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odr-10 Encodes a Seven Transmembrane Domain Olfactory Receptor Required for Responses to the Odorant Diacetyl

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Summary

Olfactory signaling is initiated by interactions between odorants and olfactory receptors. We show that the C. elegans *odr-10* gene is likely to encode a receptor for the odorant diacetyl. *odr-10* mutants have a specific defect in chemotaxis to diacetyl, one of several odorants detected by the AWA olfactory neurons. *odr-10* encodes a predicted seven transmembrane domain receptor; a green fluorescent protein–tagged Odr-10 protein is localized to the AWA sensory cilia. *odr-10* expression is regulated by *odr-7*, a transcription factor implicated in AWA sensory specification. Expression of *odr-10* from a heterologous promoter directs behavioral responses to diacetyl, but not to another odorant detected by the AWA neurons. These results provide functional evidence for a specific interaction between an olfactory receptor protein and its odorant ligand.

Introduction

The remarkable discriminatory power of the olfactory system allows animals to generate diverse behavioral responses to odorants. Subtle alterations in chemical structures between two odorants can result in substantial differences in perceived olfactory quality. For example, two enantiomers of carvone produce the distinct sensations of spearmint and caraway. This perceptual difference suggests that distinct neuronal pathways are preferentially activated by each enantiomer, probably owing to selective binding of the odorants to different olfactory receptor proteins.

The receptors that interact with odorants are likely to be G protein–coupled seven transmembrane domain proteins. Vertebrate olfactory neurons produce cAMP in response to odorants (Pace et al., 1985; Sklar et al., 1986), which activates a cyclic nucleotide–gated channel in the olfactory cilia (Nakamura and Gold, 1987; Firestein et al., 1991). The cAMP pathway is thought to be the major vertebrate olfactory transduction pathway, but odorants also stimulate IP_3 production in invertebrate and vertebrate olfactory neurons (Boekhoff et al., 1990, 1994; Breer et al., 1990). Candidate receptor molecules have been identified in several sensory systems. In mammals, putative olfactory receptors are encoded by a large family consisting of ~1000 receptor genes (Buck and Axel, 1991) that may interact with odorants (Raming et al., 1993). An additional family of genes might encode mammalian pheromone receptors (Dulac and Axel, 1995). These genes are expressed in the neurons of the olfactory epithelium and the vomeronasal organ, respectively. In the nematode Caenorhabditis elegans, several different families of candidate chemosensory receptor genes have been identified (the *sr* genes, which consist of *sra*, *srb*, *srd*, *sre*, *srg*, and *sro* families) (Troemel et al., 1995). These gene families encode potential chemosensory receptors based on their similarity to known G protein–coupled receptors and their preferential expression in chemosensory neurons.

It is not known how any of these sensory receptors interacts with its ligands. It is possible that each receptor interacts with only a small number of odorants, so that specific olfactory stimuli activate only a few receptors. Alternatively, the receptors might be able to recognize many different odorant molecules, with discrimination arising through later processing and integration of information by the nervous system. To address these issues, it is necessary to identify the odorant ligands that activate a given olfactory receptor protein under physiological conditions.

In the nematode C. elegans, molecular and functional properties of individual chemosensory neurons can be analyzed in the whole animal. An adult C. elegans hermaphrodite has 302 neurons in its nervous system, including 14 types of chemosensory neurons (White et al., 1986). In most cases, one type of neuron consists of a bilaterally symmetric pair of neurons with similar functions. The functions of 10 types of chemosensory neurons have been defined through behavioral analysis of animals in which particular cells have been killed. Two types of chemosensory neurons, called AWA and AWC neurons, are similar to vertebrate olfactory neurons in that they detect volatile (airborne) attractants (Bargmann et al., 1993). Each pair of neurons detects several different odorants: the AWA olfactory neurons detect diacetyl, pyrazine, and thiazoles, whereas the AWC olfactory neurons detect benzaldehyde, butanone, isoamyl alcohol, and thiazoles. In the presence of saturating levels of one odorant, animals can still respond to other odorants that are sensed by the same olfactory neuron (Bargmann et al., 1993; Colbert and Bargmann, 1995). These behavioral experiments suggest that one olfactory neuron has several distinct odorant-binding sites.

The expression patterns of the candidate *sr* receptor genes in C. elegans provide a possible explanation for the diverse functions mediated by single sensory cell types (Troemel et al., 1995). Multiple candidate receptor genes are expressed in a single chemosensory neuron, suggesting that the ability of these neurons to recognize many chemicals arises through the expression of several different receptors. However, the ligands for the *sr* receptors have not been defined.

A genetic approach to chemosensation provides a way to relate the functions of individual genes to particular behavioral responses. To identify the functions of genes involved in olfaction in C. elegans, we have conducted behavioral screens for mutants with specific olfactory defects (*odr*, or odorant-response mutants). Mutations in some *odr* genes affect many or all olfactory

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responses, whereas others have more selective effects on olfaction (Bargmann et al., 1993). Here we describe the genetic and molecular characterization of the *odr-10* gene, which functions in the response to a specific odorant, diacetyl. *odr-10* encodes a predicted seven transmembrane receptor that might interact directly with diacetyl in olfactory transduction.

Results

odr-10 Mutants Are Defective in Chemotaxis to Diacetyl

When placed in a gradient of an attractive odorant, C. elegans moves toward the peak of the gradient (Bargmann et al., 1993). Over 90% of wild-type animals accumulate at a point source of a strong attractant such as diacetyl in chemotaxis assays. A modified version of this assay was used to identify mutants with defects in their diacetyl responses. Mutagenized animals were given a choice between the attractants diacetyl and pyrazine, which are both recognized by the AWA olfactory neurons (Bargmann et al., 1993). Under the conditions used, wild-type animals preferentially accumulated at the diacetyl, animals that did not sense either odorant distributed randomly across the plate, and animals that did not sense diacetyl accumulated at the pyrazine. After two rounds of this behavioral enrichment and subsequent retesting, a single strain with a mutation in the *odr-10* gene was isolated.

odr-10(ky32) mutants were tested for chemotaxis to a panel of volatile odorants. The mutants were defective in their response to diacetyl, but exhibited wild-type behavior in their responses to all other tested odorants (Figure 1A). They also displayed normal responses to water-soluble attractants such as NaCl and lysine, to water-soluble repellents, and to mechanical stimuli (data not shown). Their development, locomotion, and fertility appeared to be normal.

