genes and from one another. However, they have secondary structures and key residues that define them as members of the G protein–coupled receptor superfamily. The receptor genes are clustered in the genome, with up to nine genes present in a single cluster. Most of these genes are expressed in sensory neurons, and multiple receptors can be expressed by a single sensory neuron.

Results

A Large Family of Potential Seven Transmembrane Receptors

In C. elegans, genes with related functions are often found clustered in the genome in operons: a primary transcript encoding several genes is cleaved to produce multiple mature mRNAs (Zorio et al., 1994). Therefore, we examined regions around potential olfactory signaling molecules in the sequenced DNA of C. elegans for genes that might be chemosensory receptors. Immediately adjacent to a transmembrane guanylyl cyclase on chromosome II, we found nine novel genes that were related to one another. Although these genes were not homologous to any known genes, they encoded proteins with multiple predicted transmembrane domains, as would be expected of receptors. These sequences were used to search databases for related genes, which were then used in further sequence searches (Altschul et al., 1990). From this analysis, we identified 41 potential C. elegans receptor genes that fell into six families based on sequence similarity with one another. The gene families were named *sra*, *srb*, *srg*, *srd*, *sre*, and *sro* (for serpentine receptor classes a, b, g, d, e, and o). The chromosomal locations of these genes are shown on Figure 1A; their sequences are presented in Figure 2.

The *sra*, *srb*, *srg*, *srd*, and *sre* genes were not significantly similar to any known gene in homology searches. However, when their sequences were manually aligned with consensus sequences for seven transmembrane receptors, they were found to contain features that are characteristic of that family. Each gene displayed approximately seven hydrophobic peaks that could be transmembrane domains (Figure 1B) (Kyte and Doolittle, 1982) as well as some key residues that are usually present in G protein–coupled receptors (Probst et al., 1992). The regions of conservation in each subfamily were most pronounced in predicted transmembrane domains 3 and 7 and in linker regions between transmembrane domains. These regions tend to be most conserved among related seven transmembrane receptors, supporting the hypothesis that the *sra*, *srb*, *srg*, *srd*, *sre*, and *sro* genes encode G protein–coupled receptors from this superfamily (Probst et al., 1992).

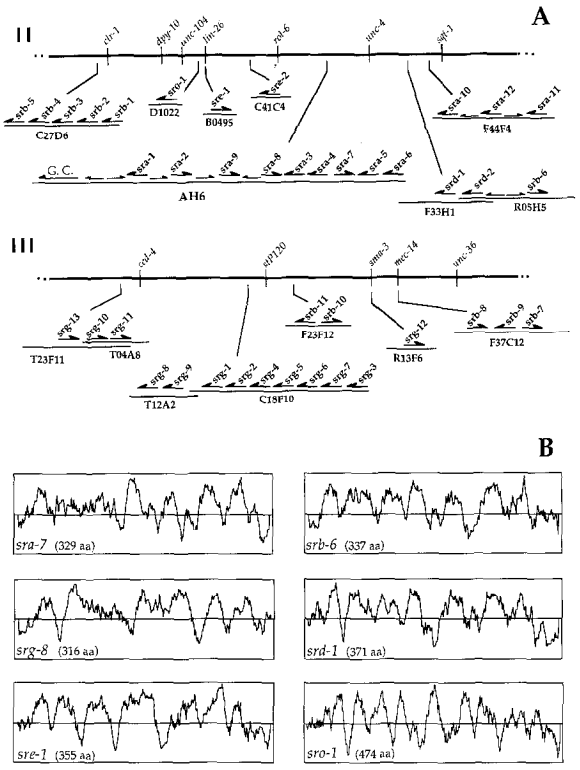


Figure 1. Genomic Organization and Structure of Predicted Receptor Genes

(A) Genomic organization of predicted receptor genes (not to scale). Approximately two thirds of chromosome III and one third of chromosome II were available through GenBank when these genes were discovered. At the top is shown the approximate genetic map position of each set of genes. In insets are shown the cosmids from which each gene was derived. Predicted receptor genes are indicated by the thicker lines; interspersed genes that did not belong in these receptor families are indicated by the thinner lines. In the *sra-1–sra-9* cluster, the interspersed genes were AH6.5, a zinc finger–containing protein, AH6.15, a transposon, and AH6.13, a fragment of an *sra*-like gene. G. C. is the guanylyl cyclase AH6.1. In the *sra-10–sra-12* cluster, the interspersed genes were F44F4.6, a β-1,6-N-acetylglucosaminyltransferase, and F44F4.8, a transposase. In the *srd-1–srd-2–srb-6* cluster, the interspersed genes were R05H5.2, a phosphatase, and R05H5.7, a novel protein.

(B) Hydrophobicity plots of representative genes. Six of the genes whose expression patterns are presented in Figures 3–6 are shown. Hydrophobic peaks predicted by Kyte–Doolittle analysis (Kyte and Doolittle, 1982) appear above the center line in each graph. Similar plots were obtained for all family members shown in Figure 2.

The three largest families of genes were the *sra*, *srb*, and *srg* genes. The *sra* family included the nine genes that initiated the search, *sra-1* through *sra-9*, which shared about 35% amino acid identity overall, and three other genes, *sra-10* through *sra-12*, which were about 20%–25% identical with *sra-1* through *sra-9*. The eleven *srb* genes were distantly related to the *sra* genes (about 10%–15% amino acid identity) but significantly more closely related to one another (about 30% identity). The thirteen *srg* genes were essentially unrelated to the *sra* and *srb* genes by sequence, but between 10%–30% identical to one another. The *srg* genes were independently predicted to be G protein–coupled receptors by E. Sonnhammer of the Sanger Center (personal communication).

Fewer members of the *srd*, *sre*, and *sro* families of genes were detected. *srd-1* and *srd-2* were 48% identical to one another at the amino acid level. *sre-1* and *sre-2* were 28% identical to one another at the amino acid level, and marginally similar to members of the *srb* gene family. While the *sra–sre* genes were unrelated in sequence to known sensory receptors, the single *sro-1* gene displayed distant similarity to opsin genes (Figure 2). *sro-1* is highly diverged from known opsins; it lacks the lysine that forms a Schiff base with retinal, so its sequence similarity with opsins might not reflect functional similarity (Thomas and Stryer, 1982).

Most of the *sra*, *srb*, *srg*, and *srd* genes were found in clusters of two to nine related genes (see Figure 1A). Contrary to the initial rationale of the search, the nine original *sra* genes were not organized into a single operon with the guanylyl cyclase gene or each other. Although they were all found within a 30 kb region, different members were transcribed from different DNA strands and presumably had different promoters (see Figure 1A). The three additional *sra* genes *sra-10*, *sra-11*, and *sra-12* were also closely linked to one another, but they were far from *sra-1–sra-9* on chromosome II. In both cases, the *sra* genes were not strictly clustered: unrelated genes were interspersed among the *sra* genes. By contrast, many of the *srb*, *srg*, and *srd* clusters were uninterrupted and might encode polycistronic transcripts, since the genes were transcribed in the same orientation within 1 kb of each other (see Figure 1A) (Zorio et al., 1994). The five genes *srb-1–srb-5* and the nine genes *srg-1–srg-9* might each arise from a single transcript. With the exception of the *srd-1–srd-2–srb-6* cluster, closely linked genes fell within one sequence family (e.g., all *sra* or all *srb* genes), and with the further exception of two *srb* clusters (*srb-7–srb-9* and *srb-10–srb-11*), the linked genes were always the most closely related genes within a family.

