Neuron, Vol. 22, 339–347, February, 1999, Copyright ©1999 by Cell Press

The Odor Specificities of a Subset of Olfactory Receptor Neurons Are Governed by Acj6, a POU-Domain Transcription Factor

Peter J. Clyne,* Sarah J. Certel,† Marien de Bruyne,* Lina Zaslavsky,* Wayne A. Johnson,†‡ and John R. Carlson*§

*Department of Molecular, Cellular, and Developmental Biology
Yale University
New Haven, Connecticut 06520
†Genetics Program
‡Department of Physiology and Biophysics
University of Iowa
College of Medicine
Iowa City, Iowa 52242

Summary

Little is known about how the odor specificities of olfactory neurons are generated, a process essential to olfactory coding. We have found that neuronal identity relies on the *abnormal chemosensory jump 6* (*acj6*) gene, originally identified by a defect in olfactory behavior. Physiological analysis of individual olfactory neurons shows that in *acj6* mutants, a subset of neurons acquires a different odorant response profile. Certain other neurons do not respond to any tested odors in *acj6*. Molecular analysis of *acj6* shows that it encodes a POU-domain transcription factor expressed in olfactory neurons. Our data suggest that the odor response spectrum of an olfactory neuron, and perhaps the choice of receptor genes, is determined through a process requiring the action of Acj6.

Introduction

Olfactory coding depends on the existence of a functionally diverse population of olfactory receptor neurons (ORNs) (Shepherd, 1994; Buck, 1996; Hildebrand and Shepherd, 1997). Each class of ORN exhibits a distinct odor response profile, as revealed by physiological measurements of individual ORNs. The odor specificity of these classes presumably reflects the expression of different odorant receptor genes. Little is known, however, about how the odor specificities of ORNs are generated during development.

The olfactory system of *Drosophila melanogaster* allows convenient genetic, molecular, and physiological analysis of this problem (Siddiqi, 1987; Carlson, 1996). The fly contains two olfactory organs, the third segment of the antenna and the maxillary palp. Each organ is covered with sensory hairs that contain ORNs, with each hair on the maxillary palp housing two ORNs and hairs on the antenna containing up to four. Olfactory response can be measured in vivo, either behaviorally or physiologically. One means of physiological measurement is to record the receptor potentials of populations of ORNs in the vicinity of an extracellular electrode, in recordings called electroantennograms (EAGs) or electropalpograms (EPGs). Another means is to record the action potentials of the neurons within a single sensory hair, in single-unit recordings (Siddiqi, 1991; Clyne et al., 1997).

We previously isolated a *Drosophila* olfactory mutant, *acj6* (*abnormal chemosensory jump 6*), using an olfactory-driven behavioral screen (McKenna et al., 1989). Wild-type flies jump in response to a sudden pulse of odor, presumably as an escape response; *acj6* was isolated by virtue of a reduced jump response to odor stimuli. Further analysis revealed that *acj6* flies have severe physiological deficits in response to some, but not all odors, as revealed by reductions in amplitudes of EAGs and EPGs (Ayer and Carlson, 1991, 1992). One kind of defect that might have different effects on responses to different odors is a defect affecting the specification of a subset of olfactory neurons.

How are the identities of olfactory neurons specified? By analogy to other processes, such as early embryonic development in *Drosophila*, the generation of ORN phenotypes is likely to be governed largely by combinatorial interactions of transcription factors. Attractive candidates for key regulatory molecules in olfactory system development are POU-domain transcription factors. POU-domain proteins consist of a highly conserved ~75 amino acid POU-specific domain, tethered by a linker of variable length and sequence to a 60-amino-acid homeodomain (Ruvkun and Finney, 1991; Herr and Cleary, 1995). A large number of POU-domain proteins have now been identified from a broad range of organisms, and members of the class IV subfamily are required for the development of specific phenotypes in subsets of sensory system neurons (reviewed by Ryan and Rosenfeld, 1997). In the mouse retina, three such class IV POU-domain proteins, Brn-3a, Brn-3b, and Brn-3c, are expressed in overlapping subsets of ganglion cells (Xiang et al., 1995, 1996; Erkman et al., 1996). Targeted disruption of *Brn-3b* leads to a selective loss of 70% of retinal ganglion cells; other neurons in the retina and brain are affected little if at all (Erkman et al., 1996; Gan et al., 1996). *Brn-3c* is also expressed in hair cells in the auditory system, and a null mutation causes a failure of hair cells to differentiate (Erkman et al., 1996). In *C. elegans*, the class IV POU-domain transcription factor encoded by the *unc-86* gene is required for the proper differentiation of mechanosensory neurons (Chalfie et al., 1981; Finney et al., 1988; Finney and Ruvkun, 1990).

Four POU-domain genes have been identified in *Drosophila*, and all four are expressed in the nervous system (Billin et al., 1991; Dick et al., 1991; Lloyd and Sakonju, 1991; Treacy et al., 1991; Yang et al., 1993; Anderson et al., 1995; Poole, 1995). The sole *Drosophila* representative of class IV POU-domain proteins has been called I-POU (Treacy et al., 1991, 1992). I-POU is expressed in embryos, larvae, pupae, and adults, as determined in Northern and Western blots, but its spatial localization has been determined only in the embryo, where it is neuron specific (Treacy et al., 1991). No mutants of I-POU have been reported.

§To whom correspondence should be addressed (e-mail: john.carlson@yale.edu).

Figure 1. Isolation of New *acj6* Alleles with the "Bump Assay"

We mutagenized CS-5 (Helfand and Carlson, 1989) wild-type males and crossed them to *y w cv cm l(1)ogre^{in1} g acj6^{1} sd f*/FM6 females. Approximately 300 progeny of all genotypes were placed in a fresh culture bottle. The bottle was then bumped on a benchtop to knock the flies to the bottom of the bottle. The lid was then removed for 15 s, and flies remaining in the bottle were saved for further analysis. The bumping and removing of the lid was done inside a large box in order to contain the escaping flies. We typically carried out three cycles of the bump procedure and then took receptor potential recordings from olfactory organs of the remaining CS-5/*y w cv cm l(1)ogre^{in1} g acj6^{1} sd f* females to identify new *acj6* mutants.

Here, we describe the identification of Acj6 as the POU-domain transcription factor I-POU. We show it is expressed in olfactory organs during development, beginning at the time of ORN differentiation, and also in mature ORNs. We show that loss of Acj6 function causes specific changes in the responses of individual ORNs, with one set of neurons acquiring a novel odor sensitivity and a second set of neurons losing odor sensitivity. These results indicate a role for the Acj6 POU-domain transcription factor in determining the odor specificity of ORNs.

