Two Neuronal G Proteins are Involved in Chemosensation of the *Caenorhabditis elegans* Dauer-Inducing Pheromone

Richard R. Zwaal,[1,2] Jane E. Mendel,*[,2] Paul W. Sternberg* and Ronald H. A. Plasterk

*The Netherlands Cancer Institute, Division of Molecular Biology, Amsterdam, The Netherlands and *California Institute of Technology, Howard Hughes Medical Institute, Division of Biology, Pasadena, California 91125*

Manuscript received August 8, 1996
Accepted for publication December 2, 1996

ABSTRACT

Caenorhabditis elegans uses chemosensation to determine its course of development. Young larvae can arrest as dauer larvae in response to increasing population density, which they measure by a nematode-excreted pheromone, and decreasing food supply. Dauer larvae can resume development in response to a decrease in pheromone and increase in food concentration. We show here that two novel G protein alpha subunits (GPA-2 and GPA-3) show promoter activity in subsets of chemosensory neurons and are involved in the decision to form dauer larvae primarily through the response to dauer pheromone. Dominant activating mutations in these G proteins result in constitutive, pheromone-independent dauer formation, whereas inactivation results in reduced sensitivity to pheromone, and, under certain conditions, an alteration in the response to food. Interactions between *gpa-2*, *gpa-3* and other genes controlling dauer formation suggest that these G proteins may act in parallel to regulate the neuronal decision making that precedes dauer formation.

HETEROTRIMERIC guanine nucleotide-binding proteins (G proteins) act as molecular switches in the transduction of signals, functioning between seven transmembrane receptors and intracellular second messenger pathways. G proteins are involved in many cellular functions including the reception of sensory stimuli such as light, tastes, and odors (*e.g.*, STRYER 1986; BUCK and AXEL 1991; MARGOLSKEE 1993). Although the biochemical pathways for certain sensory transductions have been described, the ways in which multiple sensory pathways are integrated and regulated have yet to be understood. Such understanding can be enhanced through genetic studies in a relatively simple organism. In this paper we describe two G proteins that are involved in responding to environmental signals that regulate the formation of dauer larvae in *Caenorhabditis elegans*.

Under conditions that favor growth, *C. elegans* develops through four larval stages to become a reproductive adult. Formation of the dauer stage, an alternative third larval stage, is induced under circumstances of high population density and insufficient food (for review, see RIDDLE 1988; THOMAS 1993). The dauer larva, specialized to survive harsh environmental conditions (CASSADA and RUSSELL 1975), is arrested in development as well as aging, does not feed, and has an altered energy metabolism. Dauer larvae can be distinguished from other larvae by both morphology and behavior (CASSADA and RUSSELL 1975; RIDDLE 1988).

The presence of a dauer-inducing pheromone is both necessary and sufficient for initiation of dauer development. The pheromone, a mixture of closely related, hydroxylated, short-chain fatty-acid-like compounds, is continuously excreted by the nematodes (GOLDEN and RIDDLE 1982, 1984b) and thus can serve as a measure of population density. Other environmental cues attenuate the efficiency of pheromone-induced dauer larva formation. A food signal favors growth and suppresses dauer formation and thus counteracts the effect of pheromone. Also, animals are more prone to forming dauer larvae at higher temperature (GOLDEN and RIDDLE 1984a,b). Dauer formation therefore represents the end result of the integration of multiple chemosensory pathways as well as thermosensation, and the ability of an animal to undergo dauer formation can be used as a means of scoring its ability to sense the different types of signals involved.

The signals for dauer formation are recognized by and transduced through the ciliated chemosensory neurons of the amphids, which are also involved in recognition of soluble attractants and repellents, and of regions of high osmotic strength (WARD 1973; CULOTTI and RUSSELL 1978). Mutants in which these neurons do not contact the environment are not capable of forming dauer larvae because they are not able to transduce the proper inducing signals (LEWIS and HODGKIN 1977; ALBERT *et al.* 1981; PERKINS *et al.* 1986). The individual amphid neurons ADF and ASI produce a signal that represses dauer formation under growth promoting

Corresponding author: Ronald H. A. Plasterk, Division of Molecular Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands. E-mail: rplas@nki.nl

[1] *Present address:* University of Texas Southwestern Medical Center, Department of Biochemistry, 5323 Harry Hines Blvd., Dallas, TX 75235-9038.

[2] These authors contributed equally to this work.

conditions (BARGMANN and HORVITZ 1991b). Presence of pheromone could repress this signal, derepressing the dauer pathway.

Many genes involved in the induction of dauer formation have been identified, with mutants falling into two classes: those that form dauer larvae even in the absence of inducing conditions (Daf-c for dauer-formation constitutive) and those that fail to form dauer larvae in response to dauer pheromone (Daf-d for dauer-formation defective) (RIDDLE *et al.* 1981; SWANSON and RIDDLE 1981). The properties of double mutant phenotypes are consistent with either a linear (RIDDLE *et al.* 1981; VOWELS and THOMAS 1992) or a parallel (THOMAS *et al.* 1993) pathway of signal processing. One branch of the pathway has been shown to involve homologs of TGF-β regulated signaling proteins (GEORGI *et al.* 1990; ESTEVEZ *et al.* 1993).

The dauer stage is completely reversible; animals recover and resume development when circumstances become beneficial (CASSADA and RUSSELL 1975; KLASS and HIRSH 1976). The same environmental cues that control dauer formation also influence the recovery process: pheromone inhibits recovery, whereas food and low temperature promote recovery (GOLDEN and RIDDLE 1982, 1984a,b). Recovery from the dauer stage is mediated, at least in part, by the ciliated amphid neuron ASJ (BARGMANN and HORVITZ 1991b). The cilium-structure mutants that block entry into the dauer stage also prevent recovery of Daf-c dauer larvae, presumably by blocking the nematode's ability to sense the food signal (ALBERT *et al.* 1981).

We provide evidence that the entry of young larvae into and the recovery of dauer larvae from the dauer stage is under G-protein control. Two G protein α subunits, encoded by *gpa-2* and *gpa-3* (FINO SILVA and PLASTERK 1990; LOCHRIE *et al.* 1991), are necessary for full response to dauer pheromone. Activated forms of either of these genes result in a Daf-c phenotype. Both genes are apparently expressed in chemosensory and possibly interneurons, and both interact genetically with previously identified mutations known to affect dauer formation. Since, under strong inducing conditions (less food or more pheromone), *gpa-2* and *gpa-3* loss-of-function mutants form dauer larvae up to wild-type levels, we conclude that GPA-2 and GPA-3 are involved in sensation or integration of signals regulating dauer formation, thus altering the balanced decision to initiate dauer differentiation.

MATERIALS AND METHODS

Strains: Nematodes were cultured as described by WOOD (1988). *syls* refers to integrated transgenes. Strains used in this work are Bristol N2, CB1362 (*dpy-20(e1362)* IV), PS2105 (*dpy-20(e1362)*; *syls13*[*pJMG2QL,pMH86*] IV), PS2022 (*dpy-20(e1362)*; *syls30*[*pJMG2QL,pMH86*] X), PS1922 (*dpy-20(e1362)*; *syls24*[*pJMG3QL,pMH86*] IV), PS1926 (*dpy-20(e1362)*; *syls25*[*pJMG3QL,pMH86*] X), MT3126 (*mut-2(r459)* I), CB1489 (*him-8(e1489)* IV), PS1455 (*dpy-20(e1362)*; *him-5(e1490)*; *syls7*[*pJMG2BV.28,pMH86*]), PS1939 (*dpy-20(e1362)*; *him-5(e1490)*; *syls28*[*pJMG3BP.11,pMH86*]), DR47 (*daf-11(m47)* V), DR183 (*dpy-11(e224) daf-11(m47)* V), JT6130 (*daf-21(p673)* V), DR40 (*daf-1(m40)* IV), CB1393 (*daf-8(e1393)* I), CB1124 (*che-3(e1124)* I), CB1386 (*daf-5(e1386)* II), NL334 (*gpa-2(pk16)* V), NL335 (*gpa-3(pk35)* V), NL348 (*gpa-2(pk16) gpa-3(pk35)* V). Strains are described by BRENNER (1974), CULOTTI and RUSSELL (1978), HODGKIN *et al.* (1979), HODGKIN (1985), THOMAS *et al.* (1993), ZWAAL *et al.* (1993), and this work.

Activated constructs: The plasmid pJMG2, derived from cosmid PS#01C2 (LOCHRIE *et al.* 1991), has an 8.0-kb insert in pBSK$^+$ (Stratagene) containing the *gpa-2* coding sequence (FINO SILVA and PLASTERK 1990) flanked by 2.3 kb of DNA at the 5′ end and 2.6 kb at the 3′ end. pJMG2 was used as a substrate for mutagenesis in vitro (MutaGene phagemid kit, Bio-Rad) using the primer 5′-TATCTCCGACCGGAGACCTCCTACGTC-3′, corresponding to nucleotides 2000–2027 in *gpa-2*. The mutation was confirmed by limited sequencing of the resulting plasmid, pJMG2QL. The plasmid pJMG3, derived from cosmid PS#14A3 (LOCHRIE *et al.* 1991), has a 10.0-kb insert in pBSK$^+$ containing the *gpa-3* coding sequence flanked by 5.9 kb of DNA at the 5′ end and 2.4 kb at the 3′ end. pJMG3 was used as a substrate for mutagenesis *in vitro* using the primer 5′-TCTCTCTGATCGAAGACCTCCAACGTC-3′, corresponding to nucleotides 2205–2031 in *gpa-3*. The mutation was confirmed by limited sequencing of the resulting plasmid, pJMG3QL.