The candidate vertebrate olfactory receptors are encoded by genes that are similar enough to cross-hybridize with one another at high stringency (Buck and Axel, 1991), but their level of sequence similarity is higher than that of the families of genes described here. To determine whether many other genes might belong to the *sra*, *srb*, and *srg* gene families, we used the coding regions of *sra-6*, *sra-7*, *srb-1*, *srb-8*, *srb-10*, and *srg-8* to probe genomic Southern blots of C. elegans DNA at high (65°C) or reduced (55°C) stringency. Each gene appeared to detect only its own sequence at both high and lowered stringency (data not shown).

Expression of Seven Transmembrane Receptors in Sensory Neurons

The large number of related sequences in the *sra*, *srb*, and *srg* gene families, their relatively small size (311–371 amino acids), and their clustering in the genome (Ben-Arie et al., 1994) were reminiscent of vertebrate olfactory receptors. To ask whether these genes might be expressed by chemosensory neurons, upstream regions of 22 genes were fused to the reporter gene *GFP* (green fluorescent protein) and introduced into the germline of C. elegans to produce transgenic animals (see Experimental Procedures) (Chalfie et al., 1994). Interestingly, many of these reporter gene constructs yielded highly specific expression patterns (see Figures 3–6; Table 1). By aligning *GFP* fluorescence with differential interference Nomarski images, expression could be localized to single cell types (Figure 4).

Of thirteen genes whose expression was observed in hermaphrodites, seven were expressed only in small subsets of chemosensory neurons. Fusion genes derived from the two linked *srg* genes *srg-2* and *srg-8* and the two linked *sra* genes *sra-7* and *sra-9* were expressed exclusively in the two ASK sensory neurons (Figure 3A). The ASK neurons, which are implicated in chemotaxis to the amino acid lysine and in sensory regulation of egg laying, are easily recognized by their positions in a trio of cell bodies at the dorsal midline (Figure 4A).

Three other fusion genes were also localized strictly to sensory neurons. *srd-1::GFP* was expressed in the sensory neuron ASI, which detects water-soluble attractants and the dauer pheromone that regulates nematode development (Figures 3B and 4C). *sre-1::GFP* was expressed in the ADL neuron, which is required for the response to some repellents (Figures 3C and 4B; Table 2; B. E. Kimmel, C. I. B., and J. H. Thomas, unpublished data). In addition, a lower level of *sre-1::GFP* expression was detected in the sensory neuron ASJ, which is implicated in pheromone detection. *srg-13::GFP* was expressed in the PHA neurons in the tail (Figure 5D). The function of these neurons is unknown, but their morphology is characteristic of chemosensory neurons.

In addition to the seven genes that were expressed exclusively in chemosensory neurons, three additional fusion genes were expressed predominantly in chemosensory neurons. *srb-6::GFP* was expressed in five types of sensory neurons, three in the head and two in the tail (see Table 1; Figures 5A and 5B). In addition to this neuronal expression, a low level of expression of the reporter gene was observed in the egg laying structures in the mid-body region. *sra-6::GFP* also showed both sensory and nonsensory expression. The ASH and ASI sensory neurons expressed the fusion gene (Figures 5C and 5E), as did the PVQ interneurons (Figure 5E). *sro-1::GFP* was expressed mainly in the ADL sensory neurons (see Figure 3C), but lower expression was observed in the SIA neurons, which have unknown functions.

Three gene fusions were expressed predominantly outside the chemosensory system (see Table 1; data not shown). The linked genes *sra-10* and *sra-11* were expressed in some sensory neurons, interneurons, and pharyngeal neurons and muscle. *srg-12*, the most divergent *srg* gene, was expressed in the excretory cell and the gut.

Expression of the gene fusions was examined in animals of all developmental stages. In all cases, *GFP* expression was observed in animals from the first larval stage through the adult, though some variability was apparent (for example, *srg-8* was consistently expressed more strongly in young larvae than in adults). A total of 22 predicted genes were tested by this approach (see Experimental Proce-