Results

New *acj6* Alleles Isolated with a Novel Behavioral Paradigm

To investigate the molecular basis of *acj6* function, we first screened for new alleles. We designed a novel behavioral paradigm based on an additional phenotype of *acj6*, reduced mobility (Figure 1). Several hundred flies were placed in a culture bottle and allowed to run up the sides. The bottle was then bumped against a lab bench such that the flies fell to the bottom of the bottle. The lid of the bottle was then removed for 15 s, during which time most wild-type flies escaped, whereas *acj6^{1}* flies did not. Specifically, in reconstruction experiments with a mixture of wild-type and *acj6^{1}* flies, 90%–95% of the wild-type flies left the bottle in 15 s while 0% of the *acj6^{1}* flies left the bottle.

Following mutagenesis, we used the bump paradigm to carry out three cycles of enrichment for new alleles of *acj6*. We isolated three new X-ray-induced alleles (*acj6^{2}*, *acj6^{3}*, *acj6^{5}*) and one new ethyl methane sulfonate–induced allele (*acj6^{6}*), all of which are homozygous viable. All four new alleles have both impaired mobility and reduced amplitudes in recordings of receptor potentials from adult olfactory organs (Figure 2A). In tests of olfactory function using EPG recordings, *acj6^{6}* is a genetic null (Figure 2B), the three X-ray-induced alleles are also likely to be null mutations (Figure 2A), *acj6^{1}* is a partial loss-of-function mutation (Figures 2A and 2B), and all *acj6* alleles are recessive (Figures 2C and 2D).

Cytological mapping with 14 deficiency and duplication chromosomes placed *acj6* between cytogenetic positions 13B7–8 and 13C2–3 on the X chromosome, and meiotic recombination mapping placed *acj6* 0.02 cM proximal to the P element AS438, which is in 13C1–2 (De Cicco and Spradling, 1984). We conclude that *acj6* maps to region 13C1–3.

Acj6 is a POU-Domain Transcription Factor

A partial cDNA of the class IV POU-domain transcription factor gene, I-POU, was previously shown to hybridize to 13C1–2 on polytene chromosomes (Treacy et al., 1991). We cloned ~100 kb of genomic DNA flanking P(AS438) and determined the genomic organization of the I-POU gene. We found that all three X-ray-induced mutations of *acj6* disrupted the I-POU locus (Figure 3A),

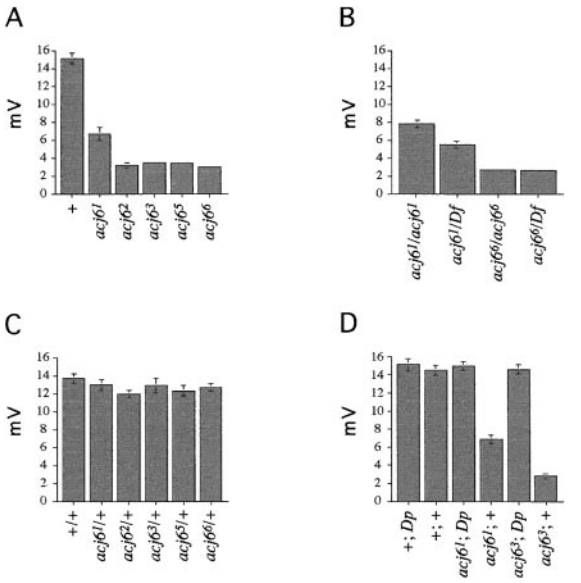

Figure 2. Genetic Analysis of *acj6*

Values indicate electropalpogram (EPG) responses in millivolts to ethyl acetate.

(A) *acj6* alleles. Two- to seven-day-old males were tested. "+" refers to CS-5, the parental strain from which the alleles were derived. n = 10 for each genotype. Error bars are too small to be seen in some cases.

(B) *acj6^{6}* is a null allele and *acj6^{1}* is a hypomorphic, or partial loss-of-function, allele. Since the phenotype of *acj6^{6}*/*acj6^{6}* is indistinguishable from that of *acj6^{6}*/*Df*, *acj6^{6}* meets the genetic criterion for a null allele. By contrast, *acj6^{1}*/*acj6^{1}* is less severe than *acj6^{1}*/*Df*. Two- to six-day-old females were tested. "Df" refers to *Df(1)M34-1Cs*, which extends from 13B7–8 to 14B15–18. n = 8 for each genotype.

(C) The alleles of *acj6* are recessive. Two- to seven-day-old females were tested. "+" indicates the balancer chromosome *FM6*, except that "+/+" refers to CS-5/*FM6*. n = 10 for each genotype.

(D) *acj6^{1}* and *acj6^{3}* are loss-of-function alleles. Three- to six-day-old males were tested. "Dp" refers to *Dp(1;2)eag^{x6}*, which extends from 13A1 to 13E14-15. n = 8 for each genotype.

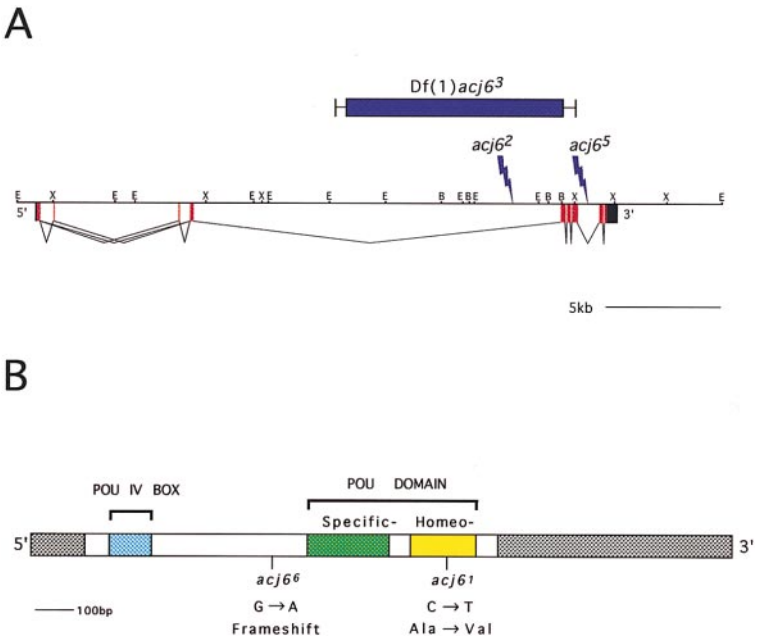

Figure 3. The Structure of the *acj6* Gene

(A) The genomic structure of *acj6*. The translated regions of the *acj6* transcript are indicated in red and the untranslated regions in black. We have identified nine exons and have found five new alternative splice forms in addition to the two described previously (Treacy et al., 1992). There is a series of alternative splices at the 5′ end of the transcript that either disrupt or leave intact the POU IV box protein motif (Theil et al., 1993) (see [B] below). The purple lightning bolts indicate the positions of the chromosomal breakpoints in $acj6^2$ and $acj6^5$. The positions of these breakpoints are accurate to within ~0.5 kb. $acj6^3$ is a deletion whose left and right breakpoints are shown by the purple box, with uncertainty indicated by the bars. We have found alternative splice junctions in exon 5 and exon 8 that are not visible at this scale. Abbreviations: B, BamHI; E, EcoRI; X, XhoI.

