Dauer Formation Induced by High Temperatures in *Caenorhabditis elegans*

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ABSTRACT

Dauer formation in *Caenorhabditis elegans* is regulated by several environmental stimuli, including a pheromone and temperature. Dauer formation is moderately induced as the growth temperature increases from 15° to 25°. Here we show that dauer formation is very strongly induced at a temperature of 27° in both wild-type animals and mutants such as *unc-64*, *unc-31*, and *unc-3*, which do not form dauers at 25°. A 27° temperature stimulus is sufficient to induce dauer formation in wild-type animals independent of pheromone. Analysis of previously described dauer mutants at 27° reveals a number of surprising results. Several classes of mutants (*dyf*, *daf-3*, *tax-4*, and *tax-2*) that are defective in dauer formation at lower temperatures reverse their phenotypes at 27° and form dauers constitutively. Epistasis experiments place *unc-64* and *unc-31* at a different position in the dauer pathway from *unc-3*. We also uncover new branches of the dauer pathway at 27° that are not detected at 25°. We show that epistatic gene interactions can show both quantitative and qualitative differences depending on environmental conditions. Finally, we discuss some of the possible ecological implications of dauer induction by high temperatures.

UNDER favorable environmental conditions, the nematode *Caenorhabditis elegans* life cycle consists of four larval stages (L1–L4) in the progression to an adult. However, if environmental conditions are unfavorable, a worm may arrest development following the L2 stage and become a dauer larva. Dauers have several morphological and physiological alterations that make them well adapted for long-term survival and resistant to harsh environmental conditions (Cassada and Russell 1975; Riddle and Albert 1997). Upon the return of favorable environmental conditions, dauers can recover and complete normal development. Since environmental conditions outside of the laboratory presumably are frequently unfavorable, correct regulation of dauer formation is likely to be of considerable ecological importance.

Three environmental cues are known to regulate the decision to form a dauer. The most critical is the concentration of a pheromone that is constitutively secreted throughout the life cycle, serving as an indicator of population density (Golden and Riddle 1982; Ohba and Ishibashi 1982). The pheromone has been partially purified and consists of several related molecules similar to hydroxylated fatty acids (Golden and Riddle 1984c). Temperature and food signals modulate the dauer decision, with higher temperatures and lower amounts of food increasing the frequency of dauer formation (Golden and Riddle 1984a,b). However, it has been thought that pheromone is both necessary and sufficient for dauer formation. The fact that pheromone is capable of inducing dauer formation at low temperatures in the presence of ample food suggests that it is sufficient to induce dauer formation. Evidence for the necessity of pheromone comes from analysis of the *daf-22* mutant, which does not produce pheromone (Golden and Riddle 1985). *daf-22* mutants do not form dauers if crowded and starved (while wild-type worms do) and a pheromone extract prepared from *daf-22* mutants is not capable of inducing dauer formation in wild-type animals. Furthermore, *daf-22* mutants are capable of forming dauers in response to exogenously supplied pheromone.

Pheromone is sensed by chemosensory neurons that have endings directly exposed to the environment in the bilateral amphid organs at the tip of the worm's nose (Perkins *et al.* 1986). By killing cells with a laser, researchers have shown that several different amphid neurons regulate pheromone response (Bargmann and Horvitz 1991; Schackwitz *et al.* 1996). The ASI and ADF neurons repress dauer formation in the absence of pheromone and derepress dauer formation in the presence of pheromone. Killing these cells leads to inappropriate dauer formation. In contrast, the ASJ neuron promotes dauer formation in the presence of pheromone. Killing this cell leads to reduced responsiveness to pheromone. All the other amphid sensory neurons have been killed with little documented effect on dauer formation.