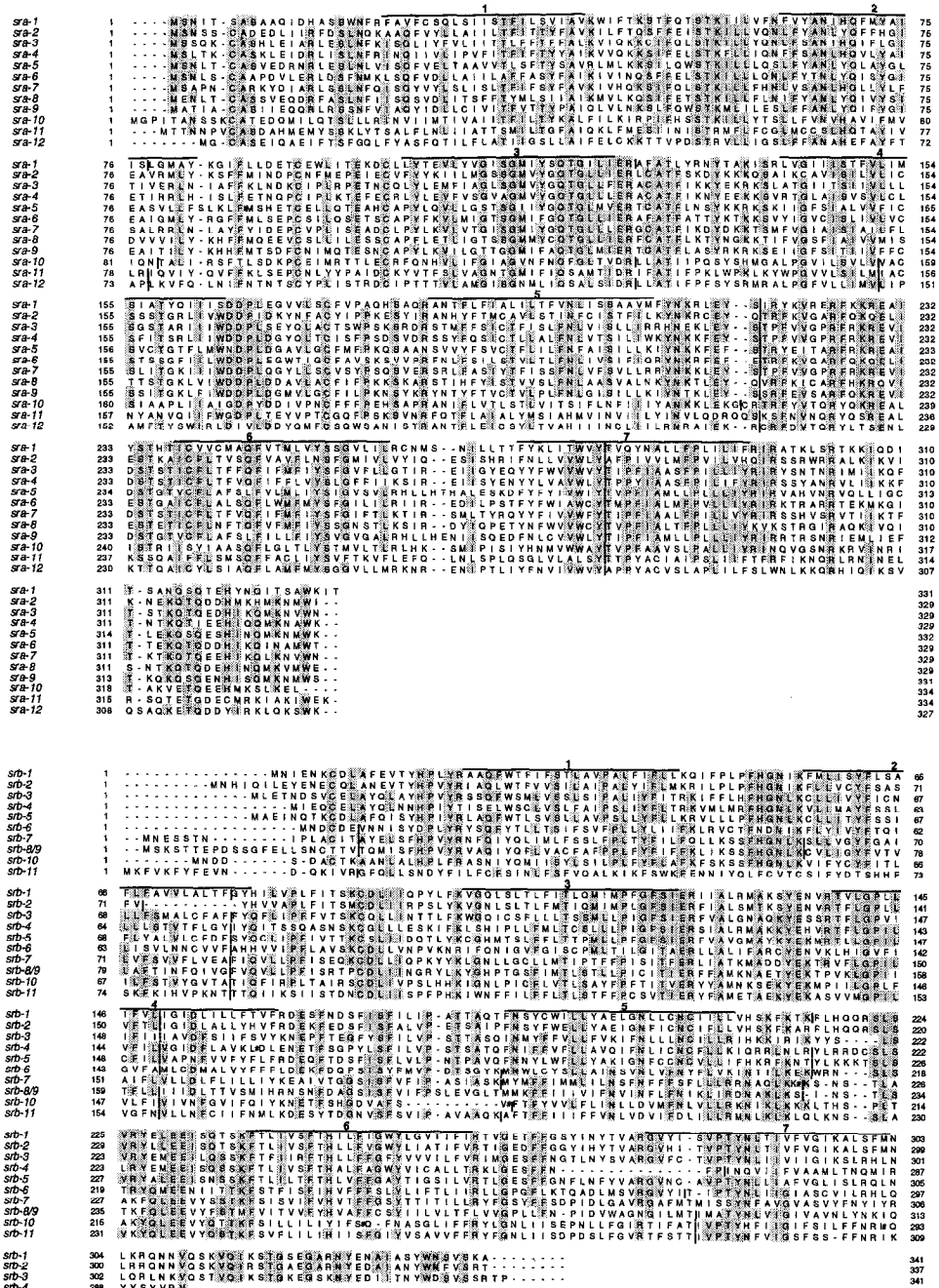


Figure 2. Sequence Alignments of Predicted Seven Transmembrane Receptors

Unless otherwise noted, all genes were as predicted by the C. elegans sequencing consortium. Residues conserved in ≥50% of the clones are shaded in grey. The approximate locations of predicted transmembrane domains 1 through 7 are noted. Exon/intron boundaries are denoted by slash marks.

The *sra* family: *sra-5* contained a single frameshift, which was introduced at the position marked with a number symbol in its sequence to align it with the other genes (see Experimental Procedures). *sra-11* and *sra-12* were modified from the previously predicted genes (see Experimental Procedures).

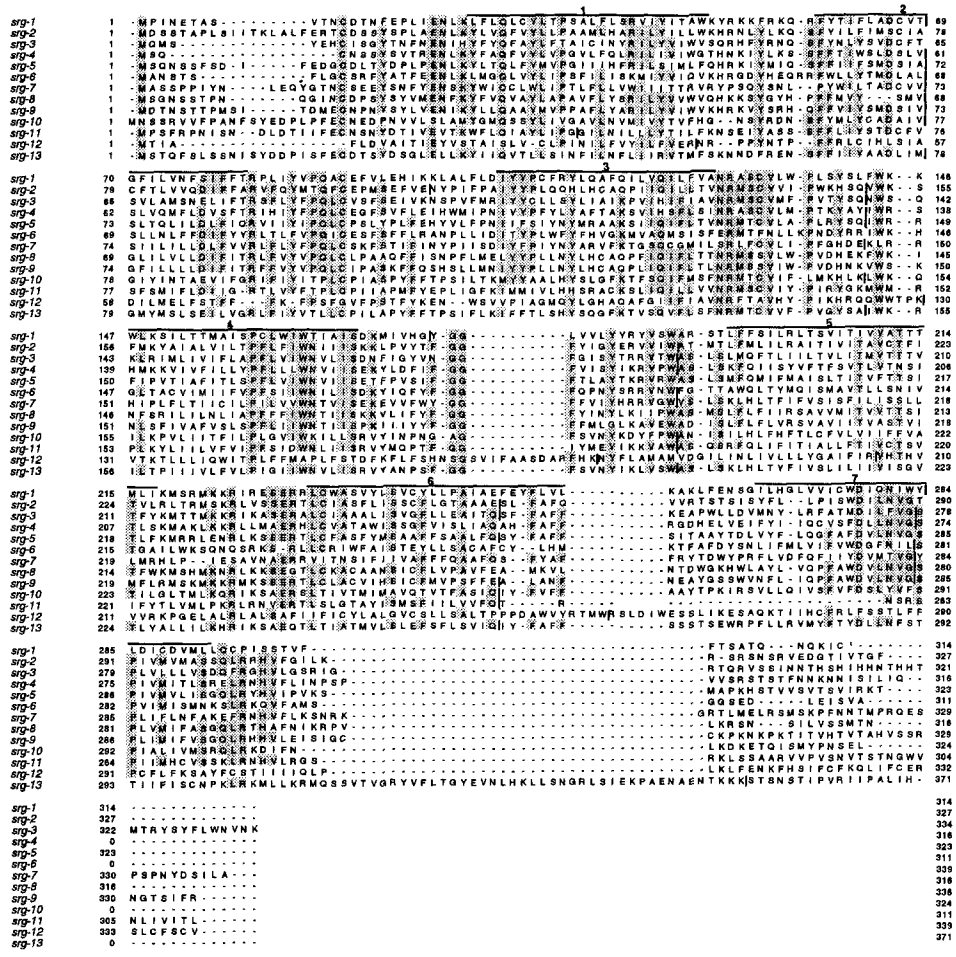
The *srb* family: *srb-1*, *srb-2*, *srb-3*, *srb-4*, and *srb-5* were all different from the previously predicted genes. *srb-8* and *srb-9* were also different from the previously predicted genes; these two genes are identical at the amino acid level. *srb-4*, *srb-7*, and *srb-10* could only be aligned with these sequences by introducing frameshifts, denoted by a number symbol.

The *srg* family: *srg-1*, *srg-2*, *srg-3*, and *srg-9* were all modified from previously predicted genes. *srg-4*, *srg-5*, *srg-6*, and *srg-7* were identified on the basis of searches of genomic regions in the C18F10 cosmid.

The *srd* family: *srd-2* was modified from the previously predicted gene.

The *sre* family: *sre-2* was modified from the previously predicted gene.

sro-1: alignment of *sro-1* with the rh2 ocellar opsin from Drosophila pseudoobscura (Carulli and Hartl, 1992) and the rh1 photoreceptor opsin from Calliphora vicina (Huber et al., 1990). The lysine that forms a Schiff base with retinal is denoted with an asterisk.