(B) Structure of the *acj6* gene and the locations of the *acj6* point mutations. The 5′ and 3′ untranslated regions are indicated in gray. Blue indicates the POU-IV box, green the POU-specific domain (Ryan and Rosenfeld, 1997), and yellow the POU homeodomain (Ryan and Rosenfeld, 1997). This figure depicts a splice form that lacks both exon 2 and exon 3. In $acj6^6$, the mutation lies in a splice acceptor site at the 5′ end of exon 6 and effectively shifts the splice position by one nucleotide, thereby causing a frameshift. Specifically, the AG dinucleotide immediately 5′ to the G at position 733 is mutated to an AA; thus, a new AG dinucleotide is created one base downstream. In $acj6^1$, the mutation lies at nucleotide position 1201.

and sequence analysis of the two EMS-induced alleles of *acj6* revealed that they also altered the predicted I-POU gene products (Figure 3B), with $acj6^1$ mutating a highly conserved alanine in the homeodomain, and $acj6^6$ resulting in a frameshift. With the evidence that all five mutations of *acj6* alter the I-POU locus, we concluded that both the olfactory and mobility phenotypes of *acj6* flies stem from disruptions of the I-POU gene and that *acj6* and I-POU are the same gene.

Abnormal Odor Sensitivity in Individual Olfactory Receptor Neurons of *acj6* Mutants

To extend our functional and phenotypic analysis of the *acj6* mutations to the level of the single cell, we used the single-unit recording technique (Huber, 1957; Siddiqi, 1987; Clyne et al., 1997), to record from *Drosophila* olfactory hairs. Single-unit recording is an extracellular measure of the action potentials of the neurons housed in an individual sensillum, or sensory hair. Based upon its amplitude and shape, each action potential from a recording can be assigned to a single neuron in the sensillum under study (Guillet and Bernard, 1972; Fujishiro et al., 1984; Arora et al., 1987; Getz and Akers, 1997). We have used this technique first to identify the various wild-type ORNs based on their odor-induced responses and then to determine whether these neurons are modified in the *acj6* mutants.

We began our single-unit studies with the maxillary palp, one of the two olfactory organs in the adult fly, because of its numerical simplicity. On the surface of the maxillary palp, there are 60 olfactory sensilla, with each sensillum housing just two ORNs (Singh and Nayak, 1985). We have mapped the entire maxillary palp and found that the 120 ORNs can be grouped into six classes of ~20 neurons each, based upon their odor response spectra (Figure 5; an independent, detailed description of the odorant response profiles is provided in M. deB., P. J. C., and J. R. C., submitted). There is also a second level of organization: these six classes of neurons are housed in characteristic pairs in three types of sensilla, which we call types 1, 2, and 3. We call these six classes of neurons 1A, 1B, 2A, 2B, 3A, and 3B. Thus, each 1A neuron, for example, has a particular odor response spectrum, and is always paired with a 1B neuron, which has a distinct spectrum (Figure 4, first and third traces, and Figure 5, "+" column).

We then examined the odor specificities of single ORNs in null mutants of *acj6* to determine if they differed from wild type. The results clearly show that of the two neurons in the type 1 sensillum, only the 1A neuron is altered in *acj6* flies (Figure 4). In wild type, the 1A neuron responds to several of the tested stimuli: it responds most strongly to ethyl acetate and moderately to several others, of which an illustrative subset is shown in Figure 5. The wild-type 1B neuron, by contrast, is narrowly tuned: it responds strongly to 4-methylphenol, but shows no response to the other tested odors. In *acj6*, we found sensilla with neurons that show the same response spectrum as the 1B neuron, i.e., a strong response to 4-methylphenol and nothing else. However, we never found a neuron with a response spectrum like 1A in *acj6* (Figures 4 and 5).

What happens to the 1A neuron in *acj6* mutants? Our recording data suggest that 1A can take on two distinct identities. First, in some *acj6* sensilla two 1B neurons are found, as if the 1A neuron is transformed into a second 1B neuron (Figure 5). Second, in other *acj6* sensilla a neuron that does not respond to any of the tested

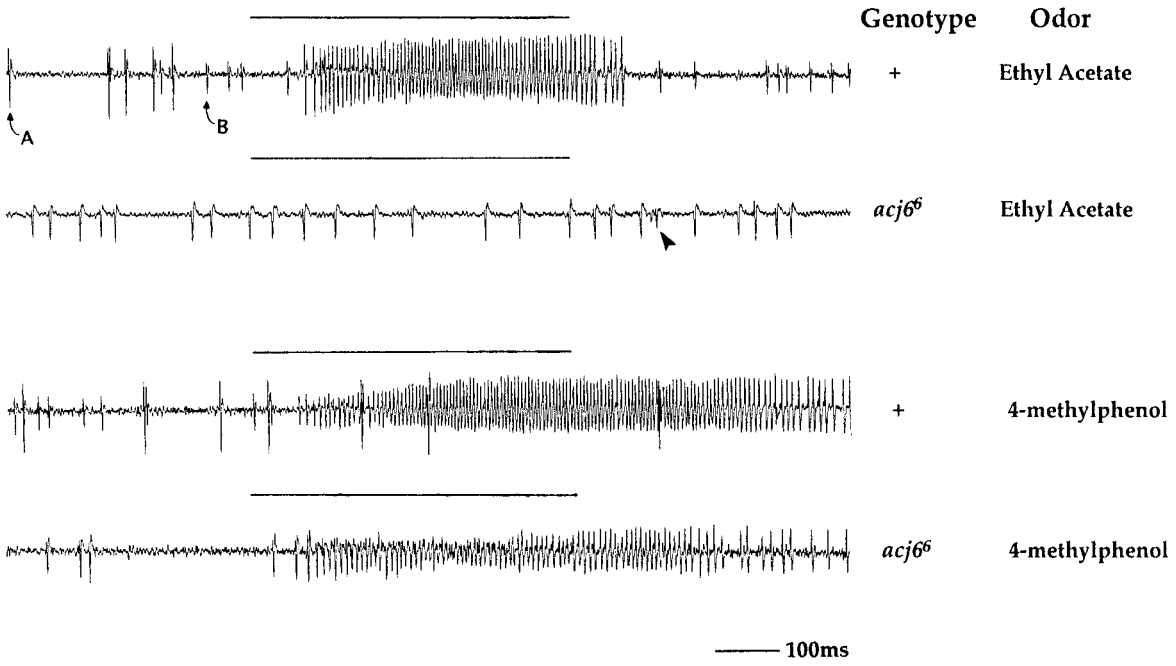

Figure 4. Typical Single-Unit Traces of Recordings from Wild-Type and *acj6*^{6} Type 1 Sensilla