Generation of transgenic strains: Transgenic *C. elegans* were generated by injection of DNA into adult hermaphrodites (MELLO *et al.* 1991). Test DNA was injected into *dpy-20(e1362)* along with pMH86, a plasmid containing wild-type *dpy-20* DNA (HAN and STERNBERG 1991). In all cases, similar phenotypes were observed for multiple stable transgenic lines. Transgenic arrays were chromosomally integrated as described by MENDEL *et al.* (1995). Chromosomally integrated transgenic strains were outcrossed three times to *dpy-20(e1282)*; *him-5(e1490)*, and the transgene reisolated in a *dpy-20(e1362)* background. Chromosomal integration of transgenic arrays stabilized but did not alter the observed phenotypes. For each transgene, two independent chromosome insertions were analyzed to distinguish transgene-mediated from random background effects. To establish *syls13* and *syls30*, pJMG2QL was injected at 200 ng/μl along with pMH86 at 10 ng/μl. To establish *syls24* and *syls25*, pJMG3QL was injected at 200 ng/μl along with pMH86 at 10 ng/μl. To establish *syls7*, pJMG2BV.28 (see below) was injected at 50 ng/μl along with pMH86 at 10 ng/μl and pBSK$^+$ at 50 ng/μl. To establish *syls28*, pJMG3BP.11 (see below) was injected at 50 ng/μl along with pMH86 at 10 ng/μl and pBSK$^+$ at 50 ng/μl.

Deletion mutants: The isolation of Tc1 insertion mutants and deletion derivatives has been described previously (ZWAAL *et al.* 1993). Primers used to identify a Tc1 insertion in *gpa-2* were 2384 5′-CTAATTCTCGTTATCGCGAAGC-3′ and 3357 5′-AAGTCGTGCAATTGATAAGGAG-3′ at the 5′ end of *gpa-2* and left 1 and left 2 (ZWAAL *et al.* 1993) in Tc1. Deletions were scored using 2384 and 3357 at the 5′ end and 5623 5′-GTGCTAGTTTCAATCCAAGATC-3′ and 3550 5′-GCAACTGCTCGAAACCTCCGCAAC-3′ at the 3′ end. Primers used to identify a Tc1 insertion in *gpa-3* were 1511 5′-TATGGGATTATGCCAATCTGC-3′ and 2519 5′-TGCAGAACCATATGTCACAGC-3′ at the 5′ end of *gpa-3* and left 1 and left 2 in Tc1. Deletions were scored with 1511 and 2519 at the 5′ end and 1512 5′-ACCGCATCCTTGCAAGTTGGC-3′ and 1870 5′-GATCTGTATCTGTTGCACACG-3′ at the 3′ end. The deletion mutants were backcrossed seven times to N2 before character-

ization of the phenotypes. *gpa-2(pk16) gpa-3(pk35)* double mutants were obtained by analyzing progeny of *gpa-2* +/+ *gpa-3* animals by PCR for homozygosity for one of the deletions linked to heterozygosity of the other one.

Expression constructs: A 3.7-kb *Bam*HI-*Pvu*II fragment from cosmid PS#01C2 (see above), extending 2.3 kb 5′ from and including the *gpa-2* translational start as well as exons one, two and a portion of three (FINO SILVA and PLASTERK 1990), was cloned into the *lacZ* expression vector pPD21.28 (FIRE *et al.* 1990) and named pJMG2BV.28. The same restriction fragment was cloned into the *GFP* expression vector pPD95.75 (A. FIRE, J. AHNN, G. SEYDOUX and S. XU, personal communication) and named pJMG2BV95.75. A 6.0-kb *Bam*HI-*Pst*I fragment from cosmid PS#14A3 (see above), extending 5.9 kb 5′ from and including the *gpa-3* translational start as well as a portion of exon one (LOCHRIE *et al.* 1991), was cloned into the *lacZ* expression vector pPD22.11 (FIRE *et al.* 1990) and named pJMG3BP.11. The same restriction fragment was cloned into the *GFP* expression vector pPD95.75 (A. FIRE, J. AHNN, G. SEYDOUX and S. XU, personal communication) and named pJMG3BP95.75. For both *gpa-2* and *gpa-3*, promoter fragments that extended ~2 kb farther 5′ were tested and found to give the same expression pattern as those described above. pJMG2BV.28 and pJMG3BP.11 were used to establish the transgenic strains *syls7* and *syls28*, respectively. pJM2BV95.75 and pJM3BP95.75 were used to establish multiple transgenic lines. Derivatives of pJMG2BV.28 and pJMG3BP.11 that lack a nuclear localization signal were constructed by excising the *Kpn*I cassette in each of these plasmids. F_1 transgenic nematodes transiently expressing the plasmids lacking the nuclear localization signal were used to visualize processes of *gpa-2-* and *gpa-3*-expressing cells. Transgenic nematodes expressing *lacZ* reporter genes were fixed and stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) or Salmon-Gal (6-chloro-3-indolyl-β-D-galactopyranoside) (Biosynth) as described (FIRE *et al.* 1990) and were examined with Nomarski optics. Transgenic nematodes expressing *GFP* reporter genes were examined, comparing the fluorescence image with Nomarski images of the same animal (CHALFIE *et al.* 1994). Twenty to 30 animals from each of two to four independent transgenic lines were scored for either *gpa-2*::*GFP* or *gpa-3*::*GFP*. Cell identifications were made by the size, shape, and position of cell bodies, their nuclei and processes (WHITE *et al.* 1986; WOOD 1988; HALL and RUSSELL 1991).

Dauer assays: Dauer larvae were identified by both morphology and behavior (CASSADA and RUSSELL 1975). The ability of each strain to form typical dauer larvae was confirmed by detergent treatment (CASSADA and RUSSELL 1975). The percentage of dauer larvae formed by transgenic strains bearing activated *gpa-2* and *gpa-3* was determined under non-dauer-inducing conditions on standard NG agar plates seeded with a lawn of *Escherichia coli* OP50 (WOOD 1988). Five or six adult hermaphrodites were allowed to lay eggs for 2–8 hr, until 100–200 eggs were laid, and then removed. Plates were incubated at 15° or 25°, checked frequently for transient dauers and the number of dauer and non-dauer larvae counted after 60 hr at 25° or 120 hr at 15°. The percentage of dauer larvae was averaged over at least three trials.

Dauer-inducing pheromone was isolated as described by VOWELS and THOMAS (1994). One unit was defined as the amount required to induce 33% dauer formation in a wild-type population at 25°. Pheromone assays were performed as described in GOLDEN and RIDDLE (1984), except that 50 mg/ml streptomycin was added to the agar plates and plates were seeded with 20 ml of pelleted *E. coli* OP50 resuspended in water. Adult hermaphrodites were allowed to lay eggs for 2–8 hr and then removed. After removal of the adults, plates were incubated at 15° for 80 hr or 25° for 40 hr before dauer and non-dauer larvae were counted. Plates were checked frequently during the incubation, and larvae counted when most were either dauer or L4 larvae. The weighted mean (total number of dauer larvae on all plates/total number of nematodes on all plates) and standard error of the mean of all tests were determined.

The frequency of dauer formation by strains doubly mutant for *daf-11, daf-21, daf-1* or *daf-8,* and *gpa-2(pk16)* or *gpa-3(pk35)*, as well as by *gpa-3(pk35)*; *syls30* and *gpa-2(pk16)*; *syls25,* was determined under non-dauer-inducing conditions at 25° as described above for pheromone assays except that no pheromone was added to the agar plates.

Other assays: Living animals were stained with 5-fluorescein isothiocyanate (Sigma) as described by HEDGECOCK *et al.* (1983) and DiO (Sigma) as described by HERMAN and HEDGECOCK (1990).

Genetics: Double mutants of *daf-11(m47)* or *daf-21(p673)* and *gpa-2(pk16)* or *gpa-3(pk35)* were constructed as follows. For *daf-11,* non-Dpy dauer progeny of *dpy-11* + *daf-11*/+ *gpa* + animals at 25° were recovered at 15° and tested for heterozygosity of *gpa* by PCR. Non-Dpy-segregating F_2 animals were tested by PCR for homozygosity for *gpa.* For *daf-21,* F_1 *daf-21* homozygotes from *gpa* +/+ *daf-21* animals were identified at 25° by the presence of dauer larvae in the F_2. These were tested for heterozygosity for *gpa* by PCR. F_2 were tested by PCR for homozygosity. Double mutants of *daf-1(m40)* or *daf-8(e1393)* and *pk16* or *pk35* were isolated from the progeny of *daf*/+; *gpa*/+ animals by testing for homozygosity for *gpa* by PCR and for *daf* by inspecting F_2 at 25°.

syls13, syls30, syls24, and *syls25* were genetically mapped as dominant suppressors of the Dpy phenotype of *dpy-20* using standard methods (BRENNER 1974).

Double mutants between *syls30, syls24* or *syls25* and *che-3(e1124), che-13(e1805) daf-5(e1386)* or *daf-8(e1393)* were constructed by balancing the Daf mutation in *trans* with a tightly linked visible marker (*unc-13(e51)* for *che-3, che-13* and *daf-8, unc-52(e444)* for *daf-5*) and tracking the transgene by its dominant non-Dpy phenotype in a *dpy-20* background. For example: *unc-13*/+; *dpy-20 syls25* males were crossed to *che-3; dpy-20* hermaphrodites. F_2 or F_3 that did not segregate Unc or Dpy were isolated from Unc-segregating F_1. The presence of *e1124* or *e1805* was confirmed by staining with DiO for *syls30* (HERMAN and HEDGECOCK 1990), and by testing for response to light nose touch for *syls24* and *syls25* (KAPLAN and HORVITZ 1993). All males were *him-5(e1490)*.

syls30; pk35 was constructed by crossing *dpy-20 syls30; him-5* males with *dpy-20; pk35* hermaphrodites. Non-dpy F_2 that did not segregate Dpy F_3 were analyzed for homozygosity of *pk35* using PCR. *syls25; pk16* was constructed by crossing *pk16; him-8* males with *dpy-20; syls25* hermaphrodites. Non-Dpy F_2 that did not segregate Dpy or males in F_3 or F_4 were analyzed by PCR for homozygosity of *pk16.* Additionally, in both cases PCR and the appearance of dauers confirmed the presence of the transgene.

syls13; syls25 and *syls24; syls30* were constructed using *unc-24(e138) mec-3(e1338 dpy-20(e1282)* to balance *syls13* or *syls24* in *trans* and the *X*-linkage of *syls25* or *syls30* to force homozygosity.