srg-1
srg-2
srg-3
srg-4
srg-5
srg-6
srg-7
srg-8
srg-9
srg-10
srg-11
srg-12
srg-13


srd-1
srd-2

sre-1
sre-2

srx-1
rh2_opsin
rh1_opsin

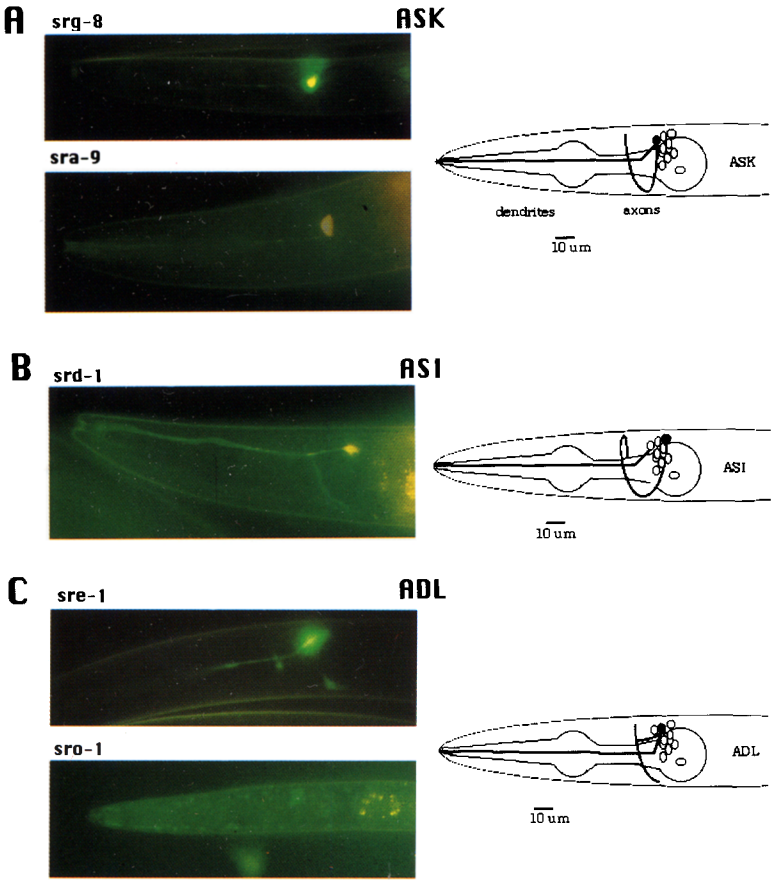


Figure 3. Expression of Reporter Gene Constructs in the ASK, ADL, and ASI Neurons

Fusions of the upstream regions of various genes were made to *GFP* and visualized in transgenic animals.

(A) Expression of *srg-8::GFP* and *sra-9::GFP* in ASK. Note the staining of axons and dendrites of the ASK neurons in the transgenic animals. Staining with *sra-7::GFP* and *srg-2::GFP* constructs was similar, but weaker.

(B) Expression of *srd-1::GFP* in the ASI neurons.

(C) Expression of *sre-1::GFP* and *sro-1::GFP* in the ADL neurons. Faint ASJ staining is also visible at lower right in *sre-1::GFP*. At right, morphology of the ASK, ASI, and ADL sensory neurons. Note the different axon morphologies, which were used to confirm the cell identifications made on the basis of position. The positions of the pharynx and other chemosensory neurons of the head are included for reference. Anterior is at left and dorsal up in all cases.

Table 1. Summary of *GFP* Expression Data

Gene	Cell	Function
Expression in chemosensory neurons		
sra-7, *sra-9*, *src-2*, *src-8*	ASK	Lysine chemotaxis; egg laying
srb-6, *sre-1*, *sro-1*	ADL	Octanol avoidance; water-soluble avoidance
srd-1, *sra-6* (faint)	ASI	Dauer pheromone; Na^+, Cl^-, cAMP, biotin, lysine chemotaxis
srb-6 (faint), *srd-1* (males only)	ADF	Dauer pheromone; Na^+, Cl^-, cAMP, biotin chemotaxis
sra-6, *srb-6*	ASH	Osmotic avoidance, nose touch avoidance, volatile avoidance
sre-1 (faint)	ASJ	Dauer pheromone (recovery)
srg-13, *srb-6*	PHA	Unknown, chemosensory
srb-6	PHB	Unknown, chemosensory
sra-1 and *sra-6* (males only)	SPD/SPV	Sex pheromones/mating
srd-1 (males only)	R8/R9?	Sex pheromones/mating
Expression in other cells		
sra-6	PVQ	Interneuron (chemosensory)
sra-11	AIY	Interneuron (chemosensory)
	AVB	Interneuron, locomotion
		One pharyngeal neuron
sra-10	URX	Sensory neuron
	ALA	Interneuron
		Additional interneurons, pharyngeal neurons, and muscle
srb-6		Vulval region
srg-12		Gut, excretory cell
sro-1	SIA	Neuron, unknown function

The cellular pattern of expression of each gene is given, along with the predicted function of those cells.
References are as follows. ASK, Bargmann and Horvitz, 1991a; E. Sawin and H. R. Horvitz, personal communication. ADL, Table 2; J. H. Thomas, personal communication. ASI and ADF, Bargmann and Horvitz, 1991a, 1991b. ASH, Bargmann et al., 1990; Kaplan and Horvitz, 1993; Table 2. ASJ, Bargmann and Horvitz, 1991b. SPD, SPV, and ray neurons, Liu and Sternberg, 1995.

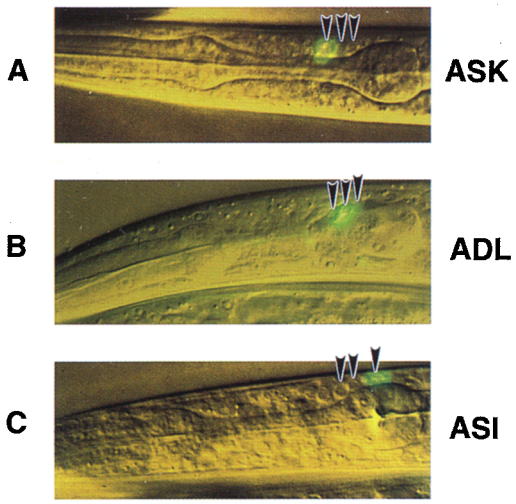


Figure 4. Alignment of Fluorescence and Nomarski Images

(A), *srg-8::GFP* staining ASK; (B), *sre-1::GFP* staining ADL; (C), *srd-1::GFP* staining ASI. In all cases, the positions of the three dorsal neurons ASK, ADL, and ASI are noted on the Nomarski image with arrowheads (compare relative positions of these three neurons in Figure 3). Slides of fluorescence and Nomarski images of the same animal were aligned with Adobe Photoshop.

Table 2. ADL and ASH Function in Avoidance of Volatile Repellents

Animals	Time to Reversal (seconds)			Number of Assays (number of animals)
	Median	Mean	SEM	
Octanol avoidance				
Intact animals	4	6.07	0.56	60 (12)
ASH killed	19.5	14.03	0.88	66 (10)
ADL killed	8	10.47	0.93	47 (11)
AWB killed	5	6.75	1.0	20 (4)
Benzaldehyde avoidance				
Intact animals	4	4.97	0.63	33 (8)
ASH killed	20	15.45	1.23	22 (5)
ADL killed	3	4.96	0.90	28 (7)
AWB killed	5	6.3	1.13	16 (4)

Median and mean time to reversal for intact, ASH-killed, ADL-killed, and AWB-killed animals in the presence of the repellents 1-octanol and benzaldehyde are given. If animals did not reverse within 20 s, their time to reversal was scored as 20 s. Avoidance of octanol was significantly impaired in both ADL and ASH-killed animals (Mann–Whitney rank sum test, $p < 0.001$), but not in AWB-killed animals, while avoidance of benzaldehyde was impaired only in ASH-killed animals ($p < 0.001$).

dures), but reporter gene expression was only observed in 14. This success rate is typical for promoter fusions with C. elegans genes (Lynch et al., 1995). The absence of expression in some cases might occur because the fusions lacked control sequences within the body of the gene, because of errors in predicting gene structure from genomic sequence, or because these genes are actually not expressed under our culture conditions (e.g., they might be expressed in alternative larval stages, or they might be pseudogenes).