The "+" refers to the wild-type CS-5 genetic background control. The 0.5 s bar above the traces indicates when a valve is open to allow for the carrier gas to pass over the odor. (Based on calculations of flow rates, there is an ~80 ms delay before the odor then reaches the fly.) The top two traces are responses of wild-type and *acj6*^{6} flies to ethyl acetate, and the bottom two traces are responses of wild-type and *acj6*^{6} flies to 4-methylphenol. In the wild-type top trace, the arrows point to action potentials of the "A" neuron and "B" neuron. The 1A neuron, but not the 1B neuron, responds to ethyl acetate. In the *acj6*^{6} ethyl acetate trace, two neurons are present. One is the 1B neuron, which, as in wild type, does not respond to ethyl acetate but does respond to 4-methylphenol (shown below). The other neuron also does not respond to ethyl acetate (or to any other odorant), and its spontaneous firing rate is so low that only a single spike (arrowhead) is observed over the time interval in this recording. The simplest interpretation of the origin of this unresponsive neuron is that it represents a transformation of the 1A neuron. In the wild-type 4-methylphenol trace, the 1B neuron responds to the odor. In the *acj6*^{6} 4-methylphenol trace, the 1B neuron responds normally. (The 1A-derived neuron did not fire over this limited time interval.) Note that the *acj6*^{6} recordings are from type 1 sensilla in which the 1B neuron is not duplicated (see Figure 5). The changing shape of the action potentials during stimulation with odors is commonly observed in insect sensory physiology (Guillet and Bernard, 1972; Fujishiro et al., 1984; Arora et al., 1987; Getz and Akers, 1997).

odors is found alongside a single 1B neuron, as if the 1A neuron is transformed into this unresponsive neuron. Similarly, in the type 3 sensillum, the 3A neuron also appears to become an unresponsive neuron that is housed alongside a normal 3B neuron (Figure 5).

acj6 alters the 2A and 2B neurons in a surprising manner. The wild-type 2A neuron responds weakly to most tested odors, and the wild-type 2B neuron is inhibited by some odors and excited by others (Figure 5, "+" column). We have found no sensilla on *acj6* flies with neurons that have response spectra like either 2A or 2B. Instead, on *acj6* flies, we find sensilla that house a neuron with a novel response spectrum unlike any of the wild-type neuronal classes (Figure 5, *acj6* column): this neuron responds strongly to benzaldehyde, moderately to 4-methylphenol, and weakly to other tested odors. The response spectrum of this new neuron, which we call 2C, cannot be explained in terms of any linear amplification or summation of the response spectra of other neuronal classes. The simplest interpretation is that the *acj6* mutations transform either the 2A or the 2B neuron into the 2C neuron. In recordings from sensilla containing the novel 2C neuron, we find electrophysiological evidence for a second neuron. This neuron produces spontaneous action potentials but does not respond to any of the tested odors (Figure 5).

Thus, in *acj6* null mutants, four of the six classes of maxillary palp ORNs are altered: wild-type 1A and 3A are never observed, and 2A and 2B are transformed into an ORN with a novel response spectrum and a second, unresponsive ORN. 1B and 3B, by contrast, are unaffected by *acj6* mutations; they retain their characteristic odor response profiles. We note finally that the unresponsive neurons in each sensillar type did not respond to any of 50 additional odors tested, nor to mechanical stimuli, nor to changes in temperature or humidity, and that the numbers of each sensillum type were normal in the *acj6*^{6} maxillary palp. Single-unit recordings from *acj6*^{1}, the partial loss-of-function mutant, showed a phenotype intermediate between that of *acj6*^{6} and wild type, in the sense that in *acj6*^{1} those neurons that are affected in *acj6*^{6} were either like those of *acj6*^{6}, normal, or in an intermediate state.

Acj6 Is Expressed Both in the Developing Olfactory System and in Mature Olfactory Receptor Neurons

Our single-unit data suggest that *acj6* functions to specify the developmental fates of a subset of ORNs. We