RESULTS

The *gpa-2* and *gpa-3* genes were cloned based on their homology with mammalian Gα subunits (FINO SILVA and PLASTERK 1990; LOCHRIE *et al.* 1991). GPA-2 and GPA-3, which are 58% identical, share 45–55% identity with all members of the mammalian subclasses: Gαs,

Gαo, Gαq and Gα12. Gα subunits within each class share at least 60% identity (SIMON *et al.* 1991). Therefore, GPA-2 and GPA-3 may have arisen by duplication and divergence after the divergence of mammals and nematodes from their common ancestor, and might function in nematode-specific signal transduction. We used two reverse genetic approaches to analyze the function of *gpa-2* and *gpa-3:* transgenic expression of presumptive dominant mutations (MENDEL *et al.* 1995) and target-selected gene inactivation (ZWAAL *et al.* 1993).

Constitutive activation of *gpa-2* or *gpa-3* results in constitutive dauer formation: Mammalian Gαs and Gαi have been shown to be constitutively activated by mutation of a conserved glutamine residue in the guanine nucleotide binding pocket (MASTERS *et al.* 1989; WONG *et al.* 1991). Expression of the *C. elegans* Gαo-encoding *goa-1* gene bearing a substitution of leucine for glutamine (Q205L) resulted in phenotypes opposite to those seen in loss-of-function *goa-1* mutants (MENDEL *et al.* 1995). Although the same result was obtained by overexpressing the wild-type gene, the phenotypes caused by the Q205L-substituted transgene were more severe, indicating that this substitution activates Gαo (MENDEL *et al.* 1995; SÉGALAT *et al.* 1995). Using *in vitro* mutagenesis, we substituted a leucine codon for that of glutamine at the equivalent position in the sequences of *gpa-2* (Q207L) and *gpa-3* (Q205L). The plasmids with these gain-of-function mutations were introduced into *C. elegans* individually as transgenes and subsequently integrated into the genome, resulting in the alleles *syIs13* (IV) and *syIs30* (X) for activated *gpa-2,* and *syIs24* (IV) and *syIs25* (X) for activated *gpa-3* (see MATERIALS AND METHODS). These mutant constructs contain the same 5′ flanking sequence as the *lacZ* and *GFP* fusions described below, as well as their full complement of introns and ~2 kb of 3′ flanking sequence.

Transgenic *C. elegans* bearing either activated *gpa-2* or *gpa-3* enter dauer development even under noninducing conditions. The percentage of animals that developed as dauer larvae is transgene-specific, perhaps due to the number of copies of each transgene or to position effects resulting from the insertion site. At standard growth conditions (25° and in abundant food), the percentage of dauer larvae formed by the two activated *gpa-2* strains was very different: ~5 ± 2.5% of *syIs13* and 99 ± 0.5% of *syIs30* formed dauer larvae. The two activated *gpa-3* strains formed ~44 ± 3% (*syIs24*) and 95 ± 2.5% (*syIs25*) dauer larvae. Wild-type animals formed no dauer larvae under these conditions.

All four transgenic strains showed appropriate responses to food and temperature: restricting food and elevating temperature resulted in the formation of more dauer larvae (data not shown). However, these strains were almost completely resistant to exogenously added pheromone. This is best illustrated at 15° and with a restricted food supply. Under these conditions,

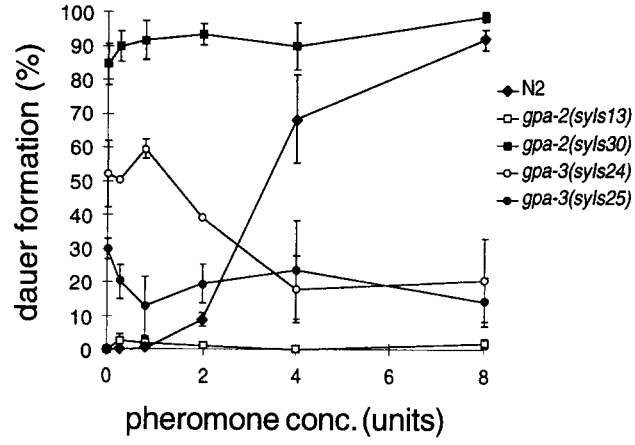

FIGURE 1.—Pheromone response by wild-type and transgenic *C. elegans* with activated *gpa-2* and *gpa-3.* Young adults were allowed to lay eggs for 4–8 hrs on culture dishes with a small lawn of 5% (w/v) *E. coli* OP50 and increasing amounts of pheromone (in units: 1 unit of pheromone is the amount required to induce 33% of a wild-type population to enter the dauer stage at 25°). Dauer and non-dauer larvae were counted after 120 hr at 15°. Each point represents the sum of three independent trials. SD, vertical bars.

wild-type *C. elegans* responded to increasing amounts of pheromone by forming increasing numbers of dauer larvae, but activated *gpa-2* or *gpa-3* transgenics formed a relatively constant percentage of dauer larvae at all pheromone levels tested (Figure 1). All transgenic strains were resistant to pheromone, independent of the relative strength of their Daf-c phenotype (*e.g., syIs13 vs. syIs30*). The resistance to exogenous pheromone, but not the Daf-c phenotype, was also seen when wild-type *gpa-2* and *gpa-3* were overexpressed as transgenes (data not shown).

Deletion of *gpa-2* or *gpa-3* reduces sensitivity to dauer pheromone: Using a strain that has natural Tc1 transposon activity and accumulates Tc1 insertions, we isolated mutants in which a copy of Tc1 had inserted in the fourth intron of *gpa-2* or the last exon of *gpa-3.* These were subsequently used to screen for mutants where Tc1 excision had resulted in loss of flanking sequences (ZWAAL *et al.* 1993). The resulting *gpa-2(pk16)* allele has lost the sequence from position 633 in the first intron through position 2823 in the presumptive 3′ untranslated region (FINO SILVA and PLASTERK 1990), so that nearly the complete coding region is removed (ZWAAL *et al.* 1993). The *gpa-3(pk35)* allele has lost genomic sequences from position 1142 in the first intron to position 2666 in the last exon (LOCHRIE *et al.* 1991), also removing most of the coding sequences (ZWAAL *et al.* 1993). Therefore, these are probably null alleles. *gpa-2* and *gpa-3* are localized within 1.5 map units of each other on chromosome *V.* Double mutants in which both *gpa-2* and *gpa-3* are deleted were obtained by recombination.

Mutants lacking *gpa-2, gpa-3,* or both showed reduced response to exogenously added dauer pheromone and

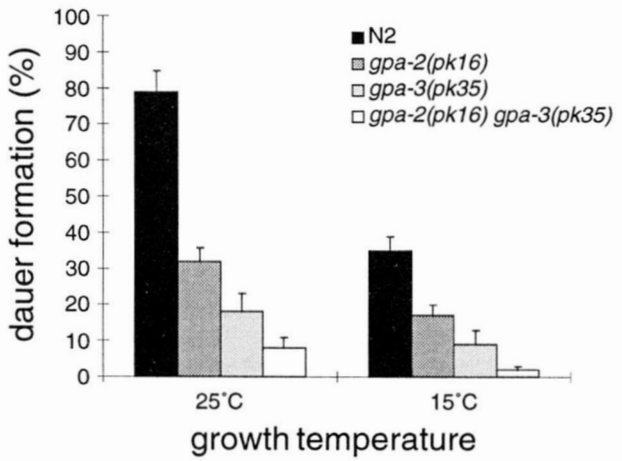

FIGURE 2.—Dauer formation by wild-type and mutants lacking *gpa-2* and *gpa-3*. Young adults were allowed to lay eggs for 4–8 hr on culture dishes with a small lawn of 6.7% (w/v) *E. coli* OP50 and 3 units of pheromone. Dauer and non-dauer larvae were counted after 40 hr at 25° or 120 hr at 15°.

thus were defective in the regulation of dauer formation. When assayed under standard conditions (25°, 3 units of pheromone, 6.67% w/v *E. coli* OP50) (GOLDEN and RIDDLE 1984a), wild-type *C. elegans* formed 79 ± 6% dauer larvae while mutants lacking *gpa-2* and *gpa-3* formed 32 ± 4% and 18 ± 5% dauer larvae, respectively (Figure 2). The *gpa-2 gpa-3* double mutant showed an additional reduction (8 ± 3% dauer larvae) relative to each single mutant. The double mutant was somewhat responsive to pheromone, indicating that there is another functional pheromone processing pathway. Both mutants and wild-type animals formed fewer dauer larvae at lower temperature, but their relative response to pheromone was unaltered by temperature (Figure 2). Therefore, it is unlikely that GPA-2 and GPA-3 are involved in the transduction of the temperature component influencing dauer formation, but instead are involved in chemosensation of the pheromone and/or food signals.

The reduced response of the mutant animals in the above assay can be explained either by reduced sensitivity to the dauer-inducing pheromone, or by hypersensitivity to the food signal. To distinguish between these possibilities, we varied one signal keeping the other constant. At a constant food concentration, the animals were exposed to increasing amounts of pheromone (Figure 3A). With no pheromone present, no dauer larvae were detected for either wild-type or any of the mutant strains. Wild type responded mildly at low concentrations of pheromone, but strongly between 0.5 and 1.5 units. The mutant animals were somewhat hyperresponsive at lower concentrations of pheromone, but were unresponsive at increasing pheromone concentrations.

We also tested whether mutants lacking *gpa-2* and *gpa-3* are able to process changes in food supply at a constant pheromone concentration. At a high concentration of pheromone (3 units), the number of wild-type dauer larvae increased with a decrease in food (Figure 3B). The mutant animals responded like wild-type to a decline in food, meaning that they could still interpret the availability of food correctly. At a low pheromone level (0.2 units), the situation was slightly different (Figure 3C). With sufficient food, the mutants formed more dauer larvae than wild type, and this was relatively unaffected by the amount of food. When the amount of food dropped below a certain threshold (~60-fold dilution), the mutants became responsive to the food signal and the number of dauer larvae formed increased with decreasing food.