Diacetyl is very similar in its chemical structure to two other attractants, butanone and 2,3-pentanedione, which are sensed primarily by the AWC olfactory neurons (Bargmann et al., 1993; P. S. and C. I. B., unpublished data) (Figure 1B). To determine whether *odr-10* mutants were subtly affected in their responses to any of these odorants, they were tested for chemotaxis to a range of different odorant concentrations (Figure 1C). *odr-10* mutants responded to high but not low concentrations of diacetyl (Figure 1C). However, their responses to all other odorants were normal for the entire range of tested concentrations.

odr-10 was mapped to the X chromosome, close to the gene *unc-6* (see Experimental Procedures). A deletion that spans the *odr-10* locus was used to determine whether the *odr-10* mutant defect was enhanced by lowering levels of *odr-10* activity. When *odr-10(ky32)* was placed in *trans* to the genetic deficiency *uDf1*, which deletes the *odr-10* region, the heterozygous animals did not exhibit additional defects (data not shown). In particular, *odr-10(ky32)/uDf1* animals displayed normal chemotaxis to pyrazine, and their chemotaxis to diacetyl was similar to that of *odr-10(ky32)* animals. No other behavioral or developmental defects were apparent in this strain. These results are consistent with the *odr-10(ky32)* mutation causing a loss of *odr-10* function.

odr-10 Encodes a Predicted Seven Transmembrane Domain Receptor

The *odr-10* gene was cloned using the C. elegans physical map and rescue of the *odr-10* behavioral phenotype (Coulson et al., 1986, 1988). In brief, genetic map data placed *odr-10* between the *unc-6* gene, which is cloned (Ishii et al., 1992), and the DNA polymorphism *stP33* (Williams et al., 1992) (Figure 2A). Cosmids from this interval were injected into *odr-10* mutants together with the *lin-15* gene as a marker to identify transgenic animals (Mello et al., 1991; Clark et al., 1994; Huang et al., 1994). The resulting transgenic strains were tested for chemotaxis to diacetyl. The cosmid C41B5 was able to complement the *odr-10* defect, as was a 3.3 kb ScaI–EcoRV subclone of the cosmid (Figure 2B).

The ScaI–EcoRV subclone was used to probe a mixed-stage C. elegans cDNA library (Barstead and Waterston, 1989), and three cDNA clones were isolated. Two cDNAs appeared to be full length, based on the fact that they began with sequences from the C. elegans splice leader SL1 (Krause and Hirsh, 1987) and ended with a polyA stretch. The full-length cDNAs were 1118 bp long, and encoded a predicted protein of 339 amino acids (Figure 3A). The corresponding genomic region has been sequenced by the C. elegans sequencing consortium (Sulston et al., 1992; Wilson et al., 1994; R. Waterston, A. Coulson, J. Sulston, et al., personal communication). The *odr-10* gene is entirely contained within the 3.3 kb rescuing fragment and includes eight exons and seven introns (Figure 3B).

Hydrophobicity analysis of *odr-10* revealed seven hydrophobic peaks that could correspond to membrane-spanning domains (Figure 3C). This structure is reminiscent of G protein–coupled receptors (Probst et al., 1992). BLAST (basic local alignment search technique) searches of GenBank and EST (expressed sequence tag) databases did not reveal a high degree of similarity between *odr-10* and any known gene (Altschul et al., 1990). However, the highest similarity that was detected in these searches was to one of the candidate vertebrate olfactory receptors, I14 (Buck and Axel, 1991), with which *odr-10* showed 12% amino acid identity over its entire length. Weak similarity was also observed between *odr-10* and *srd-1* and *srd-2*, two of the candidate chemosensory receptor genes that have recently been identified in C. elegans (Troemel et al., 1995).

To confirm that this coding region corresponded to *odr-10*, a frameshift mutation was generated within the cloned gene at an XhoI site just before transmembrane domain VI. This frameshifted clone could no longer complement the *odr-10* mutant phenotype (Figure 2B).

A Null Mutation in *odr-10* Causes a Diacetyl-Specific Chemotaxis Defect

The coding region of the *odr-10* gene was sequenced in *odr-10(ky32)* to identify the mutation responsible for its defect. The *odr-10(ky32)* mutation is a G to A transition on the noncoding strand that results in the substitution of a tyrosine for a histidine in the third predicted

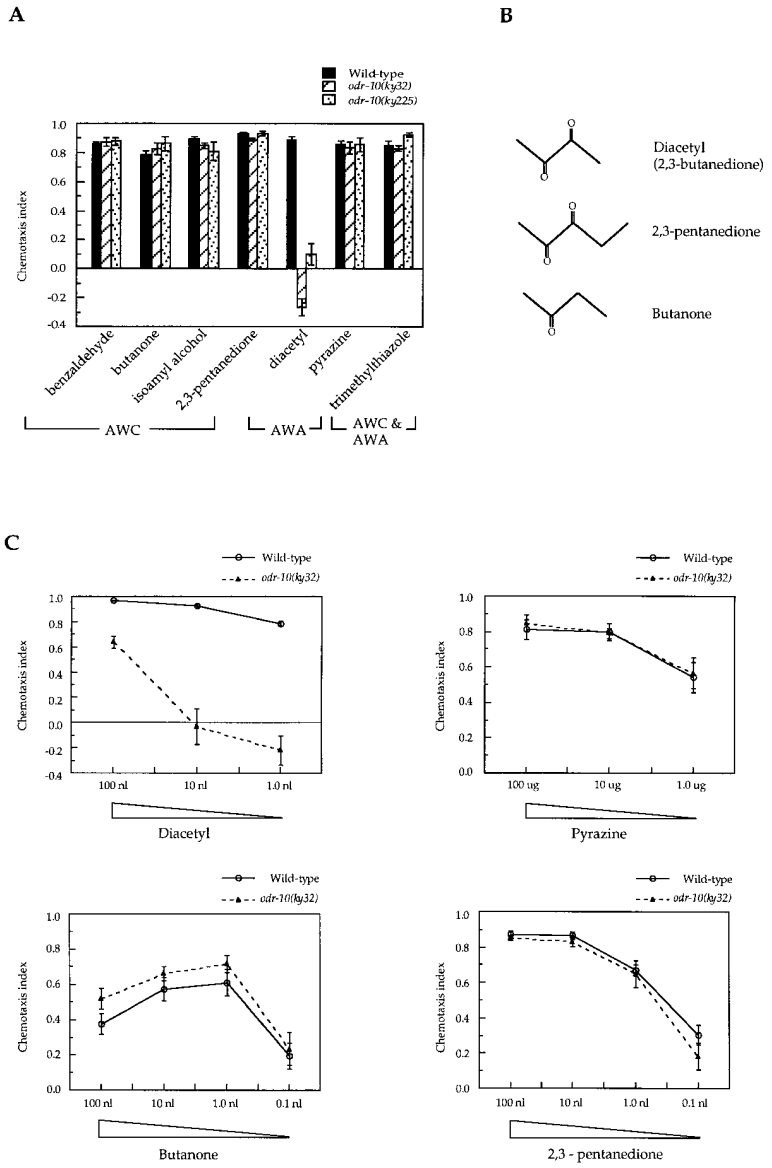

Figure 1. *odr-10* Mutants Do Not Respond to Diacetyl

(A) Chemotaxis responses of wild-type and *odr-10* mutants to volatile odorants. Responses of wild-type animals are indicated by closed bars, responses of *odr-10(ky32)* mutants by hatched bars, and responses of *odr-10(ky225)* animals by stippled bars. Chemotaxis index = (animals at the odorant − animals at the control)/total animals on the plate. A chemotaxis index of 1.0 indicates complete attraction; a chemotaxis index of −1.0 indicates complete repulsion. Each data point represents the average of six independent chemotaxis assays, using ~100 animals in each assay. Error bars equal the SEM. One microliter of diluted odorant was used per assay. Dilutions of odorants (in ethanol) were the following: 1:200 benzaldehyde, 1:1000 butanone, 1:200 isoamyl alcohol, 1:1000 2,3-pentanedione, 1:1000 diacetyl, 10 mg/ml pyrazine, and 1:1000 2,4,5-trimethylthiazole. Neurons required for the responses to these dilutions of odorants are indicated.