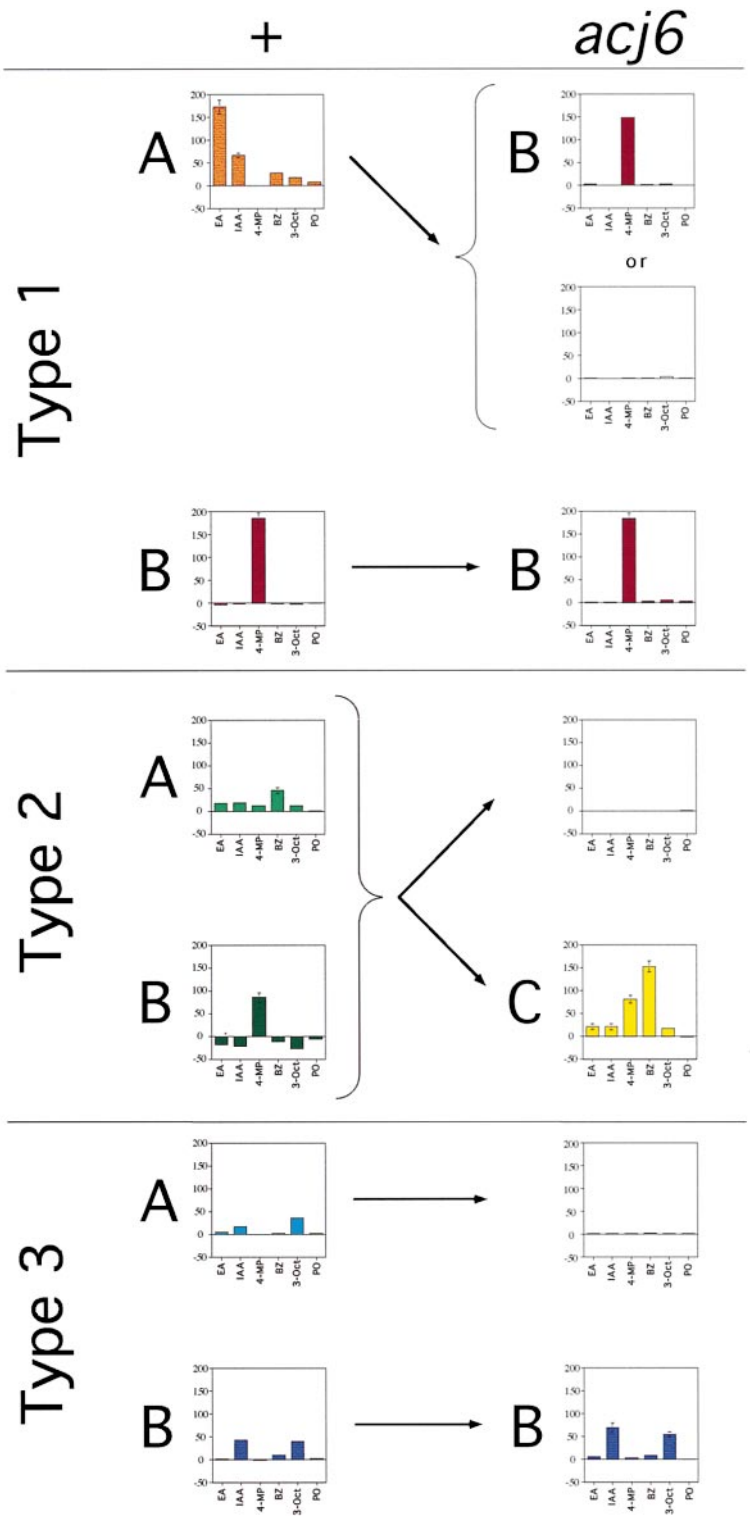

Figure 5. Fate Alterations of Specific ORNs in *acj6*

In the "+" column, each bar graph represents a specific class of neurons in the CS-5 wild type. The bar graphs in the *acj6* column indicate the classes of neurons observed in *acj6*. Arrows indicate the simplest interpretations of their origins. The graphs show the average change in action potential frequency in action potentials/s in response to a set of six odors in "+" and *acj6* sensilla. The x axis indicates the tested odors and the y axis indicates the change in the number of action potentials/s following initiation of the stimulus. n = 10–14. Error bars are too small to be seen in some cases. Abbreviations: EA, ethyl acetate; IAA, isoamyl acetate; 4-MP, 4-methylphenol; BZ, benzaldehyde; 3-Oct, 3-Octanol; and PO, paraffin oil (used as a diluent). The duplicated 1B neuron was found in 56% of 32 type 1 sensilla examined in *acj6*^{6}. The hallmarks of sensilla with duplicated 1B neurons are: action potentials all of the same amplitude but at double the frequency, and the occasional occurrence of two nearly coincident spikes with overlapping waveforms yielding an increased amplitude, reflecting the nearly simultaneous firing of two neurons. Bar graphs without odor responses in the *acj6* column indicate unresponsive neurons. In all three sensillar types, the unresponsive neurons often had a reduced frequency of spontaneous action potentials, and in some cases, spontaneous action potentials were absent altogether. The classes of neurons described in this figure were consistently found in >200 additional *acj6*^{6} sensilla from which recordings were made. On a single *acj6*^{6} fly, both a type 1 sensillum with a duplicated 1B neuron as well as a type 1 sensillum with an unresponsive neuron could be found. Results similar to those shown in the figure were also obtained from another allele, *acj6*^{2}, presumed to be a null allele (n = 5 recordings for each sensillum type, for all test odors).

therefore used immunohistochemical techniques to examine Acj6 expression patterns in developing and adult olfactory tissue. We used a monoclonal antibody generated against a subregion of the Acj6 protein that is common to all isoforms. In the developing third antennal segment, the olfactory organ whose development has been best characterized, Acj6 is first detectable in a few cells at ~16 hr after puparium formation (APF) (Figure 6a). Interestingly, ~16 hr APF is also when the earliest differentiating ORNs are found in the third antennal seg-