Some Receptor Genes Are Expressed in Sex-Specific Patterns

During mating, the C. elegans male exhibits a stereotyped series of behaviors whose progress is regulated by sensory feedback (Hodgkin, 1983; Liu and Sternberg, 1995). The mating structures in the male tail contain 75 male-specific neurons, out of a total of 79 extra neurons in the male adult (Sulston et al., 1980). Cell ablation experiments have revealed functions for many male-specific neurons in different steps of male mating (Liu and Sternberg, 1995). Over 20 of the male-specific neurons have exposed sensory endings and therefore are candidate chemoreceptor neurons that might detect pheromones during mating (Sulston et al., 1980). To investigate whether some of the *sra–sro* genes might function as mating pheromone receptors, each gene fusion was examined in adult male animals.

Most of the gene fusions had identical patterns of expression in males and hermaphrodites, but three genes showed interesting patterns of male-specific expression. The most striking difference was observed with the fusion gene *sra-1::GFP*, for which no staining was observed in hermaphrodites. In males, sensory neurons associated with the spicules stained brightly with this fusion gene (Figures 6B and 6C). The spicules are spikelike mating structures that probe the ventral surface of the hermaphrodite during mating. They contain the putative chemosensory neurons SPD and SPV, which coordinate sperm release into the vulva and have been proposed to sense vulval pheromones (Liu and Sternberg, 1995). The neuroanatomy of the male tail is not as well described as the neuroanatomy of the hermaphrodite, but the cells that stained with *sra-1::GFP* were probably the spicule neurons SPD and SPV. One of these neurons was unambiguously a spicule-associated sensory neuron, since its dendrite invaded the spicule shaft (Figure 6B).

The gene *sra-6::GFP* was also expressed in one neuron pair associated with the spicules (Figure 6D). In addition, *sra-6::GFP* males showed staining in the PVQ interneurons and the ASH and ASI head chemosensory neurons, as did hermaphrodites that contained the *sra-6::GFP* transgene.

srd-1::GFP also had a different staining pattern in males and hermaphrodites, but it was found in a sex-specific pattern in nonsex-specific neurons. Both males and hermaphrodites expressed this gene fusion in the ASI sensory neurons; in addition, in males the fusion was expressed in the ADF sensory neurons (Figure 6E). While the ADF neurons are present in both sexes, the promoter fusion reveals a potential sex-specific regulation of gene expression in these neurons. The ADF and ASI neurons detect pheromones in hermaphrodites (Bargmann and Horvitz, 1991b); these cells might participate in sex-specific pheromone detection in males. *srd-1::GFP* was also expressed in some male-specific neurons in the tail (Figure 6F). Their morphology and position did not permit unambiguous identification of the neurons, but they might be chemosensory neurons associated with the sensory rays.

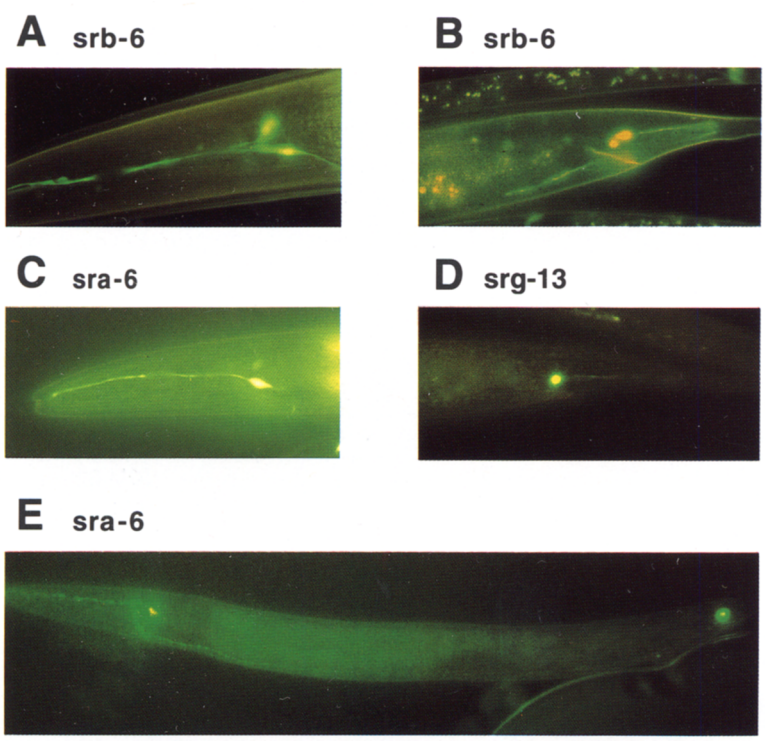


Figure 5. Expression of Additional Reporter Gene Constructs in Sensory and Nonsensory Cell Types

(A and B) Expression of *srb-6* in the ASH and ADL (A) and PHA and PHB (B) sensory neurons. ADF expression was weaker and is not obvious in this plane of focus. ASH and ADF morphologies are similar to that of ASK (Figure 3); PHA and PHB are bipolar sensory neurons in the tail with posterior dendrites and anterior axons. Gut autofluorescence is visible at the anterior edge of this photograph.

(C) Expression of *sra-6* in the ASH sensory neurons. Faint expression in the ASI neuron is visible just dorsal to the ASH neuron.

(D) Expression of *srg-13::GFP* in the PHA sensory neurons of the tail.

(E) Expression of *sra-6* in the ASH sensory neurons (anterior) and in the PVQ interneurons (posterior). Each PVQ neuron sends a single axon to the head. Anterior is at left and dorsal up in all cases.

Discussion

Novel Receptor-like Proteins from C. elegans

The *sra*, *srb*, *srg*, *srd*, *sre*, and *sro* genes have many properties reminiscent of the candidate olfactory receptor genes of vertebrates. First, they encode seven transmembrane receptors that could potentially be coupled to G proteins. The preponderance of evidence from both invertebrate and vertebrate systems supports the notion that such receptors are utilized in chemosensation (Breer et al., 1990; Buck and Axel, 1991; Boekhoff et al., 1994). Recent data implicate G protein–coupled receptors in C. elegans chemosensation as well. Animals mutant for the G proteins *gpa-2* and *gpa-3* are defective in pheromone detection (R. Zwaal and R. Plasterk, personal communication), and animals triply mutant for the G proteins *gpa-1*, *gpa-2*, and *gpa-3* are defective in chemotaxis to water-soluble attractants (E. R. T. and C. I. B., unpublished data). *gpa-1*, *gpa-2*, and *gpa-3* are expressed in sensory neurons, including those that express the *sra–sro* genes (J. Mendel and P. Sternberg, personal communication).