(B) Chemical structures of diacetyl (2,3-butanedione), 2,3-pentanedione and butanone.

(C) Chemotaxis responses of wild-type and *odr-10(ky32)* mutants to different dilutions of diacetyl, pyrazine, butanone, and 2,3-pentanedione. Responses of wild-type animals are shown by open circles and those of *odr-10(ky32)* mutants by closed triangles; *odr-10(ky225)* mutants were similar to *odr-10(ky32)* mutants (data not shown). Each data point represents the average of six independent assays. Error bars equal the SEM. The indicated amount of each odorant was diluted in 1 μl of ethanol.

membrane-spanning domain of the protein (Figures 3A and 3B). This missense mutation might cause a partial defect in *odr-10* function, or it might eliminate all *odr-10* activity.

To determine the consequences of a complete loss of *odr-10* function, we generated a mutation that deleted most of the *odr-10* coding region. The mutation *odr-10(ky225)* was generated by insertion of the transposon Tc1 into the endogenous *odr-10* gene followed by imprecise Tc1 excision (Zwaal et al., 1993). This deletion eliminated all *odr-10* coding sequences beyond the end of the third predicted transmembrane domain of the protein (Figures 3A and 3B). *odr-10(ky225)* animals were tested for chemotaxis and found to be indistinguishable from *odr-10(ky32)* animals (Figure 1A and data not shown). Like *odr-10(ky32)* mutants, they showed a strong defect in chemotaxis to diacetyl, but normal chemotaxis to other ketones and to other odorants sensed by AWA. Since this deletion allele is likely to eliminate all *odr-10* function, these results indicate that *odr-10* is uniquely important for the diacetyl response.

odr-7 Is Expressed in the Cilia of the AWA Olfactory Neurons

The Specific phenotype of *odr-10* mutants and the similarity between *odr-10* and other receptors suggested that *odr-10* might encode an olfactory receptor for diacetyl. To determine where the *odr-10* gene was expressed, the *odr-10* promoter was used to drive expression of the reporter gene GFP (green fluorescent protein) in transgenic worms (Chalfie et al., 1994; A. Fire, S. Xu, J. Ahnn, and G. Seydoux, personal communication). This fusion gene was strongly expressed in the two AWA neurons, which are the only neurons that sense low concentrations of diacetyl (Figure 4). The AWA neurons have sensory cilia that are exposed to the environment in specialized sensory organs at the tip of the

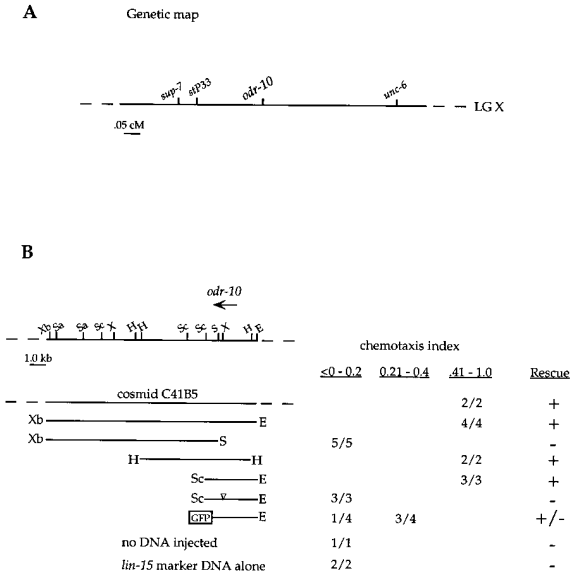

Figure 2. Genetic Map Position and Cloning of *odr-10*

(A) Genetic map position of *odr-10* on linkage group X.

(B) Localization of the *odr-10* gene. At top is a restriction map of the *odr-10* genomic region. The arrow indicates the direction of *odr-10* transcription. Below are shown the subclones that were tested for rescue of the *odr-10* mutant phenotype. The number of independent transgenic lines with an average chemotaxis index in the indicated intervals is shown as a fraction of the total lines tested; a minimum of 3 independent chemotaxis assays were conducted per line. An inverted triangle indicates the insertion of a frameshift mutation (see Experimental Procedures). The coding region of GFP was fused to *odr-10* to create a GFP-tagged Odr-10 protein (see text and Experimental Procedures). Xb = XbaI, Sa = SacII, Sc = ScaI, X = XhoI, H = HpaI, S = SmaI, E = EcoRV.

animal's nose (Ward et al., 1975; Ware et al., 1975). From these cilia, the AWA dendrites project back to cell bodies near the posterior pharynx (Figure 4A). Since GFP diffuses freely through the cytoplasm of the cells, the characteristic axon and dendrite morphology of the cells, together with the position of the cell bodies, was used to assign their identity as AWA neurons. A lower level of GFP expression was observed in the four mechanosensory neurons called CEP (e.g., see Figure 6B); this weak staining may not reflect expression of the endogenous *odr-10* gene (see Experimental Procedures).

If *odr-10* is directly involved in odorant recognition, its protein product should be localized to the sensory cilia, the site at which odorants interact with their receptors. To examine the subcellular localization of Odr-10, the GFP reporter was fused to the extreme C terminus of *odr-10* to produce a fluorescent tagged Odr-10 protein. This fusion gene partially complemented the diacetyl chemotaxis defect of *odr-10* mutants (see Figure 2B). Expression of the GFP-tagged Odr-10 protein was limited to the extreme anterior region of the AWA olfactory