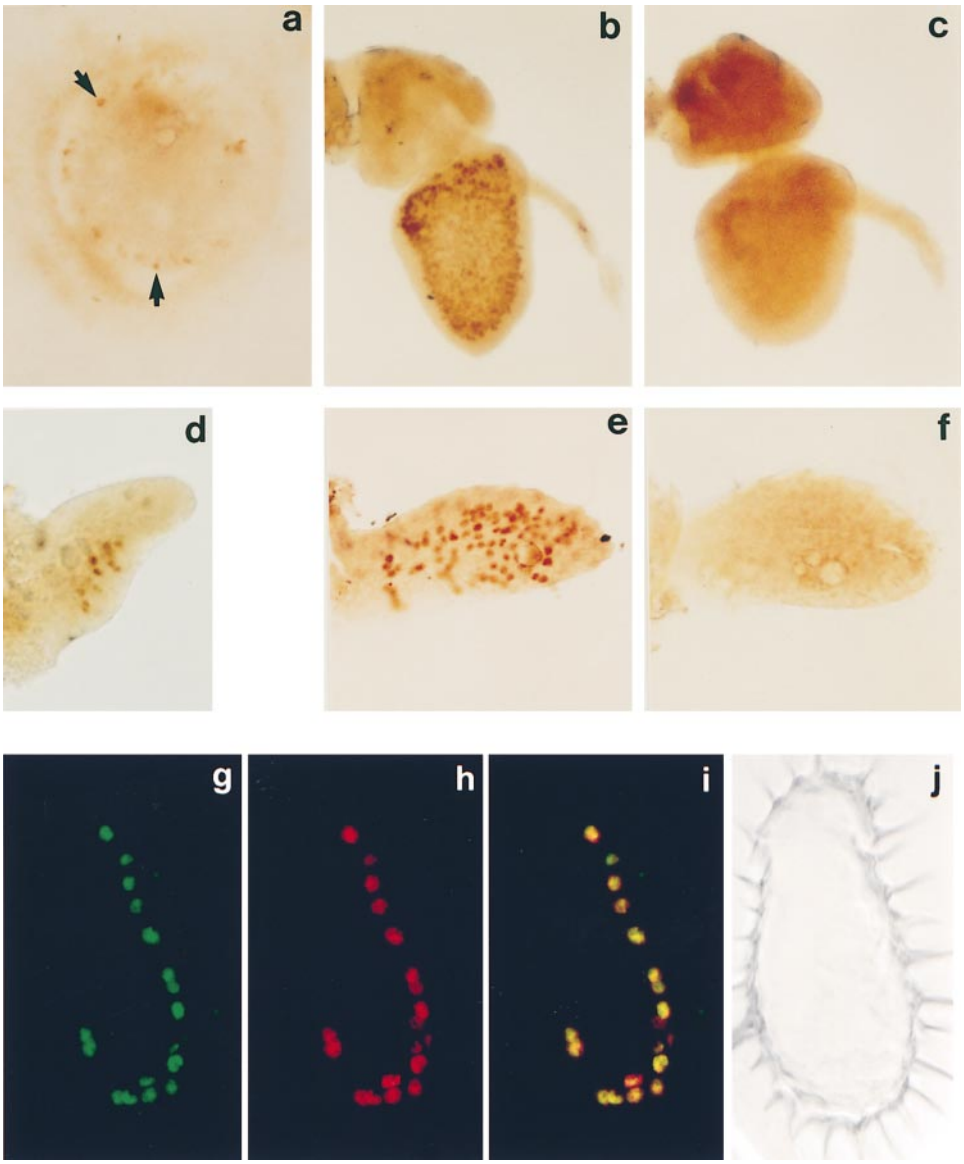

Figure 6. Immunolocalization of Acj6 in Olfactory Organs

(a) Acj6 expression in a wild-type antennal disc 16 hr APF, revealed by staining with an anti-Acj6 monoclonal antibody. Arrows indicate two of the Acj6-expressing cells, which lie on a ring of cells that form the presumptive third antennal segment.
(b) A wild-type antenna 36 hr APF. There is staining throughout the third antennal segment and none in the second antennal segment.
(c) An *acj6*^{6} antenna 36 hr APF labeled with the Acj6 monoclonal antibody. There is no detectable Acj6 protein in the mutant antenna.
(d) Acj6 expression is initiated in a small number of cells in a maxillary palp 31 hr APF. A few cells show staining.
(e) A wild-type maxillary palp 48 hr APF. Staining is found broadly over the maxillary palp.
(f) An *acj6*^{6} maxillary palp 48 hr APF labeled with the Acj6 monoclonal antibody. There is no staining.
(g–i) Confocal images of an adult maxillary palp cross section double labeled with anti-Acj6 and anti-Elav.
(g) Anti-Acj6.
(h) Anti-Elav.
(i) Superposition of the the two labels. Acj6 is expressed in each olfactory neuron.
(j) Bright-field image of the section. All the staining cells are in the portion of the maxillary palp in which the olfactory neurons are found (Singh and Nayak, 1985).

ment (Lienhard and Stocker, 1991; Ray and Rodrigues, 1995; Reddy et al., 1997). The number of Acj6-expressing neurons increases with developmental time and then stabilizes at ~36 hr APF, when Acj6 is expressed throughout the third antennal segment (Figure 6b). Expression of Acj6 is absent in the third antennal segment of *acj6*^{6} mutants (Figure 6c). Using RT–PCR and immunohistochemistry, we have found that all identified isoforms of *acj6* are expressed in the antenna throughout pupal development and in the adult (data not shown).

In the developing maxillary palp, Acj6 is first expressed in a few cells at ~31 hr APF (Figure 6d). By 48

hr APF, there are 84 ± 1 (n = 8) Acj6-expressing cells (Figure 6e). Expression of Acj6 is absent in developing maxillary palps of *acj6*^{6} mutants (Figure 6f). RT–PCR indicates that only a subset of the *acj6* isoforms are present in the adult maxillary palp (data not shown). To confirm that Acj6-expressing cells are indeed olfactory neurons, we labeled adult maxillary palp cross sections with both the neuron-specific anti-Elav antibody (O'Neill et al., 1994) and the anti-Acj6 monoclonal antibody and found that the labels colocalized (Figures 6g–6j). These studies revealed that Acj6 is expressed in all maxillary palp ORNs.

Discussion

We have shown that *acj6* is the structural gene for a POU-domain transcription factor and that its mutations cause abnormalities in the odor sensitivity of individual olfactory receptor neurons. Null mutations of *acj6* have different effects on different classes of neurons: some mutant cells exhibit a novel odor specificity, some lose all odor sensitivity, and some are unaffected. Interestingly, *acj6* is expressed in all classes of olfactory neurons in the maxillary palp, as shown by immunohistochemistry.

If *acj6* is expressed in all olfactory neurons of the maxillary palp, why does an *acj6* mutation affect only four of the six neuronal classes? Among other possibilities, *acj6* may be analogous to *sevenless*, in the sense that *sevenless* is expressed in all photoreceptor neurons but is functionally required in only a subset (Banerjee et al., 1987). *acj6* might act only in certain cells that contain a necessary cofactor; alternatively, it could act in all neuronal classes but be functionally redundant with other genes in a subset of these neurons, such that a mutant phenotype is observed in some neurons but not others.

If *acj6* is not required for specification of all neuronal types in the maxillary palp, are there counterparts of *acj6* that specify the other types? One attractive possibility is that other POU-domain genes are required for specifying other subsets of olfactory neurons. Of the four *Drosophila* POU-domain genes, we have found that at least two others, *drifter* and *pdm-1*, in addition to *acj6*, are also expressed in adult olfactory organs (unpublished data). It seems plausible, then, that multiple POU-domain proteins function in concert, perhaps combinatorially, in the specification of all types of olfactory neurons. POU-domain proteins are able to function as heterodimers with other POU-domain proteins (Voss et al., 1991), further enriching the possibilities for combinatorial coding of neuronal type.

Another degree of freedom is afforded by alternative splicing of *acj6*. We have found evidence for at least seven alternative splice forms of *acj6*, of which a subset is expressed in the adult maxillary palp. An intriguing possibility is that different isoforms of *acj6* specify different ORN classes. It is also possible that different isoforms are required at different steps in the specification of ORNs. We have found that in *acj6* null mutants, some neurons do not respond to any tested odors whatsoever (e.g., the mutant 3A cell), whereas others respond to an abnormal set of odors (e.g., the mutant 2C cell), as if different neuronal classes are disrupted at different steps in the ORN differentiation pathway. Perhaps *acj6* functions at different steps in different ORNs, establishing a cellular context capable of odorant response in one ORN class, while selecting the particular type of odorant response in another class. If so, different functions of Acj6 might be carried out by different splice forms.

Our finding that *acj6*, and perhaps other POU-domain genes, function in the determination of olfactory neuron identity is of interest in the broader context of sensory system development. Other class IV POU-domain genes have previously been implicated in the differentiation of primary neurons in auditory (Erkman et al., 1996; Vahava et al., 1998), visual (Erkman et al., 1996; Gan et al., 1996), and mechanosensory (Finney et al., 1988) systems. Our study of *acj6* now extends the functions of POU-domain genes to include a role in the olfactory system.

A regulatory hierarchy is believed to specify the odor sensitivity of each class of olfactory receptor neuron (Chess et al., 1994; Sengupta et al., 1994), and this hierarchy doubtless requires the function of a variety of transcription factors other than POU-domain proteins. For example, a zinc finger protein and helix-loop-helix proteins are likely to act in the development of all ORNs (Tsai and Reed, 1997; Wang et al., 1997). A member of the nuclear receptor superfamily in *C. elegans*, *odr-7*, functions in a single ORN class (Sengupta et al., 1994). Null mutants of *odr-7* fail to respond to all odorants detected by this ORN class. *acj6*, which functions in a subset of ORN classes, is particularly striking in that its null mutations alter odor specificity of certain ORN classes. These data suggest that in these cells Acj6 acts near the terminal level in the regulatory hierarchy that determines odorant specificity.

How might Acj6 act in controlling the identity of receptor neurons? Most interesting, how might loss of *acj6* function alter odor specificity, as in the transformation of a 2A or 2B neuron into the novel 2C neuron? It seems likely that Acj6 regulates the expression of certain downstream genes essential to establishing ORN identity, and a simple interpretation of how Acj6 determines odor specificity is that Acj6 regulates odorant receptor genes. We note that our results argue against a simple model in which an ORN expresses a single odorant receptor gene, with that receptor gene being the only one regulated by Acj6 in the cell: were this the case, then the loss of Acj6 in the 2A and 2B cells would not be expected to yield a neuron with a strong odorant response like that of 2C.