GPA-2 influences exit from the dauer stage: Dauer pheromone not only commits young *C. elegans* larvae to enter dauer development, but also represses exit from the dauer stage. In the absence of pheromone, wild-type, *gpa-2(pk16)*, *gpa-3(pk35)*, and double mutant dauer larvae quickly recover from the dauer stage at both 15° and 25° (data not shown). Since mutants lacking *gpa-2* or *gpa-3* showed a reduction in pheromone-induced dauer formation, we tested whether mutant dauer larvae were able to resume development in the presence of different concentrations of pheromone. Newly formed pheromone-induced dauer larvae were transferred to new pheromone-containing plates with plentiful food at 25° and checked frequently for recovery as indicated by pharyngeal pumping. As shown in Table 1, none, or very few, wild-type or *gpa-3(pk35)* dauer larvae recovered under these conditions. However, *gpa-3(pk16)* dauer larvae were able to recover in a concentration-dependent manner and over a prolonged period of time. At low pheromone (0.2 units), approximately half of the dauer larvae lacking *gpa-2* resumed growth within 48 hr. Even with 3 units of pheromone some of the *gpa-2(pk16)* dauer larvae were able to escape the inhibition of recovery by pheromone. The *gpa-2(pk16) gpa-3(pk35)* double mutant was similar to the *gpa-2(pk16)* mutant, indicating that the effect is independent of inactivation of *gpa-3*. The fact that, although both GPA-2 and GPA-3 are involved in transduction of the pheromone signal, only dauer larvae lacking *gpa-2* were able to recover in the presence of pheromone may reflect a difference in function of the different neurons expressing these two genes (see below).

The activated *gpa-2* and *gpa-3* transgenics formed dauer larvae in the absence of pheromone. Daf-c mutants apparently produce a false internal signal that mimics the pheromone signal, and Daf-c dauer larvae usually recover poorly at 15° and not at 25° (RIDDLE *et al.* 1981). In this respect dauer larvae induced by activated *gpa-2* behaved like other Daf-c mutants as they recovered slowly (24–48 hr) at 15° and even more slowly at 25°. However, dauer larvae induced by activated *gpa-3* failed to recover at 15° but recovered slowly (48–72 hr) at 25°. This differential behavior of the two G protein mutants might again reflect a difference in

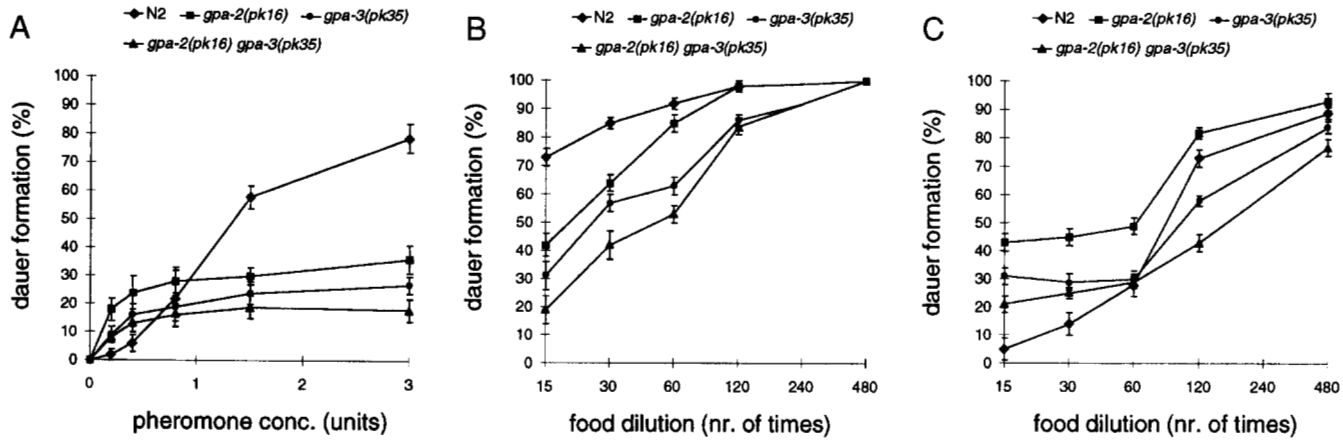

FIGURE 3.—Response of wild-type and mutants lacking *gpa-2* and *gpa-3* to increasing pheromone or decreasing food levels. (A) Percentage of larvae entering the dauer stage when exposed to increasing amounts of pheromone at 25°, with a small lawn of 6.7% (w/v) *E. coli* OP50. Young adults were allowed to lay eggs for 4–8 hr; dauer and non-dauer larvae were counted after 40 hr at 25°. (B) Percentage of larvae entering the dauer stage when exposed to 3 units of pheromone at 25°, with decreasing amounts of food. Plates were seeded with 20 ml of pelleted bacteria that were resuspended in 15, 30, 60, 120 or 480 volumes of water. Symbols are identical to A. (C) Percentage of larvae entering the dauer stage under the same conditions as B, but exposed to 0.2 units of pheromone. Symbols are identical to A.

function of the neurons expressing these two genes (see below).

Expression of *gpa-2* and *gpa-3*: To determine which cells express *gpa-2* and *gpa-3*, we made reporter constructs in which 3–6 kb of DNA containing the upstream presumptive control region of these genes directed the expression of either the *E. coli* β-galactosidase (*lacZ*) gene or the gene *GFP* (Green Fluorescent Protein; CHALFIE *et al.* 1994). The constructs were introduced as transgenes, and those containing *lacZ* were subsequently integrated into the genome. The activity of both the *gpa-2* and the *gpa-3* promoter was confined to a small, but different, subset of mainly neuronal cells. Most of the 302 neurons of *C. elegans* are situated in the head, with their cell bodies arranged around the pharynx in anterior, lateral, and ventral ganglia (WHITE *et al.* 1986). Twenty-six neurons have exposed ciliated endings consistent with a role in chemosensation. Four of these have sensory endings in the phasmid sensilla in the tail, while the remaining 22 are located in the head, sending processes to the tip of the nose through the inner labial (six cells) and amphid sensilla (16 cells) (WARD *et al.* 1975; WHITE *et al.* 1986).

gpa-2::lacZ and *gpa-2::FP* were expressed in the exposed phasmid neurons PHA and PHB, the interneuron PVT, the anal sphincter muscle, the pharyngeal neurons M1, M5 and I5, and three pairs of neurons in the head (Figure 4, A and B). One left/right lateral pair of head neurons lies in the anterior ganglion and sends processes through the labial process bundles.

TABLE 1

Recovery of the *gpa-2* and *gpa-3* loss-of-function mutants in the presence of pheromone

Strain	Pheromone concentration (U)	No. of worms	Time of recovery			
			6 hr	12 hr	24 hr	48 hr
N2	0.2	48	1	1	1	1
	0.8	36	0	0	0	0
	3	20	0	0	0	0
gpa-2	0.2	45	10	23	27	27
	0.8	36	1	2	2	5
	3	26	4	4	4	5
gpa-3	0.2	30	0	0	0	0
	0.8	25	0	0	0	0
	3	32	0	0	0	0
gpa-2 gpa-3	0.2	38	0	14	19	19
	0.8	38	0	4	6	8
	3	27	0	1	1	2

Pheromone-induced dauer larvae of each genotype were transferred to culture dishes containing food and the amount of pheromone indicated. Dauer larvae were examined for pharyngeal pumping as an indicator of resumed development. The cumulative number of recovered dauer larvae is given for each point.