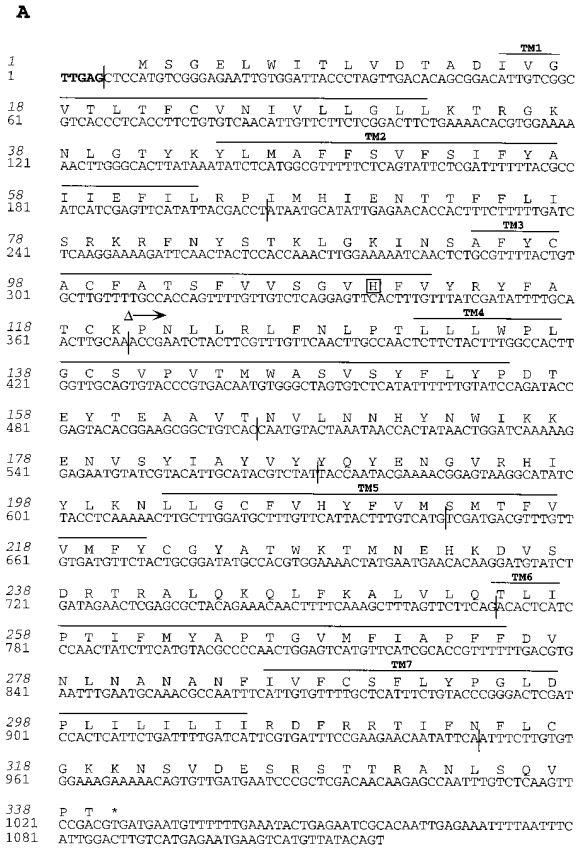

Figure 3. Sequence Analysis of *odr-10*

(A) Nucleotide and predicted amino acid sequences of the *odr-10* cDNA. Nucleotides are numbered beginning at the first nucleotide of the cDNA. Amino acids are numbered (in italics) starting at the first methionine. The STOP codon is marked by an asterisk. Splice junctions are marked by vertical lines and were determined by comparison of the sequences of the cDNA and corresponding genomic region. Nucleotides in bold at the 5′ end are predicted to be derived from the *trans*-splice leader SL1 (Krause and Hirsh, 1987). The histidine residue that is altered to a tyrosine in the *ky32* mutation is boxed. The open triangle denotes the beginning of the *ky225* deletion. Residues predicted to form transmembrane domains 1 through 7 are indicated. The GenBank accession number for *odr-10* is U49449.

(B) Structure of the *odr-10* gene. The location of the *ky32* mutation and the extent of the *ky225* deletion are indicated, along with the nucleotide and predicted amino acid alterations in *ky32*.

(C) Hydrophobicity plot of Odr-10. The plot was derived by Kyte–Doolittle hydropathic analysis of the predicted amino acid sequence of Odr-10 (Kyte and Doolittle, 1982).

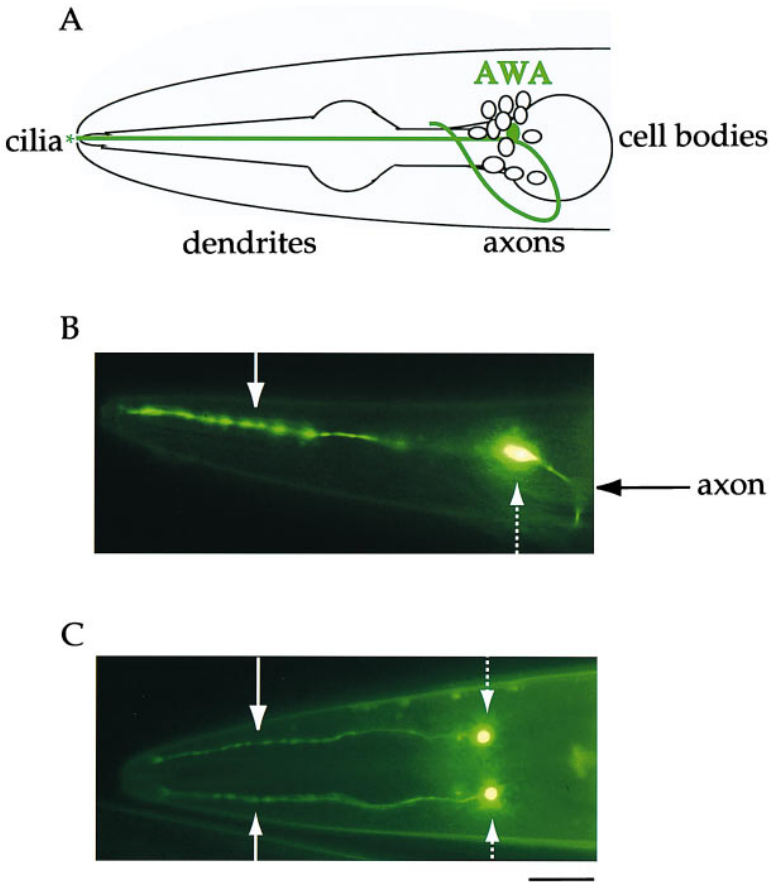

Figure 4. An *odr-10::GFP* Fusion Gene is Expressed in the AWA Neurons

(A) Schematic diagram of cell bodies in the anterior sensory ganglion (lateral view). The cell body and processes of the AWA neuron are indicated in green. Anterior is at left and dorsal is up.

(B) Lateral view of a transgenic animal expressing the integrated *odr-10::GFP* fusion gene (see Experimental Procedures). The solid arrow indicates the dendrite and the dashed arrow indicates the cell body of the AWA neuron.

(C) Dorsal view of a transgenic animal expressing the integrated *odr-10::GFP* fusion gene. The solid arrows indicate the dendrites and the dashed arrows indicate the cell bodies of the AWA neurons. Scale bar = 10 μm.

neurons, within the stereotypic branched AWA sensory cilia (Figure 5). Thus, the Odr-10 gene product appears to be localized to the sensory structures that mediate olfaction.

odr-10 Expression Is Controlled by the *odr-7* Gene, Which Regulates Olfactory Specificity

The *odr-7* gene is specifically required for olfactory function of the AWA neurons (Sengupta et al., 1994). Although their AWA neurons appear normal, *odr-7* null mutants are unable to respond to any of the odorants that are recognized by the AWA neurons. *odr-7* encodes a predicted transcription factor with similarity to the DNA-binding domains of the nuclear receptor superfamily; it has been proposed to regulate AWA sensory specificity by controlling the expression of AWA olfactory signaling molecules (Sengupta et al., 1994). If this explanation is correct, and if *odr-10* encodes the receptor for diacetyl, *odr-10* expression should be regulated by *odr-7*.

The expression of endogenous *odr-10* mRNA was examined in wild-type and *odr-7* mutant animals by reverse transcription–polymerase chain reaction. The *odr-10* message was present in wild-type animals but barely detectable in *odr-7(ky4)* null mutants (Figure 6A). The expression of the *odr-10::GFP* fusion gene was also examined in *odr-7* mutant animals. An integrated *odr-10::GFP* fusion gene was expressed in AWA in 100% of wild-type adults (n = 36). By contrast, the same integrated fusion gene was expressed in AWA in only 20% of *odr-7(ky4)* animals (n = 40), and its expression in those animals was much weaker than observed in wild-type animals (Figure 6B). This *odr-10::GFP* fusion gene included 1 kb upstream of the predicted start site of *odr-10*, indicating that sequences sufficient to confer regulation by *odr-7* are included within this region.

odr-10 Rescues Diacetyl but Not Pyrazine Responses in an *odr-7* Mutant

The loss-of-function alleles of *odr-10* indicate that this gene is necessary for chemotaxis to diacetyl, but not necessary for chemotaxis to pyrazine. Two general models could explain these results. Odr-10 protein might specifically interact with diacetyl and not pyrazine. Alternatively, Odr-10 protein might interact with both odorants but be partly redundant with a second receptor in the AWA neurons that senses pyrazine, but not diacetyl. To distinguish between these models, we asked whether *odr-10* could rescue specific odorant responses in animals that lacked all AWA functions due to a mutation in the *odr-7* gene (Sengupta et al., 1994).