Second, at least eleven of these genes appear to be expressed in small numbers of chemosensory neurons. Eight genes were expressed only in sensory neurons, and six were expressed only in a single type of sensory neuron. Our results are based on expression of reporter gene constructs and therefore may not fully reflect the endogenous expression patterns of the receptor genes. Nonetheless, the highly reproducible patterns of sensory-specific expression are likely to reflect at least some aspects of the regulation of these genes in their natural context.

Third, a substantial number of these genes are present in the genome. Hermaphrodites have 14 classes of chemosensory neurons, and males probably have more; at a minimum, one receptor gene per type of chemosensory neuron would be expected to exist. Only a subset of these genes has been examined, but expression is already suggested for eight types of hermaphrodite chemosensory neurons and three types of male-specific chemosensory neurons.

Most of these genes are found in small clusters, like the vertebrate olfactory receptors, which are found in clusters of 10–100 genes (Ben-Arie et al., 1994). The largest *sra* and *srg* clusters are particularly striking, with nine genes each included within a region of about 30 kb. Unlike the immunoglobulin or T cell receptor genes, the vertebrate olfactory gene clusters do not seem to be rearranged or precisely coexpressed, so the reason for this clustering is unknown. Perhaps this arrangement helps coordinate receptor expression so that only one or a small number of receptors are expressed per sensory neuron (Chess et al., 1994). Although the C. elegans genes are clustered, all genes within a cluster did not share identical regulatory elements; for example, three different expression patterns were observed with fusion genes to four *sra* genes from one cluster.

Several other functions could be proposed for these receptor-like proteins. Some might be neurotransmitter receptors, but relatively few synaptic connections are made onto chemosensory neurons, so they are not expected to express many neurotransmitter receptors (White et al., 1986). Alternatively, they might be receptor molecules used for axon guidance or for the selection of synaptic targets by the chemosensory neurons. Each chemosensory neuron synapses onto several classes of target neurons, and no two types of neurons share the identical

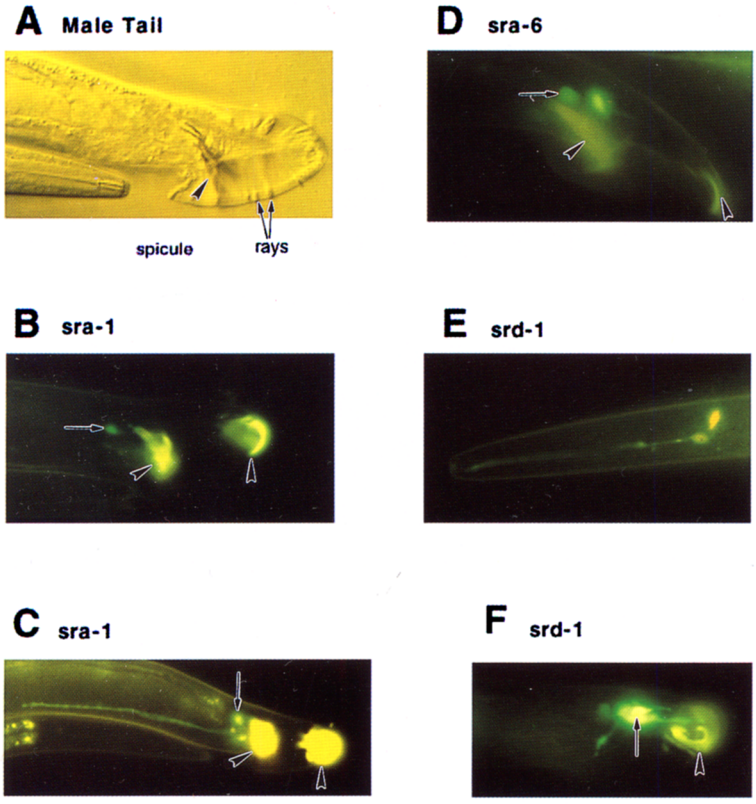


Figure 6. Male-Specific Expression of Fusion Genes

(A) Nomarski image of the male tail, with spicules and rays (compare hermaphrodite tail morphology in Figure 5). The male spicules and posterior male tail structures have an intense yellow autofluorescence (arrowheads here and in remainder of figure). Rays are located around the circumference of the male tail; only two are indicated.

(B) Expression of *sra-1::GFP* in the male tail. The *sra-1::GFP* fluorescence in SPD is green (arrow), while spicule and other autofluorescence is yellow (arrowheads). The dendrite of the spicule neuron SPD can be seen invading the shaft of the spicule.

(C) Axons of the SPD spicule neurons in the ventral nerve cord, as visualized in *sra-1::GFP* animals. An SPD cell body is denoted by an arrow.

(D) Expression of *sra-6::GFP*. One pair of male spicule neurons (arrow) and the PVQ neurons (unmarked; these neurons also stain in hermaphrodites) are visible.

(E and F) Expression of *srd-1::GFP* in males (compare Figure 3C). Two pairs of neurons, the ASI and ADF neurons, are visible in the head (e). Two additional neurons in the tail are visible, one denoted with an arrow in (F); they are bipolar neurons with one apparent sensory dendrite and one axon. These cells may be the R8 or R9 ray neurons.

downstream targets (White et al., 1986). The mechanism by which these connections are made is unknown, but it might involve specific receptors like those described here. Since there are multiple gene families, these models are not mutually exclusive; it is possible that some of the genes described here encode sensory receptors, and some of the genes have developmental or synaptic functions.

The *sra-sro* Receptors Might Sense Attractants, Repellents, or Pheromones

Since each of the sensory neurons appears to sense multiple chemicals, it is not obvious which chemical might be detected by a particular receptor gene. However, various fusion genes were expressed in chemosensory neurons that sense attractants, repellents, and pheromones.

A role in pheromone detection is suggested for the three genes for which sex-specific expression patterns were observed. Males demonstrate multiple responses to hermaphrodites that might involve chemosensation, including long-range chemotaxis to hermaphrodite pheromones, short-range behavioral changes in response to proximal hermaphrodites, and responses to vulval cues during mating (Liu and Sternberg, 1995; J. Hodgkin, E. Jorgensen, and J. H. Thomas, personal communication). Specific neurons mediate each successive step in male mating, suggesting that several sensory cues are detected during this process. Two fusion genes (to *sra-1* and *sra-6*) were expressed in sensory neurons that recognize the vulva during mating, suggesting that a mating pheromone might be detected by these receptors. Interestingly, there are indications that some of the vertebrate olfactory receptor genes are expressed in sperm (Vanderhaeghen et al., 1993). In addition, the *srd-1::GFP* fusion gene was expressed in neurons implicated in both pheromone detection and chemotaxis, suggesting that these receptors might direct male responses to hermaphrodite pheromones.