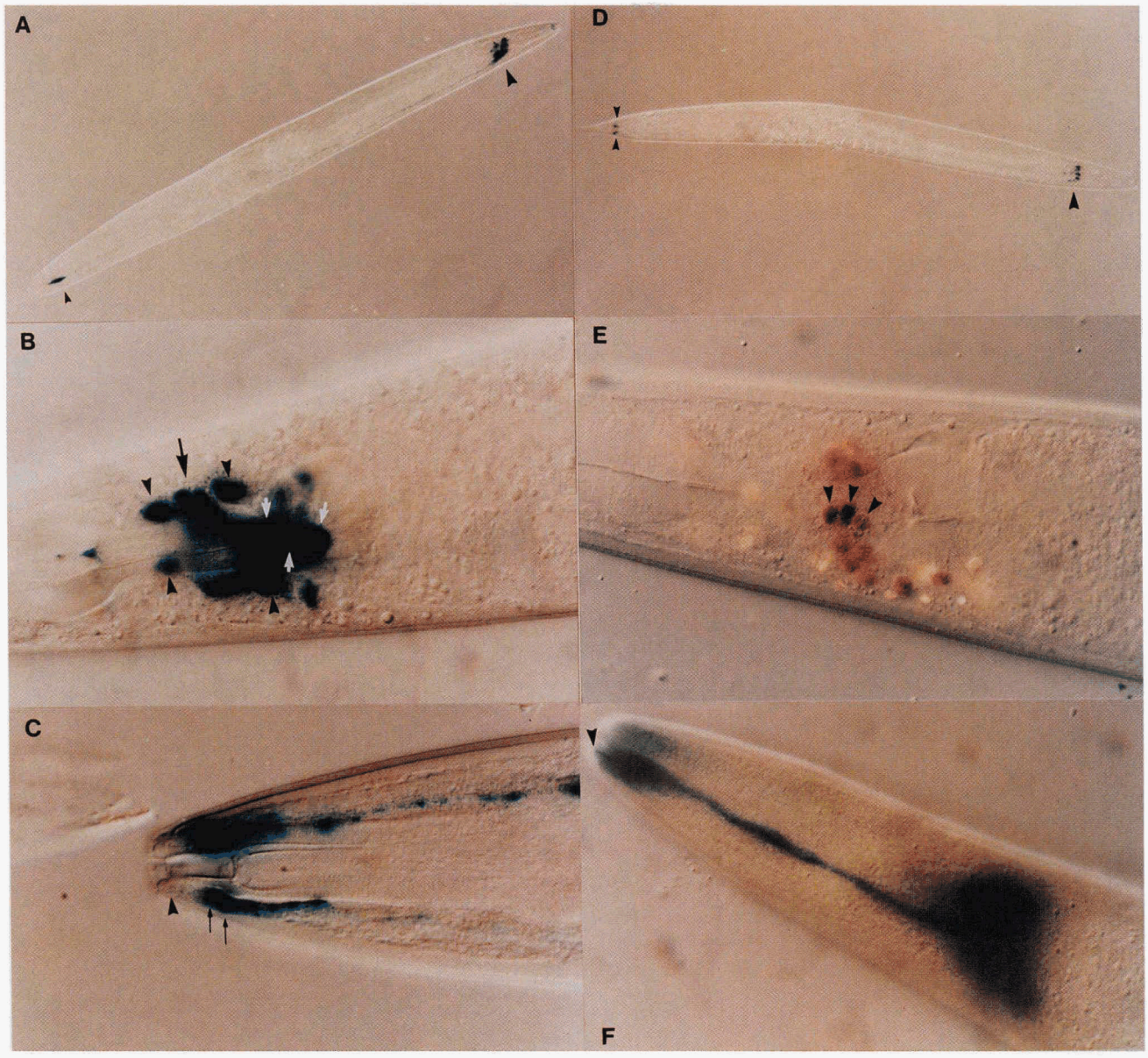

FIGURE 4.—Expression of *gpa-2::lacZ* and *gpa-3::lacZ* in adult hermaphrodites. (A) Right lateral view of a *syls7* (*gpa-2::lacZ*) adult hermaphrodite. Anterior is right, dorsal up. In the tail, the interneuron PVT is stained blue (small arrowhead). Typical staining in the phasmid neurons, PHA and PHB, and in the anal sphincter muscle does not appear in this animal. Neural nuclei and processes in the pharyngeal nerve ring can be seen anteriorly (large arrowhead). (B) Ventral view of the pharyngeal region of a *syls7* adult hermaphrodite. Anterior is left. The position of the pharyngeal nerve ring is revealed by stained processes indicated by a black arrow. Bilateral pairs of cells anterior and posterior to the nerve ring are indicated by black arrowheads. Neurons in the ventral ganglion are indicated by white arrows. See text for cell identifications. Typical staining in pharyngeal neurons does not appear in this animal. (C) Lateral view of the anterior end of an adult hermaphrodite bearing *gpa-2::lacZ* lacking a nuclear localization signal. Anterior is left. The anterior ends of the left members of two pairs of processes, one each from neurons in the anterior and lateral ganglia, projecting into the left lateral lip are indicated by arrows. One of the amphid channels is indicated by an arrowhead. (D) Right lateral view of a *syls28* (*gpa-3::lacZ*) adult hermaphrodite. Anterior is right, dorsal up. In the tail, stained nuclei of the phasmid neurons, PHA and PHB, are indicated with small arrowheads. Nine bilateral pairs of nuclei in the pharyngeal nerve ring are indicated by an arrowhead. (E) Left lateral view of the pharyngeal region of a *syls28* adult hermaphrodite. Anterior is left, dorsal up. Nine neural nuclei that occur in bilaterally symmetric pairs are stained, of which only some members of some pairs are visible in this picture. From anterior to posterior, the amphid neurons ASK, ADL, and ASI are indicated by arrowheads. See text for other cell assignments. (F) Left lateral view of the head of an adult hermaphrodite bearing *gpa-3::lacZ* lacking a nuclear localization signal. Processes projecting anteriorly from the nerve ring to the left lateral lip are stained. The amphid pore is indicated with an arrowhead. (A and D) Magnification ~40×. (B, C, E, and F) Magnification ~190×. A–D and F stained with X-Gal; E stained with Salmon-Gal. All panels were photographed on Ektar 25 film (Kodak) with Nomarski optics.

These processes terminate in the lateral lips, adjacent to the amphid channel (Figure 4C). The position of the expressing nuclei and their processes is consistent with expression in several sensory or sensory-like cells including IL1L, IL2L, OLL and URB. Of these, only IL2L is open to the outside and believed to be chemosensory (PERKINS *et al.* 1986; WHITE *et al.* 1986). A second pair of head neurons with nuclei in the lateral ganglion (Figure 4B) was seen in *gpa-2::lacZ* but not *gpa-2::GFP* lines. These cells send processes anteriorly, parallel to the amphid process bundles, but terminating prior and lateral to the amphid channel (Figure 4C). The position of the staining nuclei is consistent with expression in the nonchannel amphid neuron AWC. These neurons have branched ciliated endings that are not directly exposed to the environment (PERKINS *et al.* 1986). AWC is required for chemosensation of certain volatile odorants (BARGMANN *et al.* 1993). The remaining pair of head neurons lies in the ventral ganglion (Figure 4B) in a position consistent with expression in the amphid interneuron AIA, which receives synaptic input predominately from the amphid sensilla (WHITE *et al.* 1986).

gpa-3::lacZ and *gpa-3::GFP* were expressed in the interneuron PVT as well as several exposed chemosensory cells including the phasmid neurons PHA and PHB, and the exposed amphid neurons (Figure 4, D and E). A total of 18 head neurons, in left/right lateral pairs, showed expression (Figure 4E). Several of these send processes anteriorly that run through the amphid channel (Figure 4F). We have identified expression in the amphid neurons ADF, ADL, ASH, ASI, ASJ, and ASK. The positions of the remaining expressing nuclei are consistent with expression in the amphid neurons ASE and ASG, and the amphid interneurons AIZ. ASI and ADF have been shown to be involved in dauer formation (BARGMANN and HORVITZ 1991b), while ADL, ASI, ADF, ASH, and ASE are involved in either chemotaxis or chemical avoidance (BARGMANN *et al.* 1990; BARGMANN and HORVITZ 1991a; KAPLAN and HORVITZ 1993). Activity of the *gpa-3* promoter in the exposed amphid neurons was supported by an additional phenotype caused by the activated *gpa-3* transgene. In wild-type *C. elegans,* six of the exposed amphid neurons, ADF, ASH, ASI, ASK, ASJ, and ADL, take up fluorescein dyes, as do the phasmid neurons PHA and PHB (HEDGECOCK *et al.* 1985). Neither of the activated *gpa-3* transgenic strains took up such dyes, indicating that expression of this transgene affects the dye-filling cells. This might be an indirect effect, *e.g.,* by causing an abnormal structure of the ciliated endings, since the amphid neurons expressing the *gpa-3* reporter gene were still present in the gain-of-function mutant animals. The amphid neurons in *syls25* were visualized by injecting a plasmid encoding *gpa-3::lacZ* lacking a nuclear localization signal. F_1 offspring of injected mothers showed staining in nine pairs of neurons as well as in processes exiting through the amphid pore (data not shown), the same pattern seen in a wild-type background (Figure 4F). In addition, we tested for function of ASH, a pair of amphid neurons required for response to a light nose touch (KAPLAN and HORVITZ 1993). Both *syls24* and *syls25* displayed wild-type response to nose touch (98% and 96%, respectively, $n = 50$ animals). We also injected a *gpa-2::lacZ* plasmid into *syls30* hermaphrodites, confirming the normal morphology of the *gpa-2*-expressing neurons in transgenics bearing activated *gpa-2.*

The expression of *gpa-2::lacZ* and *gpa-3::lacZ* appeared unchanged at all stages of development, from late stage embryo to adult hermaphrodite, and was similar in adult males.

Since *gpa-2* and *gpa-3* show promoter activity in cells involved in chemotaxis to or avoidance of substances other than dauer pheromone, the response of *gpa-2* and *gpa-3* deletion mutants to a variety of substances was tested. The animals reacted normally to the soluble chemical stimuli Na^+, Cl^-, and biotin ("taste"), as well as to a concentration series of volatile agents such as diacetyl and isoamylalcohol that act via the neurons AWA and AWC ("smell") (C. BARGMANN, personal communication). They avoided osmoregulatory shock normally (by staying within a ring of 4 M NaCl; data not shown), and also acted normally in thermotaxis assays (I. MORI, personal communication).

The only sensory cells in which both *gpa-2* and *gpa-3* promoters were active are the sensory neurons of the phasmids. However, the function of these cells is unknown, and they seem not to be involved in dauer formation (VOWELS and THOMAS 1994). Therefore, it is probable the Daf-d and Daf-c phenotypes of *gpa-2* and *gpa-3* mutants are exerted by different neurons.

Pathway analysis: Animals that lack *gpa-2* or *gpa-3* show a Daf-d phenotype, whereas animals with activated *gpa-2* or *gpa-3* show a Daf-c phenotype. Previously, a number of different mutations of both phenotypic classes have been isolated. THOMAS *et al.* (1993) argued that these genes function in parallel pathways based on the following observations. Daf-c mutations in *daf-11* and *daf-21* are completely suppressed by Daf-d mutations that affect the structure of the amphid cilia, while Daf-c mutations in a second group of genes including *daf-1* and *daf-8* are suppressed by another group of Daf-d mutations including *daf-5.* Additionally, mutations in *daf-11* and *daf-21* act synergistically with the second group of Daf-c mutations, forming nearly 100% dauer larvae at 16°. To place the G proteins in the context of other genes involved in the dauer decision, we tested *gpa-2* and *gpa-3* mutants with several other Daf-c and Daf-d alleles.