In conclusion, we have used a novel behavioral paradigm to isolate new mutants of *acj6*, characterized them genetically, and determined that they contain molecular lesions in a gene encoding a POU-domain transcription factor. Through single-unit electrophysiology, we have found that the mutants contain alterations in the odor sensitivity of individual olfactory neurons. Thus, we have found evidence that the *acj6* transcription factor plays a key role in the logic by which the diverse identities of olfactory neurons are generated, which is critical to the process of olfactory coding. Most interestingly, the results suggest that *acj6* may regulate the expression of

a subset of odorant receptor genes, a suggestion addressed in the accompanying article (Clyne et al., 1999).

Experimental Procedures

Genetics

Cytogenetic mapping of *acj6* was carried out with the following chromosomes: *Dp(1;2)eag*^{x6}, *Dp(1;2)ras*^{v}, *Dp(1;f)LJ9*, *Dp(1;4)r*$^{+}$*f*$^{+}$, *Df(1)M34-1Cs*, *Df(1)M32-13C*, *Df(1)M10-A14*, *Df(1)13AC*, *Df(1)RK5*, *Df(1)RK4*, *Df(1)RK3*, *Df(1)RK2*, *Df(1)sd*^{72b}, and *T(1;Y)S29*. The position of the *acj6* locus was defined by the proximal breakpoint of *Df(1)13AC*, 13C2–3, and by the position of the *P(AS438)* insertion, at 13C1–2, to which *acj6* maps proximally. The inferred map position of *acj6*, 13C1–3, was consistent with the mapping results obtained with all 14 deficiencies and duplications.

Molecular Biology

The sequence of the *acj6* transcript in both EMS-induced mutants and wild type was determined by RT–PCR (Innis et al., 1990) using whole adult RNA as a template. The sequence of wild-type *acj6* was also independently analyzed by RT–PCR from embryonic poly(A)$^{+}$ RNA. The 5′ end of the gene was determined by 5′ RACE (Innis et al., 1990) using adult RNA as a template and by sequence analysis of an embryonic cDNA. The intron–exon boundaries of *acj6* were confirmed by sequencing genomic DNA. Sequencing was determined from both strands in all cases. The locations of the exons on the genomic map were determined by standard molecular techniques (Maniatis et al., 1982). The locations of the breakpoints of the X-ray-induced alleles of *acj6* were determined by analysis of genomic Southern blots.

Electrophysiology

Receptor potential recording techniques were performed as described previously (Ayer and Carlson, 1991, 1992). We have described the single-unit recording technique in detail elsewhere (Clyne et al., 1997; and M. deB., P. J. C., and J. R. C., submitted). Briefly, electrolytically sharpened tungsten wire electrodes were placed in the lumen of the sensillum under study using a piezo-driven micromanipulator. Once a stable contact was made and spontaneous action potentials were detectable, ultra-clean air into which pulses of odor were delivered was passed over the fly. The odor-induced responses of the neurons in a sensillum were analyzed offline using Autospike software (Syntech, Hilversum, the Netherlands). Odors were diluted 10^{-2} in paraffin oil. We do not know how many molecules evaporated from the odorant solution during the delivery process or the number of molecules that entered the lumen of the olfactory sensilla. All chemicals were from Aldrich and were of the highest purity available. In the figures, error bars indicate SEM.

Immunohistochemistry

Glutathione S-transferase–Acj6 (GST–Acj6) fusion protein was produced containing amino acids 108–237 of the Acj6 protein fused to the carboxy-terminal end of the GST polypeptide. This region does not include either the POU IV box or the POU domain, and antibodies generated against these amino acids should recognize all known isoforms of Acj6. The GST–Acj6 fusion protein was expressed and purified using glutathione–agarose according to protocols provided by the manufacturer. Monoclonal antibodies were generated by the University of Iowa College of Medicine Hybridoma Facility (Iowa City, IA).

Staining of developing antennae and maxillary palps was performed as described elsewhere (Ray and Rodrigues, 1995), except that a treatment of the tissues with 0.3% H_2O_2 in 1× PBS for 10 min was added after the fixation step. Cross sections of adult maxillary palps were prepared by fixing adult heads in 4% paraformaldehyde on ice for 2 hr followed by incubation overnight in 25% sucrose in Ringer's solution at 4°C. After embedding in OCT Embedding Compounds (Sakura Fineteck), and freezing on dry ice, 8 μm serial frontal sections were collected on polylysine-coated slides and processed as described previously (Raha and Carlson, 1994). Anti-Acj6 Mab 9C52 was used at a 1:3 dilution and rat anti-Elav 7E8A10 (O'Neill et al., 1994) at a 1:10 dilution (obtained from the Developmental Studies Hybridoma Bank, Iowa City, IA). Affinity-isolated fluorescein-conjugated goat anti-mouse (Biosource International) and rhodamine-conjugated rabbit ant-rat (Chemicon) secondary antibodies were used at a 1:200 dilution.

Acknowledgments

We thank E. Vallen, J. Paxson-Jankovsky, and M. Grams for assistance with the genomic walk; M. Grams and J. Ratchford for assistance with the mutageneses; C. Horak for assistance with RT–PCR; S. Thor and K. J. Coker for antibody purification advice; and A. Chinn, K. Certel, and members of the Carlson and Johnson labs for support and helpful discussions. This work was supported by an NSF predoctoral fellowship grant to P. J. C., an NIH Genetics predoctoral training program grant to S. C., an HFSP Fellowship to M. deB, and an NIH grant (DC-02174) to J. R. C.

Received January 19, 1999; revised January 28, 1999.

References

Anderson, M., Perkins, G., Chittick, P., Shrigley, R., and Johnson, W. (1995). drifter, a Drosophila POU-domain transcription factor, is required for correct differentiation and migration of tracheal cells and midline glia. Genes Dev. *9*, 123–137.

Arora, K., Rodrigues, V., Joshi, S., Shanbhag, S., and Siddiqi, O. (1987). A gene affecting the specificity of the chemosensory neurons of *Drosophila*. Nature *330*, 62–63.

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

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

Banerjee, U., Renfranz, P.J., Hinton, D.R., Rabin, B.A., and Benzer, S. (1987). The sevenless^{+} protein is expressed apically in cell membranes of developing Drosophila retina; it is not restricted to cell R7. Cell *51*, 151–158.

Billin, A., Cockerill, K., and Poole, S. (1991). Isolation of a family of Drosophila POU domain genes expressed in early development. Mech. Dev. *34*, 75–84.

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

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

Chalfie, M., Horvitz, H., and Sulston, J. (1981). Mutations that lead to reiterations in the cell lineages of C. elegans. Cell *24*, 59–69.

Chess, A., Simon, I., Cedar, H., and Axel, R. (1994). Allelic inactivation regulates olfactory receptor gene expression. Cell *78*, 823–834.