All of the neurons that express the *sra–sro* genes are thought to detect water-soluble compounds, although the ASH and ADL neurons can also detect some volatile compounds. At this point it is unclear whether the receptors used for volatile chemotaxis in C. elegans will belong to this gene family. The C. elegans genes have no primary sequence similarity to the candidate vertebrate olfactory receptors (Buck and Axel, 1991), nor are they similar to a novel family of genes from the rat vomeronasal organ that might encode vertebrate pheromone receptors (Dulac and Axel, 1995 [this issue of *Cell*]). Water-soluble molecules might be recognized by a different type of receptor than volatile molecules; alternatively, the chemosensory systems of nematodes and mammals may have evolved independent, though related, receptor systems. One gene, *sro-1*, was distantly related to opsin genes, but on the basis of its sequence it is unlikely to be covalently linked to a retinal chromophore (Thomas and Stryer, 1982). One possibility is that this receptor might transiently interact with a hydrophobic retinal-like chemical and act as a chemoreceptor rather than a photoreceptor.

Despite the sensory enrichment of expression observed, some of these genes were expressed predominantly in

nonsensory cell types. These genes might encode receptors for other molecules used in communication between cells. For example, the receptors for attractive amino acids in chemosensory neurons might be similar to receptors for peptide or amino acid neurotransmitters in other cells.

C. elegans Sensory Neurons Are Likely to Express Multiple Receptors

The *GFP* expression patterns predict that several receptor genes, from different gene families, will be expressed in one chemosensory cell type. Four different fusion genes were expressed in the two ASK neurons, three genes in the ADL neurons, and two genes each in the ASI neurons, the ASH neurons, and the PHA neurons. Since only fourteen genes have been examined, it is likely that the total number of genes expressed per neuron will increase.

Even considering possible problems with expression of fusion genes, it is likely that more than one of these receptor genes is expressed per sensory neuron. Over 40 candidate receptor genes were found in the sequenced DNA of C. elegans, which encompasses about 15% of the genome. Even if only half of these genes are sensory receptors, the approximate number of receptors expected in the genome would be about 100, suggesting that many receptors could be found in one cell type.

These results contrast with observations in the vertebrate olfactory epithelium, where single neurons express a very small number of receptor genes, probably one per cell (Ressler et al., 1993; Vassar et al., 1993; Chess et al., 1994). Mammalian neurons expressing a single receptor gene appear to project to common targets in the olfactory bulb, where sensory information is integrated (Ressler et al., 1994; Vasser et al., 1994). By contrast, in C. elegans much of this integration may occur within the peripheral sensory neurons. The neurons detect multiple chemically dissimilar compounds; we propose that this occurs because each neuron expresses multiple receptors with different specificities. This prediction is also consistent with the behavioral data that indicate that responses to different attractants sensed by one neuron saturate and adapt independently (Ward, 1973; Colbert and Bargmann, 1995).

In addition, the possibility that multiple cells might express one receptor gene could explain other features of the C. elegans sensory system. In cell ablation studies, it was often found that a particular molecule was sensed, not by one cell type, but by a small group of neurons (Bargmann and Horvitz, 1991a, 1991b). The expression of *sra-6*, *srb-6*, and *sre-1* in two, five, and two sensory cell types, respectively, suggest that this cellular redundancy might derive from less specific expression of some receptor genes.

One open question raised by these results is the extent to which multiple receptors expressed on a single cell type contribute to perceptual discrimination, as opposed to recognition of various compounds. Behavioral discrimination in C. elegans was demonstrated by distinct patterns of cross-saturation and adaptation between different compounds sensed by one neuron. However, cross-saturation and adaptation experiments in humans suggest that different bitter compounds are recognized by different receptors, even though the perception of all bitter compounds is the same to a human subject (McBurney et al., 1972; Lawless, 1987). Thus, the existence of several independently adapting receptors might not generate a true discrimination on the part of the animal. However, discrimination is strongly indicated in cases where one neuron mediates qualitatively different responses to different chemical stimuli. For example, the ADF and ASI sensory neurons affect both development, probably by detecting a pheromone, and chemotaxis, probably by detecting chemoattractants (Bargmann and Horvitz, 1991a, 1991b). It should be possible to ask whether this functional discrimination between chemicals arises at the level of receptors, signal transduction mechanisms, or assemblies of activated neuronal types.

Experimental Procedures

Sequence Analysis

The sequence of the cosmid AH6, which contains homology to a predicated guanylyl cyclase, was obtained from the Sanger Center Network site maintained by the C. elegans sequencing consortium (Sulston et al., 1992). AH6 was first analyzed by use of the BLASTX program, which translates sequences in all six frames before searching protein databases. All BLASTP (protein sequence) and BLASTX (DNA sequence) searches were performed with the NCBI (National Center for Biotechnology Information) BLAST network service to search databases including GenBank, SwissProt, PIR, and the Brookhaven Protein Data Bank (Altschul et al., 1990). BLASTX searches revealed at least ten different regions of AH6 that detected the previously sequenced C. elegans gene F44F4.5 (now *sra-10*). These regions were subsequently found to correspond to ten genes predicted by the C. elegans sequencing consortium, *sra-1–sra-9* and AH6.13, which appears to be a gene fragment. Each gene contained multiple potential transmembrane domains predicted by hydropathy analysis using the Kyte–Doolittle algorithm in Geneworks (Kyte and Doolittle, 1982).

The sequences of other C. elegans cosmids and coding regions predicted by the sequencing consortium were obtained through GenBank. BLASTP searches with *sra-1–sra-10* revealed more distant similarities to genes in the *srb* family. Continued searches with *sra* and *srb* genes led to the identification of the *srg*, *srd*, *sre*, and *sro* genes. In general, even weak sequence similarities were pursued if several members of a gene family recognized a new gene, and if that new gene encoded multiple predicted transmembrane domains.

Sequence alignments were produced by using the CLUSTAL W program (Thompson et al., 1994). In the initial alignments of the receptor clusters, many genes appeared to be gene fragments rather than full-length coding regions, on the basis of a smaller number of transmembrane domains. Unlike the vertebrate olfactory and vomeronasal receptors, all of the genes contain predicted introns. Analysis of the genomic organization of the receptors revealed that genes within one receptor family often shared conserved splice junctions (see, for example, *sra-2–sra-9*, all of which are generated from three similar exons [Figure 2]). Where the C. elegans sequencing project had predicted gene fragments rather than full-length gene products, we searched the genomic DNA for possible splice sites that corresponded precisely to those splice sites that were conserved in other family members. In several cases, changing the predicted genes to incorporate these conserved splice sites generated the full-length gene products shown in Figure 2.

In addition, for all genes with incomplete sequences, genomic DNA was examined for alternative exons or splice patterns. Genomic DNA was used to search the GenBank databases by using the BLASTX program as described above. This analysis revealed that many cosmids contained additional regions of homology to the receptor gene families. This information was used to predict full-length coding regions for those genes in which frameshifts or poor splice junctions were included (see below and Figure 2).