The Daf-c genes *daf-11* and *daf-21* are partially suppressed by deletion of *gpa-2* and *gpa-3* (Figure 5A). In the absence of pheromone at 25° and with plentiful food, >99% of *daf-11(m47)* larvae entered the dauer

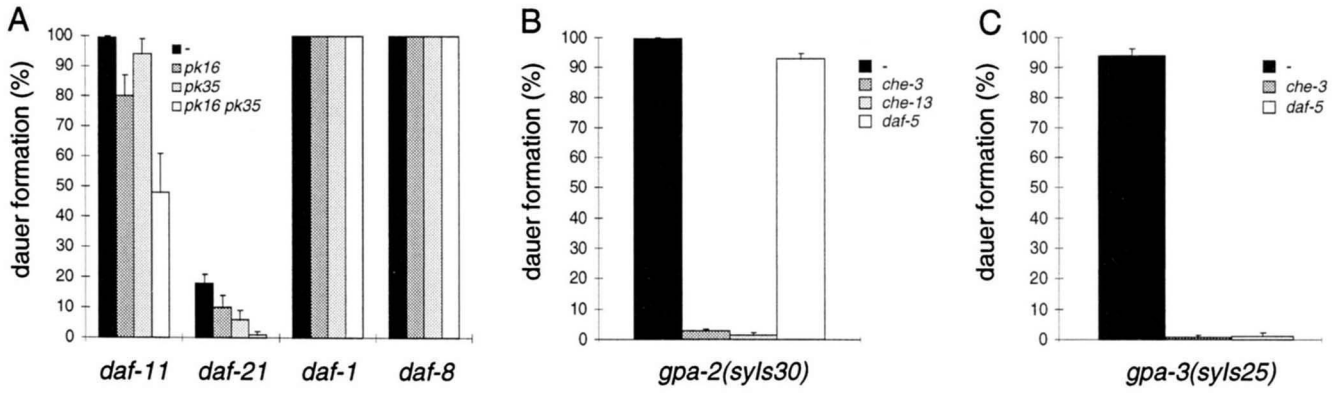

FIGURE 5.—Epistatic interactions of loss- and gain-of-function mutants of *gpa-2* and *gpa-3*. (A) *gpa-2* and *gpa-3* loss-of-function mutants partially suppress *daf-11* and *daf-21*, but not *daf-1* or *daf-8*. Assays were performed at 25° as in Figure 2 except no pheromone was added to culture dishes. The Daf-c alleles are as follows: *daf-11*(*m47*), *daf-21*(*p673*), *daf-1*(*m40*) and *daf-8*(*e1393*). (B) Activated *gpa-2*(*syls30*) is suppressed by the cilium-structure dauer defective allele *che-3*(*e1124*) and *che-13*(*e1805*), but not by a dauer defective allele with normal sensory cilia, *daf-5*(*e1386*). (C) Activated *gpa-3*(*syls25*) is suppressed by *che-3*(*e1124*) as well as by *daf-5*(*e1386*).

program. Double mutants of *daf-11*(*m47*) with *gpa-2*(*pk16*) or *gpa-3*(*pk35*) showed a reduction in dauer formation: 80 ± 6% of *gpa-2 daf-11* and 94 ± 5% of *gpa-3 daf-11* developed into dauer larvae. Only 50 ± 1% of the triply mutant *gpa-2 gpa-3 daf-11* formed dauer larvae. Similar effects were seen between *daf-21*(*p673*) and *gpa-2* or *gpa-3* (Figure 5A). Deletion of *gpa-2* and *gpa-3* was not able to suppress *daf-1* or *daf-8* (Figure 5A). Therefore, *gpa-2* and *gpa-3* act genetically downstream of or in parallel to *daf-11* and *daf-21* and upstream of or in parallel to *daf-1* and *daf-8*. We did observe a synergistic effect between *gpa-2* and *daf-1*(*m40*) or *daf-8*(*e1393*) at 15°. Although fewer than 10% of *daf-1*(*m40*) or *daf-8*(*e1393*) became dauer larvae at this temperature, in combination with *pk16* >90% became dauer larvae (data not shown). Similar effects have been observed previously in combinations of *daf-1* and *daf-8* with *che-3*(*e1124*) and *osm-5*(*p813*) (VOWELS and THOMAS 1992), two mutations that affect amphid neuron morphology (PERKINS *et al.* 1986).

Suppression of *daf-11* and *daf-21* by mutations affecting the amphid cilia implies that the products of these genes act within the cilia (THOMAS *et al.* 1993). We tested the ability of the cilium-structure mutations *che-3*(*e1124*) and *che-13*(*e1805*) to suppress the activated *gpa-2* transgene *syls30*. As with *daf-11* and *daf-21*, both cilium-structure mutations suppressed *syls30* (Figure 5B), suggesting that GPA-2 acts within the sensory cilia. The candidate *gpa-2*-expressing cells affected by mutations in *che-3* and *che-13* are IL2L and the amphid associated neuron AWC (ALBERT *et al.* 1981; PERKINS *et al.* 1986). Activated *gpa-2* is not suppressed significantly by a member of the noncilium structure group of Daf-d mutations, *daf-5*(*e1386*) (Figure 5B), while mutations in both *daf-11* and *daf-21* are partially suppressed by this allele (THOMAS *et al.* 1993). As *daf-11* and *daf-21*, *syls30* showed a synergistic effect with *daf-8*(*e1393*) (data not shown). Therefore, activated *gpa-2* shows genetic interactions similar but not identical to *daf-11* and *daf-21* (THOMAS *et al.* 1993). The interactions of activated *gpa-3* with other genes in the dauer pathway are also unique among Daf-c mutations. *syls25* was suppressed by *che-3*(*e1124*) (Figure 5C), suggesting that GPA-3 also acts in sensory cilia. However, *syls25* was also suppressed by *daf-5*(*e1386*) (Figure 5C). Similar results were obtained with *syls24* (data not shown). No other previously described Daf-c allele is completely suppressed by mutations in both *che-3* and *daf-5*. It is possible that activated *gpa-3* interacts nonspecifically with a pathway requiring *daf-5*, perhaps due to cross-talk between G-protein-mediated pathways, or misexpression of the transgene. Both activated *gpa-3* transgenic lines showed a synergistic effect with *daf-8*(*e1393*) (data not shown) as do *daf-11* and *daf-21* (THOMAS *et al.* 1993). Altogether, *gpa-2* and *gpa-3* cannot be classified strictly into the categories previously described for genes involved in the dauer formation pathway.

In response to pheromone as well as in suppression of *daf-11* and *daf-21*, *gpa-2* and *gpa-3* exhibit some functional redundancy since the effect of the *gpa-2 gpa-3* double deletion was more severe than that of either single mutation. However, the effect of the activated transgenes was neither additive nor synergistic, but instead showed mutual suppression. At 25° and in abundant food, *syls13* (activated *gpa-2*) partially suppressed *syls25* (activated *gpa-3*), and *syls24* (activated *gpa-3*) partially suppressed *syls30* (activated *gpa-2*) (Figure 6, A and B).

To determine whether GPA-2 and GPA-3 act in series or in parallel, we constructed strains having an activated transgene for one G protein and deletion of the other G protein gene. Deletion of *gpa-3* did not suppress constitutive dauer formation in *syls30*, and deletion of *gpa-2* did not suppress *syls25* (Figure 6, C and D), suggesting that these G proteins act in parallel to transduce the dauer pheromone signal.

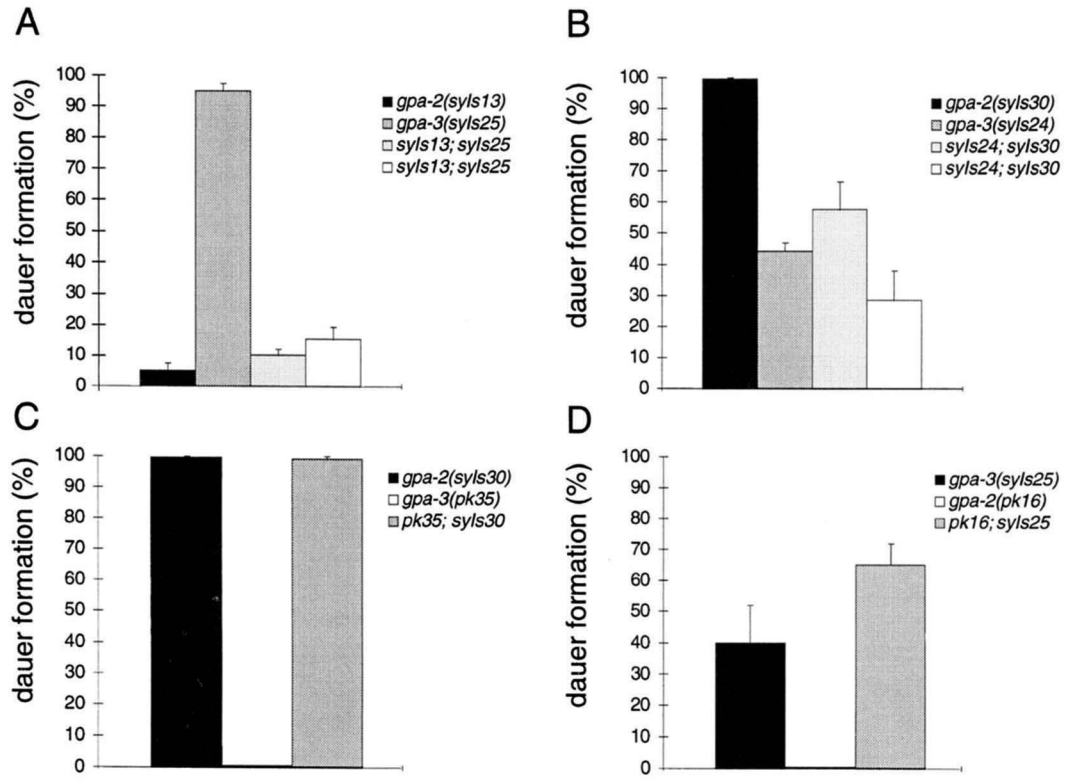

FIGURE 6.—Interactions between *gpa-2* and *gpa-3* gain- and loss-of-function mutants. Activated *gpa-2* and *gpa-3* show mutual suppression. (A) The percentage of dauer larvae formed under noninducing conditions by *syls13, syls25,* and two independently isolated *syls13; syls25* strains. (B) The percentage of dauer larvae formed under noninducing conditions by *syls24, syls30,* and two independently isolated *syls24; syls30* strains. (C) Deletion of *gpa-3* does not suppress activated *gpa-2.* The percentage of dauer larvae formed under noninducing conditions by *syls30, gpa-3*(*pk35*) and *gpa-3*(*pk35*; *syls30* is shown. (D) Deletion of *gpa-2* does not suppress activated *gpa-3.* The percentage of dauer larvae formed under noninducing conditions by *syls25, gpa-2*(*pk16*) and *gpa-2*(*pk16*); *syls25* is shown.