The wild-type *odr-10* cDNA was introduced into *odr-7* mutants under control of the *odr-3* promoter, which drives expression in both the AWA and the AWC sensory neurons (K. Roayaie and C. I. B., unpublished data). *odr-7* mutants did not chemotax to diacetyl or pyrazine, but transgenic *odr-7* animals expressing the *odr-3::odr-10* fusion gene were able to chemotax to diacetyl, but not pyrazine (Table 1). Thus, the *odr-10* gene product was sufficient to restore the diacetyl response in an

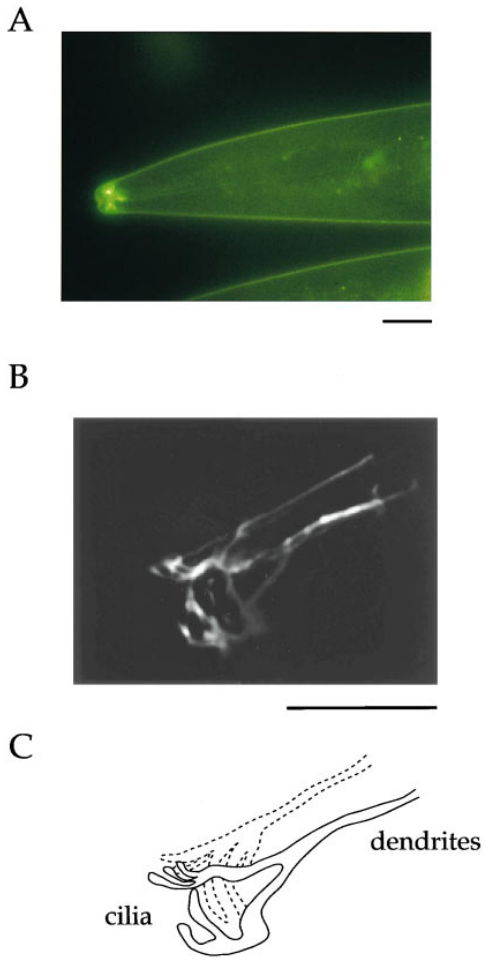

Figure 5. The Odr-10 Protein is Localized to the Sensory Cilia of the AWA Neurons

(A) Expression of the Odr-10 protein tagged with GFP (see Experimental Procedures). Strong GFP expression is seen at the tip of the nose, and faint expression is seen in the dendrites and cell bodies of the AWA neurons. Scale bar = 10 μm.

(B) Higher magnification view of the cilia of a transgenic animal expressing the Odr-10 protein tagged with GFP. The AWA cilia are identified by their characteristic branched pattern. Three dimensional images were acquired using wide-field fluorescence microscopy and constrained iterative deconvolution (Hiraoka et al., 1990). Scale bar = 10 μm.

(C) Schematic diagram of the sensory cilia of the AWA neurons. The image of the sensory cilia in (B) is shown. The solid and dashed lines represent the cilia of the two bilaterally symmetrical AWA neurons on the near and far sides of the animal, respectively.

odr-7 mutant, but not sufficient to restore the pyrazine response. These results indicate that expression of Odr-10 can confer a specific responsiveness to diacetyl on either the AWA or the AWC olfactory neurons.

Discussion

odr-10 Encodes a Candidate Receptor for Diacetyl

Based on their sequences and expression patterns, several families of vertebrate and invertebrate seven transmembrane domain proteins have been proposed to encode olfactory receptors (Buck and Axel, 1991; Ngai et al., 1993b; Dulac and Axel, 1995; Troemel et al., 1995). The data presented here provide direct evidence that a single C. elegans receptor is required for the physiological response to an odorant, and indicate that the *odr-10* gene product is likely to be a receptor for the odorant diacetyl.

Two genetic results implicate *odr-10* as a critical component of the olfactory response to diacetyl. First, mutations in *odr-10* lead to a selective loss in the animal's ability to sense diacetyl. The mutants exhibit normal responses to other odorants recognized by the AWA olfactory neurons, and thus are not completely defective in AWA function. Second, expression of a wild-type *odr-10* cDNA specifically restores diacetyl sensitivity to a mutant (*odr-7)* that has lost its response to several odorants.

The pattern of *odr-10* expression suggests that *odr-10* is directly involved in sensory transduction. *odr-10::GFP* gene fusions are expressed at high levels in the AWA neurons, the single type of olfactory neuron that senses low concentrations of diacetyl. In addition, a tagged protein that contains the entire *odr-10* coding region is localized to the AWA sensory cilia. These cilia are probably used mainly for sensory transduction, and expression here suggests sensory function for *odr-10*. Because all AWA synapses are located on the AWA axons, which are distant from the cilia, *odr-10* is unlikely to participate in synaptic transmission (White et al., 1986).

Sequence analysis predicts that *odr-10* encodes a seven transmembrane domain receptor of the G protein–coupled superfamily, with distant similarity to the candidate mammalian olfactory receptors. At least 10 additional C. elegans genes that are similar to *odr-10* have been found in the sequenced regions of the C. elegans genome (Wilson et al., 1994; J. H. C., E. Troemel, and C. I. B., unpublished data; J. Sulston, A. Coulson, R. Waterston et al., personal communication). The predicted *odr-10* sequence includes several residues that are conserved in the G protein–coupled receptor superfamily, particularly residues around the third and seventh transmembrane domains (Probst et al., 1992). As yet there is no direct evidence for G protein involvement in C. elegans olfaction, but G proteins have been shown to function in pheromone detection and in other forms of chemosensation in worms (R. Zwaal and R. Plasterk, personal communication; E. Troemel and C. I. B., unpublished data). Since G protein signaling pathways are used in olfaction in vertebrates and other invertebrates (Jones and Reed, 1989; Breer et al., 1990), it is likely that they also function in the C. elegans olfactory system.

In mammals, it has been suggested that olfactory receptors may be part of an axon guidance system for olfactory neurons (Singer et al., 1995; Sullivan et al., 1995); neurons that express a single olfactory receptor project to common target glomeruli in the olfactory bulb (Ressler et al., 1994; Vassar et al., 1994). Two observations make it unlikely that *odr-10* plays a direct role in axon guidance of C. elegans olfactory neurons. First, *odr-10* mutants have morphologically normal AWA axons, so this receptor is not required for normal axon outgrowth and guidance. Second, the Odr-10 gene product appears to be localized to the sensory cilia of the AWA neurons, while axon outgrowth occurs from a distant part of the cell.