Genes that correspond exactly to genes predicted by the C. elegans sequencing project were as follows (the names for these genes given

by the sequencing project are shown in parentheses): *sra-1* (AH6.4), *sra-2* (AH6.6), *sra-3* (AH6.7), *sra-4* (AH6.8), *sra-6* (AH6.10), *sra-7* (AH6.11), *sra-8* (AH6.12), *sra-9* (AH6.14), *sra-10* (F44F4.5), *srb-6* (R05H5.6), *srb-11* (F23F12.10), *srg-8* (T12A2.9), *srg-10* (T04A8.1), *srg-12* (R13F6.3), *srg-13* (T23F11.5), *srd-1* (F33H1.5), *sre-1* (B0495.1), and *sro-1* (D1022.6).

Genes that differ in their splicing pattern from previously predicted genes include *srb-8* (modified from F37C12.6), *srb-9* (F37C12.8), *srg-1* (C18F10.4), *srg-2* (C18F10.5), *srg-3* (C18F10.6), *srg-9* (T12A2.10), *srd-2* (R05H5.1), and *sre-2* (C41C4.2). Similar analysis of a single predicted gene, C27D6.2, revealed five internal regions that could each encode a full-length receptor protein (renamed *srb-1–srb-5*). Analysis of the predicted gene F44F4.7 on the advice of S. Jones from the Sanger Center (personal communication) revealed that it most likely consisted of a false fusion of two genes, which were renamed *sra-11* and *sra-12*.

A few of the genes could only be aligned well to the other sequences if unfavorable splices or frameshifts were incorporated into their sequences. These genes might be pseudogenes or they might have sequencing errors; their alignments are shown in Figure 2 with incorporated frameshifts denoted by number symbols. They include *sra-5* (AH6.9, one frameshift), *srb-4* (one frameshift), *srb-7* (F37C12.5, one unfavorable splice, one frameshift), *srb-10* (F23F12.5, four frameshifts), and *srg-11* (T04A8.2, one unfavorable splice). BLASTP searches also revealed several predicted gene fragments that could not be aligned with complete coding regions of these genes; these genes have not been included here.

If a putative receptor gene was found near a region of 2 kb or more with no predicted coding regions, those regions were scanned for additional receptor genes with the BLASTX program. From this analysis, we found four additional genes in the C18F10 cluster (*srg-4*, *srg-5*, *srg-6*, and *srg-7*). Genes were predicted on the basis of a combination of amino acid similarity to other family members and conserved splice junctions.

Expression Constructs

Promoter fusions for the genes *sra-7* and *sra-11* were generated by using standard molecular biology methods to subclone these genes from cosmid clones (Sambrook et al., 1989). Four kilobases upstream of *sra-7* were included in a fusion to the reporter gene *GFP* (Chalfie et al., 1994); the fusion site was a HindIII site 31 amino acids into the predicted coding region of *sra-7*. Six kilobases upstream of *sra-11* and approximately two thirds of its coding region were included in an XhoI–XbaI fragment for a translational fusion to *GFP*.

All other promoter fusions were generated by using the polymerase chain reaction (PCR) to join *GFP* to the first 7–20 predicted amino acids of a given gene product. These fusions included 3–4 kb upstream of the predicted translational start site (2 kb of upstream region were used for *srg-8*). The downstream PCR primer was engineered to end in a BamHI site and the upstream primer in an SphI or PstI site, and the fragment was inserted into the C. elegans *GFP* expression vectors TU#61 and TU#62 (Chalfie et al., 1994). Promoters were amplified from purified cosmid DNA or C. elegans genomic DNA and checked for predicted restriction sites, and junctions were confirmed by DNA sequencing.

Most primers were amplified with Taq polymerase by PCR in 50 mM KCl, 10 mM Tris (pH 8.0), 2.5 mM $MgCl_2$, 200 μM each dNTP, and 50 ng of total genomic DNA or 10 ng of cosmid DNA. Cycling conditions were 94°C for 30 s, 55°C for 60 s, 72°C for 3–4 minutes for 30 cycles on an MJ Research thermal cycler. For some primer sets, this protocol did not give good amplification; in these cases, the amplifications were conducted with the Expand long template PCR kit (Boehringer) and conditions recommended by the manufacturer.

Microinjection and Analysis of Transgenic Animals

Expression constructs (10–50 ng/μl) were injected into the germline of *lin-15(n765ts)* animals together with the *lin-15* plasmid pJM23 (30–50 ng/μl), which was used as a coinjection marker to facilitate identification of transgenic animals (Mello et al., 1991; Huang et al., 1994). *lin-15* mutants have a multivulval phenotype; after injection, transgenic F1 animals were recognized by their normal vulval morphology at 20°C. These animals were used to establish transmitting lines of transgenic animals that expressed both plasmids from unstable arrays. Typically, 40%–90% of the F2 or F3 animals in these lines would be rescued for the *lin-15* phenotype and therefore presumed to be transgenic. At least four independent lines were established for each expression construct, and *GFP*-expressing cells were identified in at least ten independent animals (in most cases, 20–30 animals were examined for each fusion gene). For the genes *sra-6*, *sra-7*, *sra-9*, *sra-10*, *sra-11*, *srb-6*, *srg-12*, *srg-13*, *srd-1*, *sre-1*, and *sro-1*, all examined lines showed comparable expression patterns. Promoter fusions to *srg-2* and *srg-8* did not give reproducible expression in all lines; we observed staining in only 3 of 6 tested lines for both of these constructs. For both *srg-2* and *srg-8*, all of those lines showed identical expression patterns (i.e., only ASK expressed the fusion gene). Promoter fusions to the genes *sra-2*, *sra-3*, *sra-8*, *srb-1*, *srb-8*, *srb-9*, *srb-10*, and *srb-11* showed no detectable *GFP* expression in at least four independently derived lines. *sra-1* was expressed only in males (see below).

Cell identifications were made by comparing the fluorescence image with Nomarski images of the same animal. Particular neurons were identified by using a combination of their position and their morphology, as previously described (Bargmann and Horvitz, 1991a). In all cases, cells were observed in well-fed animals that had been grown on standard nematode culture plates under sparse conditions (Brenner, 1974).

To identify staining cells in males, wild-type males were crossed to adult transgenic hermaphrodites and the resulting cross-progeny viewed by fluorescence optics. Only a fraction of the male cross-progeny carried the transgenic array, but since *lin-15* is on the X chromosome, all males that did not carry the array were multivulval at high temperature.

Behavioral Assays

Avoidance assays for volatile odorants were conducted by presenting concentrated odorant in front of the animal's nose in a 20 μl microcapillary pipette, or dipping an eyebrow hair into the odorant and presenting it in front of the animal's nose. Animals were scored for the time until their next reversal. Typically, wild-type animals reversed less than once per minute in the absence of odorant, while they reversed in approximately 4 s when presented with octanol or benzaldehyde. Wild-type and laser-operated animals were presented with odorants for four to ten trials per animal, with at least 5 min of rest between trials, and scored for reversal responses. If animals did not reverse within 20 s, the odorant was removed and the animal scored as negative. Results were compared by use of a Mann-Whitney rank sum test in the Statview II program. Cell ablations were conducted with a nitrogen-pumped dye laser and standard methods (Avery and Horvitz, 1987).

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