DISCUSSION

GPA-2 and GPA-3 are involved in the response to dauer pheromone: To study the function of *gpa-2* and *gpa-3,* we used a reverse genetics approach and found that both are involved in the response to a pheromone that promotes formation of the dauer larva, an alternate larval form. As a first approach, we introduced mutations in the sequences of *gpa-2* and *gpa-3* that lock the mutant proteins in the active GTP-bound state. Animals with either of these mutations enter the dauer program even under noninducing conditions. Although these mutants reacted normally to food and temperature changes, they were unresponsive to changes in the amount of pheromone present. Overexpression of wild-type *gpa-2* or *gpa-3* also led to reduced pheromone response. This lack of response may result from either activation of negative regulatory pathways or adaptation of downstream components of the signaling cascade. Alternatively, it may be indirect, for example, due to degeneration of the pheromone responsive tissue. The presumed pheromone responsive tissue, the amphid channel neurons, are morphologically normal in activated *gpa-2* transgenics and show normal fluorescein dye uptake. However, neither of the activated *gpa-3* transgenic strains took up such dyes. The amphid neurons were present in these strains, and at least one pair, ASH, functioned normally in the nose touch response. In addition, constitutive dauer formation induced in wild type by the ablation of ADF and ASI is not affected by disruption of the amphid cilium structures (BARGMANN and HORVITZ 1991b), whereas *gpa-3* transgenics were suppressed by cilium-structure mutations. Thus, the Daf-c phenotype of the *gpa-3* mutants is probably not caused by degeneration of the ADF or ASI cell bodies.

As a second approach, a transposition-based strategy (ZWAAL *et al.* 1993) was used to inactivate *gpa-2* and *gpa-3.* While animals lacking *gpa-2* or *gpa-3* were still capable of developing into the dauer stage, fewer dauer larvae were formed in the presence of high levels of dauer-inducing pheromone. Under strong inducing conditions, when pheromone is high or food is low, the deletion mutants responded appropriately to food but had a reduced response to pheromone. Under these conditions, *gpa-2* and *gpa-3* appear to be primarily involved in chemosensation of the dauer pheromone. Thus, opposite mutations in these G proteins seem to induce opposite effects: inactivation of *gpa-2* or *gpa-3* presumably inactivates a signal transduction pathway involved in the response to elevated levels of pheromone,

whereas constitutively active GPA-2 or GPA-3 seems to lead to continuous stimulation of this pathway.

***gpa-2* and *gpa-3* may also have an effect on the response to food:** When exposed to weak dauer-inducing conditions (abundant food and low pheromone), the deletion mutants were hypersensitive to pheromone but insensitive to changes in food levels. As food dropped below a certain threshold, they responded appropriately to diminishing food by forming more dauers. One possible interpretation is that mutant animals may be unable to detect saturating amounts of food, and thus promote dauer formation in the presence of little pheromone. This effect may be indirect though, for example, through elevated levels of free $\beta\gamma$ in these mutants. The response to temperature seems unaffected, both in the gain- and in the loss-of-function mutants.

Since under the strongest inducing conditions (high temperature, low food, and high pheromone) mutants lacking both *gpa-2* and *gpa-3* were able to form dauer larvae as efficiently as wild-type *C. elegans,* we propose that GPA-2 and GPA-3 are involved in the decision to enter the dauer formation pathway and not in the execution of dauer development.

***gpa-2* and *gpa-3* likely function in neurons:** Using *lacZ* and *GFP* reporter genes, we found that *gpa-2* and *gpa-3* promoter fusion constructs are expressed in small, but different, subsets of neurons. Due to the limitations of determining expression patterns with reporter genes, we may not have discovered the native expression of *gpa-2* and *gpa-3.* However, expression of *gpa-3* in the exposed neurons of the amphids correlates well with a function of *gpa-3* in dauer formation. Dauer pheromone is believed to be detected by the amphid neurons since mutations in nine genes that share the property of disrupting the structure or exposure of the amphid cilia are nonresponsive to dauer pheromone (see THOMAS 1993 for review). Also, ablation of the amphid sheaths leads to loss of pheromone response (VOWELS and THOMAS 1994). Additionally, ablation of the individual amphid neurons ADF and ASI induces dauer formation in the absence of pheromone, suggesting a default state to form dauer larvae that is suppressed by the activity of these neurons (BARGMANN and HORVITZ 1991b). We did see unambiguous expression of *gpa-3* reporter gene fusions in ASI (Figure 4E). Since activated *gpa-3* was suppressed by mutations disrupting ciliated neural endings, it is likely that GPA-3 functions in the amphid channel cilia. This location is consistent with coupling to the pheromone receptor, suggesting that the receptor for dauer pheromone is of the seven transmembrane family. A number of divergent seven transmembrane receptors have been discovered in the *C. elegans* genomic sequence, some of which are expressed in individual amphid neurons (TROEMEL *et al.* 1995).

We found no evidence that *gpa-2* is expressed in the exposed amphid cilia since we did not see reporter gene activity in the amphid channel as we did for *gpa-3* promoter fusion constructs. Constitutive dauer formation by activated *gpa-2* was suppressed, however, by mutations that disrupt all classes of sensory cilia in *C. elegans* (ALBERT *et al.* 1981; PERKINS *et al.* 1986). Therefore, GPA-2 may also transduce some component of the pheromone signal. A role for the candidate *gpa-2*-expressing neurons in pheromone detection has not been described previously. Indeed, a role for IL2L and the nonchannel amphid neuron AWC in pheromone sensation has been excluded based on the pheromone resistence of mutants having abnormal amphid channel neurons and apparently wild-type labial and nonchannel amphid neurons (VOWELS and THOMAS 1994). Although the morphology of the relevant neurons appears unaffected in such mutants, functional defects have not been tested. Previous work focused exclusively on the amphid channel neurons, and the role of individual amphid neurons in dauer formation and recovery was identified under growth-promoting conditions (BARGMANN and HORVITZ 1991b). Any additional neurons required for response to pheromone could not have been determined this way. Alternatively, GPA-2 may indeed function in the amphid channel cilia but our promoter fusion constructs lack intron or downstream sequences that direct expression in those tissues.

In addition to functioning in dauer formation, GPA-2 also plays a role in dauer recovery since dauer larvae lacking *gpa-2* were able to recover in the presence of pheromone, presumably because they do not fully transduce the inhibiting pheromone signal. Mutants lacking *gpa-3* are also impaired in the ability to transduce the pheromone signal, but they were not able to recover in the presence of pheromone. This observation can be explained by assuming that the neurons expressing *gpa-2* are involved both in pheromone-induced dauer formation and pheromone-repressed recovery, whereas the amphid neurons expressing *gpa-3* are involved only in the former. One amphid neuron, ASJ, has been implicated in dauer recovery (BARGMANN and HORVITZ 1991b). However, most likely ASJ is activated under noninducing conditions to initiate dauer recovery in response to food. The neurons expressing *gpa-2* may represent additional sensory neurons that repress recovery from the dauer stage under dauer-inducing conditions. Another possibility is that the role of GPA-2 in dauer recovery is exerted in interneurons.

The Daf-c mutations *daf-11(m47)* and *daf-21(p673)* are completely suppressed by mutations that disrupt sensory cilia (VOWELS and THOMAS 1992), suggesting that their products function in the sensory endings. Inactivation of *gpa-2* and *gpa-3* only partially suppressed *daf-11* and *daf-21* mutations, suggesting that the *daf-11* and *daf-21* gene products may normally act through G proteins. Alternatively, this suppression could be indirect. For example, GPA-2, GPA-3, and the *daf-11* and *daf-21* gene products could act in different neurons that

regulate each other's activity or that trigger the same target interneuron.

GPA-2 and GPA-3 may act in parallel: The suppression of activated *gpa-2* and *gpa-3* by cilium structure mutations suggests that both GPA-2 and GPA-3 functions in sensory neural cilia. Since deletion of *gpa-2* did not suppress activated *gpa-3*, and deletion of *gpa-3* did not suppress activated *gpa-2*, it is likely these G proteins function in parallel. Conceivably, GPA-3 might couple to the pheromone receptor in the amphid sensillum, while GPA-2 couples to it in other sensory cells. Although the 58% identity shared by GPA-2 and GPA-3 argues against coupling to the same receptor, the pheromone may be a mixture of closely related compounds (GOLDEN and RIDDLE 1982) and could act via multiple receptors. It is also possible that GPA-2 and GPA-3 both act downstream of the initial signal transduction resulting from interaction of dauer pheromone with its receptor.

Chemosensation of dauer pheromone involves a network of signal tranduction pathways: The roles of the GPA-2- and GPA-3-mediated pathways in dauer formation appear to be complex. Mutants lacking either gene seemed hyperresponsive under mildly inducing conditions, whereas they were hyporesponsive under strongly inducing conditions. This might be caused by changes in different pathways in which heterotrimeric G proteins function. Moreover, constitutive activation of either *gpa-2* or *gpa-3* completely repressed response to exogenous pheromone, even though wild-type copies of both genes were present in the transgenic animals. It is likely, therefore, that the GPA-2- and GPA-3-mediated signal transduction pathways are subject to a common negative regulatory pathway such that hyperactivation of either pathway results in down-regulation of both.

The analysis of *gpa-2* and *gpa-3* mutants presented here indicates that *C. elegans* dauer formation is controlled by multiple parallel chemosensory pathways. Since deletion of both *gpa-2* and *gpa-3* does not completely eliminate the response to pheromone, additional pathways certainly exist. All of these pathways must ultimately be integrated so that dauer larvae are produced and maintained only when necessary.

We thank BETSY MALONE and JIM THOMAS for help and advice on the pheromone response assays and for sending us the strain JT6130, and ANDY FIRE for providing us with the *lacZ* and *GFP* expression vectors. We thank CORI BARGMANN and IKUE MORI for additional phenotypic analysis of deletion mutants, HENRI VAN LUENEN for help in preparing the manuscript and RIK KORSWAGEN, LORNA BRUNDAGE and DAVE SONNENBORN for critical reading of the manuscript. Some of the strains were provided by the Caenorhabditis Genetic Center. This work was supported by a PIONIER grant from the Netherlands Organization for Scientific Research (NWO) to R.H.A.P. and from the Human Frontiers in Science Program (HFSP) to R.H.A.P. and P.W.S., an investigator with the Howard Hughes Medical Institute. The transposon mutant library from which the Tc1 mutants were isolated is currently supported by grant 5 RO1 RR10082 from the National Institutes of Health National Center for Research Resources.