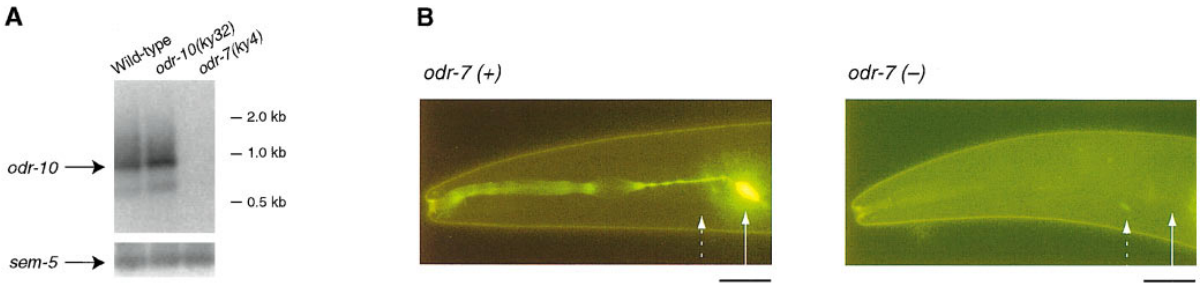

Figure 6. Expression of *odr-10* is Regulated by *odr-7*

(A) Expression of *odr-10* in wild-type, *odr-10(ky32)*, and *odr-7(ky4)* mutant animals. *odr-10* message was amplified by nested reverse transcription–polymerase chain reaction and detected by hybridization to an *odr-10* cDNA probe (see Experimental Procedures). *sem-5* message was amplified in parallel as a positive control.

(B) Expression of an *odr-10::GFP* integrated fusion gene in wild-type and *odr-7(ky4)* animals (lateral views). (Left) Expression of the fusion gene in an *odr-7*(+) background. The solid arrow indicates the position of the AWA cell body showing bright expression. The dashed arrow indicates the position of the CEP neurons. (Right) Expression of the integrated fusion gene in *odr-7(ky4)* mutants. The solid arrow indicates the expected position of the cell body of the AWA neuron. The dashed arrow indicates faint expression in one of four CEP neurons (see text and Experimental Procedures). This is the same fusion gene that is shown in Figure 4. Scale bar = 10 μm.

Odorant Specificity of the *odr-10* Gene Product

Individual olfactory neurons in C. elegans can sense several different odorants, including odorants that can be distinguished by the animal in behavioral assays (Bargmann et al., 1993; Colbert and Bargmann, 1995). In principle, each neuron could express one olfactory receptor that detects many odorants, or several more specific olfactory receptors. The expression patterns of the putative chemosensory receptors encoded by the *sra*, *srb*, *srd*, *sre*, *srg*, and *sro* genes indicate that one sensory neuron can express many different candidate receptors (Troemel et al., 1995). Similarly, the genetic properties of *odr-10* favor the model that several olfactory receptors are expressed per neuron. The AWA olfactory neurons sense several structurally dissimilar odorants that are discriminated by the animal, including diacetyl and pyrazine. *odr-10* null mutants have defective responses to diacetyl but not pyrazine, suggesting the existence of at least one additional receptor on the AWA neurons.

Diacetyl specificity of Odr-10 was also indicated by the observation that the *odr-10* cDNA rescues the diacetyl but not the pyrazine defect of *odr-7* mutants. Presumably, at least one additional gene controlled by the candidate transcriptional regulator *odr-7* is required for a normal pyrazine response.

The AWA neurons sense diacetyl at low concentrations, probably in the nanomolar range. However, they detect 2,3-pentanedione 10- to 100-fold less efficiently, and they do not detect 2-butanone at all (Bargmann et al., 1993; P. S. and C. I. B., unpublished data). Therefore, the diacetyl-sensing pathway of the AWA neurons selects among molecules with similar structures. If *odr-10* encodes the diacetyl receptor, this selectivity might be an intrinsic property of Odr-10, and the biochemical sensitivity of the Odr-10 protein to odorant agonists or antagonists could generate behavioral specificity.

Although many other tested odorant responses are normal in *odr-10* mutants, Odr-10 might sense odorants other than diacetyl. The AWA neurons are redundant with the AWC neurons for a number of olfactory responses, and a defect in the AWA component of those responses might not be apparent in behavioral assays.

Table 1. *odr-10* Expression Driven by the *odr-3* Promoter Rescues Diacetyl but Not Pyrazine Responses in *odr-7(ky4)* Mutants

		Chemotaxis	
Strain	Fusion Gene Injected	Diacetyl	Pyrazine
odr-7(ky4)	none	–	–
odr-7(ky4)	*odr-10* cDNA (no promoter)	–	–
odr-7(ky4)	*odr-3::odr-10*	+	–
odr-10(ky32)	none	–	+
odr-10(ky32)	*odr-3::odr-10*	+	+

Fusion genes were microinjected into an *odr-7(ky4) lin-15(n765ts)* X strain or an *odr-10(ky32) lin-15(n765ts)* X strain together with *lin-15* marker DNA. At least four independent transgenic lines carrying each fusion gene were tested. Chemotaxis indices of 0–0.3 were scored as negative; indices of 0.3–1.0 were scored as positive. A minimum of three chemotaxis assays were conducted per transgenic line.

Parallels between Vertebrate and Invertebrate Olfaction

The sequence similarity between *odr-10* and the vertebrate olfactory receptors is limited to a few residues in the predicted proteins (~10% overall amino acid identity). *odr-10* is more similar to these receptors than to other G protein–coupled receptors, but it is unclear whether the vertebrate and invertebrate olfactory receptors are derived from a common ancestor.

Vertebrate olfactory neurons have been reported to respond to many different odorants. For example, as many as 25% of all salamander olfactory neurons generate an electrical response to cineole (Firestein et al., 1993). If these salamander neurons are like mammalian olfactory neurons and express only one olfactory receptor gene per neuron, then that one receptor might recognize a broad variety of odorants. One rat olfactory receptor clone, OR5, has been shown to generate an

IP_3 response to many different odorants when expressed in SF9 cells (Raming et al., 1993). These studies suggest that vertebrate odorant–receptor interactions are relatively promiscuous. However, they were conducted at micromolar odorant concentrations, whereas physiological levels are in the nanomolar or picomolar range. At lower odorant concentrations, vertebrate receptors might also show the striking specificity observed with Odr-10.

Whereas individual C. elegans neurons probably express several receptor genes, mammalian olfactory neurons probably express only one receptor gene per neuron (Ressler et al., 1993; Vassar et al., 1993; Chess et al., 1994). Why should this difference exist? C. elegans has only 32 chemosensory neurons, but chemosensation is its most complex sensory modality and the main mechanism by which the animal makes qualitative judgments of its environment. Given the apparent chemical specificity of Odr-10 and the small number of sensory neurons, each cell might need to express many receptors for the animal to recognize the full spectrum of salient odorants. It will be interesting to determine whether olfactory systems of intermediate complexity, such as those of insects (Siddiqi, 1987), lobsters (Boekhoff et al., 1994), and fish (Ivanova and Caprio, 1993; Ngai et al., 1993a; Kang and Caprio, 1995), follow the mammalian pattern or the nematode pattern of receptor expression.

Experimental Procedures

Strains and Genetics

Wild-type worms were C. elegans variety Bristol, strain N2. Worms were grown using standard methods (Brenner, 1974).