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

Clyne, P.J., Warr, C.G., Freeman, M.R., Lessing, D., Kim, J., and Carlson, J.R. (1999). A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in *Drosophila*. Neuron *22*, this issue, 327–338.

De Cicco, D., and Spradling, A. (1984). Localization of a *cis*-acting element responsible for the developmentally regulated amplification of Drosophila chorion genes. Cell *38*, 45–54.

Dick, T., Yang, X., Yeo, S., and Chia, W. (1991). Two closely linked Drosophila POU domain genes are expressed in neuroblasts and sensory elements. Proc. Natl. Acad. Sci. USA *88*, 7645–7649.

Erkman, L., McEvilly, R., Luo, L., Ryan, A., Hooshmand, F., O'Connell, S., Keithley, E., Rapaport, D., Ryan, A., and Rosenfeld, M. (1996). Role of transcription factors Brn-3.1 and Brn-3.2 in auditory and visual system development. Nature *381*, 603–606.

Finney, M., and Ruvkun, G. (1990). The unc-86 gene product couples cell lineage and cell identity in C. elegans. Cell *63*, 895–905.

Finney, M., Ruvkun, G., and Horvitz, H. (1988). The C. elegans cell lineage and differentiation gene unc-86 encodes a protein with a homeodomain and extended similarity to transcription factors. Cell *55*, 757–769.

Fujishiro, N., Kijima, H., and Morita, H. (1984). Impulse frequency and action potential amplitude in labelar chemosensory neurons of *Drosophila melanogaster*. J. Insect Physiol. *30*, 317–325.

Gan, L., Xiang, M., Zhou, L., Wagner, D., Klein, W., and Nathans, J. (1996). POU domain factor Brn-3b is required for the development of a large set of retinal ganglion cells. Proc. Natl. Acad. Sci. USA *93*, 3920–3925.

Getz, W.M., and Akers, R.P. (1997). Response of American cockroach (*Periplaneta americana*) olfactory receptors to selected alcohol odorants and their binary combinations. J. Comp. Physiol. [A] *180*, 701–709.

Guillet, J.C., and Bernard, J. (1972). Shape and amplitude of the spikes induced by natural or electrical stimulation in insect receptors. J. Insect Physiol. *18*, 2155–2171.

Helfand, S., and Carlson, J. (1989). Isolation and characterization of an olfactory mutant in *Drosophila* with a chemically specific defect. Proc. Natl. Acad. Sci. USA *86*, 2908–2912.

Herr, W., and Cleary, M. (1995). The POU domain: versatility in transcriptional regulation by a flexible two-in-one DNA-binding domain. Genes Dev. *9*, 1679–1693.

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

Huber, D.H. (1957). Tungsten microelectrode for recording from single units. Science *125*, 549–550.

Innis, M., Gelfand, D., Sninsky, J., and White, T. (1990). PCR Protocols: A Guide to Methods and Applications (San Diego, CA: Academic Press).

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

Lloyd, A., and Sakonju, S. (1991). Characterization of two Drosophila POU domain genes, related to oct-1 and oct-2, and the regulation of their expression patterns. Mech. Dev. *36*, 87–102.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

McKenna, M., Monte, P., Helfand, S., Woodard, C., and Carlson, J. (1989). A simple chemosensory response in *Drosophila* and the isolation of *acj* mutants in which it is affected. Proc. Natl. Acad. Sci. USA *86*, 8118–8122.

O'Neill, E.M., Rebay, I., Tjian, R., and Rubin, G. (1994). The activities of two ETS-related transcription factors required for Drosophila eye development are modulated by the Ras/MAPK pathway. Cell *78*, 137–147.

Poole, S. (1995). Conservation of complex expression domains of the pdm-2 POU domain gene between Drosophila virilis and Drosophila melanogaster. Mech. Dev. *49*, 107–116.

Raha, D., and Carlson, J. (1994). OS9: a novel olfactory gene of *Drosophila* expressed in two olfactory organs. J. Neurobiol. *25*, 169–184.

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

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

Ruvkun, G., and Finney, M. (1991). Regulation of transcription and cell identity by POU domain proteins. Cell *64*, 475–478.

Ryan, A.K., and Rosenfeld, M.G. (1997). POU domain family values: flexibility, partnerships, and development codes. Genes Dev. *11*, 1207–1225.

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

Shepherd, G. (1994). Discrimination of molecular signals by the olfactory receptor neuron. Neuron *13*, 771–790.

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

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

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

Theil, T., McLean-Hunter, S., Zornig, M., and Moroy, T. (1993). Mouse Brn-3 family of POU transcription factors: a new amino terminal domain is crucial for the oncogenic activity of Brn-3a. Nucleic Acids Res. *21*, 5921–5929.

Treacy, M., He, X., and Rosenfeld, M. (1991). I-POU: a POU-domain protein that inhibits neuron-specific gene activation. Nature *350*, 577–584.

Treacy, M., Neilson, L., Turner, E., He, X., and Rosenfeld, M. (1992). Twin of I-POU: a two amino acid difference in the I-POU homeodomain distinguishes an activator from an inhibitor of transcription. Cell *68*, 491–505.

Tsai, R., and Reed, R. (1997). Cloning and functional characterization of Roaz, a zinc-finger protein that interacts with O/E-1 to regulate gene expression. J. Neurosci. *17*, 4159–4169.

Vahava, O., Morell, R., Lynch, E., Weiss, S., Kagan, M.E., Ahituv, N., Morrow, J.E., Lee, M.K., Skvorak, A.B., Morton, C., et al. (1998). Mutation in transcription factor *POU4F3* associated with inherited progressive hearing loss in humans. Science *279*, 1950–1954.

Voss, J.W., Wilson, L., and Rosenfeld, M.G. (1991). POU-domain proteins Pit-1 and Oct-1 interact to form a heteromeric complex and can cooperate to induce expression of the prolactin promoter. Genes Dev. *5*, 1309–1320.

Wang, S.S., Tsai, R.Y.L., and Reed, R.R. (1997). The characterization of the Olf-1/EBF-like HLH transcription factor family: implications in olfactory gene regulation and neuronal development. J. Neurosci. *17*, 4149–4158.

Xiang, M., Zhou, L., Macke, J., Yoshioka, T., Hendry, S., Eddy, R., Shows, T., and Nathans, J. (1995). The Brn-3 family of POU-domain factors: primary structure, binding specificity, and expression in subsets of retinal ganglion cells and somatosensory neurons. J. Neurosci. *15*, 4762–4785.

Xiang, M., Zhou, H., and Nathans, J. (1996). Molecular biology of retinal ganglion cells. Proc. Natl. Acad. Sci. USA *93*, 596–601.

Yang, X., Yeo, S., Dick, T., and Chia, W. (1993). The role of a Drosophila POU homeo domain gene in the specification of neural precursor cell identity in the developing embryonic central nervous system. Genes Dev. *7*, 504–516.

GenBank Accession Number

The *acj6* sequence is deposited in the GenBank database under accession number AF086816.