LITERATURE CITED

ALBERT, P. S., S. J. BROWN and D. L. RIDDLE, 1981 Sensory control of dauer larva formation in *Caenorhabditis elegans*. J. Comp. Neurol. **198:** 435–451.

BARGMANN, C. I., and H. R. HORVITZ, 1991a Chemosensory neurons with overlapping functions direct chemotaxis to multiple chemicals in *C. elegans*. Neuron **7:** 729–742.

BARGMANN, C. I., and H. R. HORVITZ, 1991b Control of dauer development by chemosensory neurons in *Caenorhabditis elegans*. Science **251:** 1243–1246.

BARGMANN, C. I., E. HARTWEIG and H. R. HORVITZ, 1993 Odorant-selective genes and neurons mediate olfaction in *C. elegans*. Cell **74:** 515–527.

BARGMANN, C. I., J. H. THOMAS and H. R. HORVITZ, 1990 Chemosensory cell function in the behavior and development of *Caenorhabditis elegans*. Cold Spring Harbor Symp. Quant. Biol. **50:** 529–538.

BRENNER, S., 1974 The genetics of *Caenorhabditis elegans*. Genetics **77:** 71–94.

BUCK, L., and R. AXEL, 1991 A novel multigene family may encode odorant receptors: a molecular basis of odor recognition. Cell **65:** 175–187.

CASSADA, R. C., and R. L. RUSSELL, 1975 The dauer larva, a post-embryonic developmental variant of the nematode *Caenorhabditis elegans*. Dev. Biol. **46:** 326–342.

CHALFIE, M., Y. TU, G. EUSKIRCHEN, W. W. WARD and D. C. PRASHER, 1994 Green fluorescent protein as a marker for gene expression. Science **263:** 802–805.

CULOTTI, J. G., and R. L. RUSSELL, 1978 Osmotic avoidance defective mutants of the nematode *Caenorhabditis elegans*. Genetics **90:** 243–256.

ESTEVEZ, M., L. ATTISANO, J. L. WRANA, P. S. ALBERT, J. MASSAGUE *et al.*, 1993 The *daf-4* gene encodes a bone morphogenetic protein receptor controlling *C. elegans* dauer larva development. Nature **365:** 644–649.

FINO SILVA, I., and R. H. A. PLASTERK, 1990 Characterization of a G-protein alpha-subunit gene from the nematode *Caenorhabditis elegans*. J. Mol. Biol. **215:** 483–487.

FIRE, A., S. WHITE HARRISON and D. DIXON, 1990 A modular set of *lacZ* fusion vectors for studying gene expression in *Caenorhabditis elegans*. Gene **93:** 189–198.

GEORGI, L. L., P. S. ALBERT and D. L. RIDDLE, 1990 *daf-1*, a *C. elegans* gene controlling dauer larva development, encodes a novel receptor protein kinase. Cell **61:** 635–645.

GOLDEN, J. W., and D. L. RIDDLE, 1982 A pheromone influences larval development in the nematode *Caenorhabditis elegans*. Science **218:** 578–580.

GOLDEN, J. W., and D. L. RIDDLE, 1984a The *Caenorhabditis elegans* dauer larva: developmental effects of pheromone, food and temperature. Dev. Biol. **102:** 368–378.

GOLDEN, J. W., and D. L. RIDDLE, 1984b A pheromone-induced developmental switch in *Caenorhabditis elegans:* temperature-sensitive mutants reveal a wild-type temperature-dependent process. Proc. Natl. Acad. Sci. USA **81:** 819–823.

HALL, D. H., and R. L. RUSSELL, 1991 The posterior nervous system of the nematode *Caenorhabditis elegans:* serial reconstruction of identified neurons and complete patterning of synaptic interaction. J. Neurosci. **11:** 1–22.

HAN, M., and P. W. STERNBERG, 1991 Analysis of dominant-negative mutations of the *Caenorhabditis elegans let-60 ras* gene. Genes Dev. **5:** 2188–2198.

HEDGECOCK, E. M., J. G. CULOTTI, J. N. THOMSON and L. A. PERKINS, 1985 Axonal guidance mutants of *Caenorhabditis elegans* identified by filling sensory neurons with fluorescein dyes. Dev. Biol. **111:** 158–170.

HERMAN, R. K., and E. M. HEDGECOCK, 1990 Limitation of the size of the vulval primordium of *Caenorhabditis elegans* by *lin-15* expression in surrounding hypodermis. Nature **348:** 169–171.

HODGKIN, J., 1985 Novel nematode amber suppressors. Genetics **111:** 287–310.

HODGKIN, J., H. R. HORVITZ and S. BRENNER, 1979 Nondisjunction mutants of the nematode *Caenorhabditis elegans*. Genetics **91:** 67–94.

KAPLAN, J. M., and H. R. HORVITZ, 1993 A dual mechanosensory

and chemosensory neuron in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA **90:** 2227–2231.

KLASS, M., and D. HIRSH, 1976 Non-ageing developmental variant of *Caenorhabditis elegans*. Nature **260:** 523–525.

LEE, Y.-J., S. SHAH, E. SUZUKI, T. ZARS, P. M. O'DAY *et al.*, 1994 The *Drosophila dgq* gene encodes a Gα protein that mediates phototransduction. Neuron **13:** 1143–1157.

LEWIS, J. A., and J. A. HODGKIN, 1977 Specific neuroanatomical changes in chemosensory mutants of the nematode *Caenorhabditis elegans*. J. Comp. Neurol. **172:** 489–510.

LOCHRIE, M. A., J. E. MENDEL, P. W. STERNBERG and M. I. SIMON, 1991 Homologous and unique G-protein alpha subunits in the nematode *Caenorhabditis elegans*. Cell Regul. **2:** 135–154.

MARGOLSKEE, R. F., 1993 The molecular biology of taste transduction. BioEssays **10:** 645–650.

MASTERS, S. B., R. T. MILLER, M.-H. CHI, F.-H. CHANG, B. BEIDERMAN *et al.*, 1989 Mutations in the GTP-binding site of Gs α alter stimulation of adenylyl cyclase. J. Biol. Chem. **264:** 15467–15474.

MELLO, C. C., J. M. KRAMER, D. STINCHCOMB and V. AMBROS, 1991 Efficient gene transfer in *C. elegans:* extrachromosomal maintenance and integration of transforming sequences. EMBO J **10:** 3959–3970.

MENDEL, J. E., H. C. KORSWAGEN, K. S. LIU, Y. M. HAJDU-CRONIN, M. I. SIMON *et al.*, 1995 Participation of the Protein Go in multiple aspects of behavior in *C. elegans*. Science **267:** 1652–1655.

PERKINS, L. A., E. M. HEDGECOCK, J. N. THOMPSON and J. G. CULOTTI, 1986 Mutant sensory cilia in the nematode *Caenorhabditis elegans*. Dev. Biol. **117:** 456–487.

RIDDLE, D. L. 1988 The dauer larva, pp. 393–412 in *The Nematode Caenorhabditis elegans*, edited by W. WOOD. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

RIDDLE, D. L., M. M. SWANSON and P. S. ALBERT, 1981 Interacting genes in nematode dauer larva formation. Nature **290:** 668–671.

SÉGALAT, L., D. A. ELKES and J. M. KAPLAN, 1995 Modulation of serotonin-controlled behaviors by Go in *Caenorhabditis elegans*. Science **267:** 1648–1651.

SIMON, M. I., M. P. STRATHMANN and N. GAUTAM, 1991 Diversity of G proteins in signal transduction. Science **252:** 802–808.

STRYER, L., 1986 Cyclic GMP cascade of vision. Annu. Rev. Neurosci. **9:** 87–119.

SWANSON, M. M., and D. L. RIDDLE, 1981 Critical periods in the development of the *Caenorhabditis elegans* dauer larva. Dev. Biol. **84:** 27–40.

THOMAS, J. H., 1993 Chemosensory regulation of development in *C. elegans*. BioEssays **15:** 791–797.

THOMAS, J. H., D. A. BIRNBY and J. J. VOWELS, 1993 Evidence for parallel processing of sensory information controlling dauer formation in *Caenorhabditis elegans*. Genetics **134:** 1105–1117.

TROEMEL, E. R., J. H. CHOU, N. D. DWYER, H. A. COLBERT and C. I. BARGMANN, 1995 Divergent seven transmembrane receptors are candidate chemosensory receptors in *C. elegans*. Cell **83:** 207–218.

VOWELS, J. J., and J. H. THOMAS, 1992 Genetic analysis of chemosensory control of dauer formation in *Caenorhabditis elegans*. Genetics **130:** 105–123.

VOWELS, J. J., and J. H. THOMAS, 1994 Multiple chemosensory defects in *daf-11* and *daf-21* mutants of *Caenorhabditis elegans*. Genetics **138:** 303–316.

WARD, S., N. THOMSON, J. G. WHITE and S. BRENNER, 1975 Electron microscopical reconstitution of the anterior sensory anatomy of the nematode *Caenorhabditis elegans*. J. Comp. Neurol. **160:** 313–338.

WHITE, J. G., E. SOUTHGATE, J. N. THOMSON and S. BRENNER, 1986 The structure of the nervous system of the nematode *Caenorhabditis elegans*. Phil. Trans. Roy. Soc. Lond. B **314:** 1–340.

WOOD, W. B., 1988 *The Nematode Caenorhabditis elegans*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

WONG, Y. H., A. FEDERMAN, A. M., PACE, I. ZACHARY, T. EVANS *et al.*, 1991 Mutant α subunits of Gi2 inhibit cyclic AMP accumulation. Nature **351:** 63–65.

ZWAAL, R. R., A. BROEKS, J. VAN MEURS, J. T. M. GROENEN and R. H. A. PLASTERK, 1993 Target-selected gene inactivation in *Caenorhabditis elegans* by using a frozen transposon insertion mutant bank. Proc. Natl. Acad. Sci. USA **90:** 7431–7435.

Communicating editor: R. K. HERMAN