Strains used in this work were: CX2111 *unc-58(e665dm)* X, MT1079 *egl-15(n484)* X, PS1032 *syDf1/unc-2(e55) lon-2(e678)* X, TY1093 *unc-42(e270)* V; *szT1/uDf1* X, CX2641 *dpy-8(e130) stDf1/lon-2(e678) egl-15(n484)* X; CB678 *lon-2(e678)* X, TY0367 *unc-18(e81) dpy-6(e14)* X, CX2629 *lon-2(e678) odr-10(ky32) unc-6(n102)* X, CX2818 *lin-15(n765ts)* X, CX2366 *odr-7(ky4) lin-15(n765ts)* X, CX2275 *odr-7(ky4) unc-9(e101)* X, CB101 *unc-9(e101)* X, MT3126 *mut-2(r459)* I; *dpy-19(n1347)* III and C. elegans variety Bergerac, strain RW7000.

Isolation of *odr-10(ky32)* and *odr-10(ky225)*

odr-10(ky32) was identified in a behavioral screen for animals that could not chemotax to diacetyl. The mutagenesis and mutant screens were performed as described previously (Bargmann et al., 1993). In brief, wild-type animals were mutagenized using the mutagen ethyl methanesulfonate (EMS). The F2 progeny of ~3600 mutagenized F1 animals were screened by giving them a choice between point sources of 1:10 dilution of diacetyl and 1 mg/ml pyrazine. After two rounds of choosing animals at the pyrazine, individual animals were placed on 6 cm plates, allowed to self-fertilize, and their progeny retested for chemotaxis defects.

odr-10(ky225) was generated by insertion and imprecise excision of the transposon Tc1 (Zwaal et al., 1993). Tc1 and *odr-10*- specific primers were used to screen 1000 frozen stocks of the mutator strain MT3126 by PCR, identifying one strain with a Tc1 insertion within the second intron of *odr-10*. The *odr-10::Tc1* strain was subjected to 68.7 mJ/cm^2 irradiation in a Stratagene UV Stratalinker to produce DNA breaks, and the progeny screened via PCR for imprecise excision of Tc1. An animal with a 1351 bp deletion that extended from the Tc1 insertion site to 428 bp past the *odr-10* stop codon was identified. The predicted product from this deletion *(odr-10(ky225))* lacks all coding sequences past the N-terminal 120 amino acids of *odr-10*.

Both mutations were backcrossed four times before behavioral testing.

Mapping of *odr-10*

The initial isolate of *odr-10* carried two mutations: *odr-10(ky32)* and *odr(ky47)*. Both mutations were linked to *unc-58(e665dm)* X. *odr(ky47)* was mapped near *egl-15(n484)* X. *odr-10(ky32)* was covered by the genetic deficiency *syDf1* X but uncovered by the genetic deficiencies *uDf1* X and *stDf1* X. These data placed *odr-10(ky32)* between *mec-2* and *unc-6* on LG X.

odr-10(ky32) was separated from *odr(ky47)* by the following strategy: a strain carrying the two linked mutations *odr-10(ky32)* and *dpy-6(e14)* X was generated by isolating Dpy non-Unc recombinants in *odr-10(ky32) odr(ky47)/unc-18(e81) dpy-6(e14)* X animals. 8/8 Dpy non-Unc recombinants segregated *odr-10(ky32)*. Males carrying the *lon-2(e678)* X mutation were mated with *odr-10(ky32) dpy-6(e14)* X animals, and recombinants carrying only the *odr-10(ky32)* mutation were isolated on the basis of their behavioral phenotype in chemotaxis assays.

Restriction fragment length polymorphisms between the Bristol strain N2 and the Bergerac strain RW7000 were used to further localize *odr-10*. The polymorphism *stP33* is a 260 bp fragment detected in RW7000 using PCR (Williams et al., 1992). RW7000 males were mated with hermaphrodites carrying the three linked mutations *lon-2(e678) odr-10(ky32) unc-6(n102)* X. 3/13 Lon non-Unc recombinants lost the *stP33* Bergerac polymorphism. Of these three, two recombinants segregated *odr-10(ky32)*. This analysis placed *odr-10* between *stP33* and the gene *unc-6* on the physical map.

Molecular Biology Methods

All general molecular biology manipulations were performed using standard methods (Sambrook et al., 1989). Sequencing was performed using the fmol sequencing system (Promega). Sequence analysis was carried out using GeneWorks (Intelligenetics). Sequence comparisons were performed using the BLAST network service (Altschul et al., 1990) and the CLUSTAL W program (Thompson et al., 1994). Preliminary sequence of the *odr-10* genomic region was obtained courtesy of the C. elegans sequencing consortium at Washington University (St. Louis) (Wilson et al., 1994).

Germline Transformation

Germline transformation was carried out as described (Mello et al., 1991; Sengupta et al., 1994). Marker *lin-15* DNA (Huang et al., 1994) was used at a concentration of 50 μg/ml and test DNA at a concentration of 30 μg/ml. Transgenic animals were identified by rescue of the *lin-15(n765ts)* multivulval phenotype at 20°C.

Isolation and Characterization of cDNAs

The 3.3 kb ScaI–EcoRV rescuing genomic fragment was used to screen approximately 1X 10^6 plaques of a mixed stage C. elegans cDNA library (Barstead and Waterston, 1989). Three positive clones were identified and partial sequences were obtained from both ends of the clones. One clone was a hybrid cDNA obtained from fusion of a partial *odr-10* cDNA with an unidentified cDNA. The remaining two cDNAs were identical and encoded *odr-10*. One of these cDNAs was sequenced completely on both strands using ^{33}P-end-labeled oligonucleotides.

Sequencing of the *odr-10(ky32)* Mutant Allele

Genomic DNA was isolated from N2 and *odr-10(ky32)* animals as described (Klein and Meyer, 1993). The genomic region containing the *odr-10* gene was amplified using the primers PS-37 (5′-CCT CGT GAA ATC AGA TTT CAG-3′) and PS-38 (5′-ACA TTC ATC ACG TCG GAA CTT-3′) flanking the open reading frame. At least one strand of the open reading frames of all eight exons, the splice junctions, and ~50 bp beyond the initiator methionine and the termination codon were sequenced. The exon containing the *ky32* mutation was sequenced on both strands.

Generation of the Frameshift Mutation

A frameshift mutation was created at the XhoI site in the sixth exon of the *odr-10* gene using the following strategy: the ScaI–EcoRV rescuing genomic fragment was cloned into the EcoRV site of the

pBluescript plasmid. The XhoI site in the polylinker was destroyed by digesting this plasmid with ApaI and SalI, blunting the overhanging ends, and religating. A frameshift was then created at the remaining XhoI site in the *odr-10* gene by digesting with XhoI, filling in the protruding ends, and religating. This results in a frameshift after Arg-241 of Odr-10. The mutation was confirmed by DNA sequencing.

Generation of *odr-10* Expression Constructs

A translational *odr-10::GFP* fusion gene was made by ligating ~1 kb of upstream promoter sequences and sequences encoding the first four amino acids of *odr-10* in-frame into the GFP expression vector pPD95.77 (Chalfie et al., 1994; A. Fire, S. Xu, J. Ahnn, and G. Seydoux, personal communication). The GFP gene used in all experiments contains five engineered introns and the mutation S65C (Heim et al., 1995). *odr-10* sequences were amplified from a subclone of the genomic region in pBluescript. PCR was performed using the T3 primer and an *odr-10*-specific primer engineered to contain a BamHI site at one end (PS-44; 5′-TAG GGT AAT GGA TCC TTC TCC CGA CAT GGA GCT GTA-3′). The resulting product was subcloned into the SalI and BamHI sites of pPD95.77. This strategy resulted in the addition of 20 amino acids between the fourth residue of the Odr-10 protein and the first methionine of GFP.

The Odr-10 protein tagged with GFP was constructed by amplifying ~1 kb of *odr-10* promoter sequences together with sequences encoding residues 1–337 of the Odr-10 protein, and ligating the PCR product in-frame into pPD95.77. Amplification was performed from a subclone of the *odr-10* genomic region in pBluescript using the primers T3 and PS-59 (5′-CAT TCA TCA GGA TCC AAC TTG AGA CAA ATT GGC-3′). PS-59 was designed to contain a BamHI site at the end. The amplified fragment was cloned into the SalI and BamHI sites of the pPD95.77 vector. This tagged protein includes 20 additional amino acids between Val-337 of Odr-10 and the initiator methionine of GFP. The sequence of the PCR product was confirmed by DNA sequencing.

The *odr-3::odr-10* expression construct was made in several steps. First, *odr-10* sequences encoding residues 1–337 of Odr-10 were amplified and inserted into pBluescript to remove the 3′ untranslated region and polyA tail. Amplification was carried out on a plasmid containing the *odr-10* cDNA using the T3 and PS-59 primers. Second, a STOP codon was inserted by ligating an NheI linker (New England Biolabs) containing Stops in all three frames at the 3′ end of the modified *odr-10* cDNA. Sequence analysis of this cDNA revealed three PCR-induced point mutations, which were removed by replacing an NsiI–BsmI fragment of the cDNA with a fragment from the original *odr-10* cDNA. Third, the GFP gene in the Tu#62 expression vector was replaced with the *odr-10* cDNA. This essentially creates a promoterless *odr-10* "expression" vector. Finally, a HindIII fragment containing approximately 3 kb of upstream sequences and sequences encoding the first 36 amino acids of *odr-3* was inserted into the SphI site in the polylinker of the *odr-10* "expression" vector by blunt-ended ligation. All junctions were verified by sequencing.

Most amplification reactions were carried out using 50–100 ng of plasmid DNA and 100 ng of each primer in 50 mM KCl, 10 mM Tris (pH 8.0), 1.5 mM $MgCl_2$, and 200 μM of each dNTP. Reaction conditions were 94°C for 30 s, 52°C for 1 min, 72°C for 1 min for 15 cycles on an MJ Research thermal cycler.

Detection of *odr-10* Transcripts by Reverse Transcription–Polymerase Chain Reaction

Total RNA was prepared from mixed stage N2, *odr-10(ky32)* and *odr-7(ky4)* animals by LiCl precipitations (Michael Finney, personal communication). First strand cDNAs for *odr-10* and *sem-5* (Clark et al., 1992) were prepared in the same reaction using the primers PS-38 and sem-5#5 (5′-TGG AGA TTA AGT AAG AGA GGG C-3′) and 10 μg of total RNA, essentially as described (Aatsinki et al., 1994). The cDNAs were used as templates for PCR amplification, using the primers SL1, PS-38, and sem-5#5 to detect *odr-10* (test) and *sem-5* (positive control) messages. The first amplification of *odr-10* and *sem-5* cDNAs was performed in the same reaction. Reactions were subjected to additional rounds of amplification using SL1 and the nested primer PS-43 (5′-TGA AAT TGG CGT TTG CAT TCA-3′) for *odr-10* and SL1 and sem-5#14 (5′-GTT GAA TTT GAC AGC C-3′) for *sem-5*. Nested reactions for *odr-10* and *sem-5* were performed separately. The conditions for PCR were as described above except that 30 cycles of amplification were carried out in each round. The reaction products were resolved by electrophoresis, transferred to a nylon membrane, and probed with ^{32}P-labeled *odr-10* cDNA or a *sem-5* genomic fragment.

Expression of *odr-10::GFP* Fusion Genes

Transgenic C. elegans lines generated by microinjection bear extrachromosomal arrays of the injected DNA that are occasionally lost during meiosis and mitosis. To confirm the expression pattern of the *odr-10::GFP* fusion gene, a strain was generated where the fusion gene (and marker *lin-15* DNA) were integrated into the genome (Mello et al., 1991). Approximately 40 L4 larvae of a transgenic line carrying the extrachromosomal array of *odr-10::GFP* and *lin-15* were irradiated with gamma rays from a ^{137}Cs source (6000 rads). Six hundred transgenic F2 progeny of the mutagenized animals (recognized by their *lin-15*+ phenotype) were cloned onto individual plates. Potential integrants were identified by the absence of *lin-15* mutant progeny. A single strain CX3260 *(lin-15(n765ts); kyIn37)* resulted from integration of the extrachromosomal array into LG II, near *rol-6*. This strain showed bright GFP expression in both AWA neurons in all animals. Although somewhat masked by this bright fluorescence, faint but consistent expression was also observed in the CEP neurons. Rarely, expression was also observed in other neurons, including ASI.

The expression pattern of the *odr-10::GFP* fusion gene was unaltered in *odr-10(ky32)* animals (data not shown). Additionally, the localization of the Odr-10 protein tagged with GFP was unaltered, and the AWA neurons had normal morphology of their sensory cilia, axons, and dendrites in *odr-10* mutants. An *odr-7::GFP* fusion gene is expressed only in the AWA neurons. Expression of this fusion gene was unaltered in *odr-10(ky32)* animals, indicating that the *odr-10(ky32)* mutation did not alter AWA cell fate (data not shown).

To visualize *odr-10::GFP* expression in *odr-7* mutants, the following strategy was used: N2 males were mated with *lin-15(n765ts); kyIn37* hermaphrodites to obtain *lin-15(n765ts); kyIn37*/+ males. Heterozygous males were mated with *unc-9(e101)* X and *odr-7(ky4) unc-9(e101)* X hermaphrodites, and cross-progeny heterozygous for both *kyIn37* and *unc-9* were cloned out. Unc animals in the F2 generation were again cloned out and homozygosed for *kyIn37* by examination under fluorescence. The presence of the *odr-7(ky4)* mutation was confirmed by complementation analysis. GFP expression was observed faintly but consistently in the CEP neurons in both wild-type and *odr-7(ky4)* animals. Since the endogenous *odr-10* message was greatly reduced or absent in *odr-7(ky4)* mutants (Figure 6A), the expression of the reporter gene fusion in CEP may not be characteristic of the endogenous *odr-10* gene.

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