Genetic analysis of dauer formation has led to the

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isolation of many mutants that fall into two general classes: dauer formation constitutive (Daf-c) mutants form dauers inappropriately under noninducing conditions while dauer formation defective (Daf-d) mutants fail to form dauers under inducing conditions. Analysis of synergistic and epistatic gene interactions in many double mutants has led to the formal genetic pathway shown in Figure 1A (Vowels and Thomas 1992; Thomas *et al.* 1993; Gottlieb and Ruvkun 1994). The parallel branches of the genetic pathway have been correlated to specific sensory neurons acting in parallel (Schackwitz *et al.* 1996). Killing ASJ suppresses the Daf-c phenotype of *daf-11* and *daf-21* mutants but has little effect on the group II Daf-c mutants, suggesting that the group I Daf-c pathway functions through this neuron. Cell isolation experiments suggest that ASI and ADF mediate the group II Daf-c pathway. Killing all the other amphid neurons in *daf-7* or *daf-1* mutants did not prevent dauer formation, suggesting that these neurons were sufficient to convey the Daf-c signal. Furthermore, a *daf-7::gfp* construct showed expression only in ASI (Ren *et al.* 1996; Schackwitz *et al.* 1996).

Mutations in the large group of Daf-d genes (*dyf*) located downstream of the group I Daf-c genes and upstream of the group II Daf-c genes affect the structure of the ciliated sensory endings of the amphid neurons, rendering them nonresponsive to pheromone (Perkins *et al.* 1986; Vowels and Thomas 1994; Starich *et al.* 1995). Mutants of this class (known as cilium-structure mutants) can easily be scored by their inability to take up the fluorescent dye FITC in their amphid neurons (Perkins *et al.* 1986), a phenotype known as dye-filling defective (Dyf). Since these mutations suppress the *daf-11* and *daf-21* Daf-c phenotypes, it is thought that *daf-11* and *daf-21* function in the sensory endings. The *daf-11* gene has been shown to encode a transmembrane guanylyl cyclase (Birnby *et al.* 2000), consistent with a role in sensory transduction. The group II genes have been shown to encode components of a TGF-β signaling pathway (Georgi *et al.* 1990; Estevez *et al.* 1993; Ren *et al.* 1996; Patterson *et al.* 1997; Inoue and Thomas 2000). Such pathways have usually been implicated as functioning in development (Kingsley 1994), so the involvement in neuronal function is unexpected. How these molecules might contribute or respond to neuronal activity is not understood, but there is evidence that *daf-7* gene expression is affected by the pheromone, temperature, and food signals that regulate dauer formation (Ren *et al.* 1996; Schackwitz *et al.* 1996). The third branch of the pathway (the insulin branch) consists of genes encoding components of an insulin receptor signaling pathway (Morris *et al.* 1996; Kimura *et al.* 1997; Lin *et al.* 1997; Ogg *et al.* 1997; Paradis and Ruvkun 1998; Paradis *et al.* 1999). It is not yet clear whether the activity of this pathway is regulated by sensory input. The final gene in the pathway *daf-12* encodes a steroid hormone receptor (Yeh 1991) and likely mediates the execution of the dauer developmental program in response to the neuronal inputs.

While much progress has been made in identifying the molecular and cellular components involved in regulating dauer formation, there is still much to be learned. For example, it is not known what cells sense temperature and food, nor at what step or branch of the genetic pathway these signals are integrated. Furthermore, while many screens have been done for genes with a strong Daf-c phenotype at 25°, there is evidence that many other genes have roles in regulating dauer formation (Avery 1993; Katsura *et al.* 1994; Malone *et al.* 1996; Iwasaki *et al.* 1997; Prasad *et al.* 1998; Take-Uchi *et al.* 1998; Ailion *et al.* 1999; Koga *et al.* 1999; Sze *et al.* 2000). Many of these genes have a synthetic Daf-c (Syn-Daf) phenotype at 25° that requires mutations in two genes to generate a detectable Daf-c phenotype, explaining why they had been missed in screens at 25°. Here we show that single mutants of several of these genes have highly penetrant Daf-c phenotypes at 27°, namely *unc-3*, which encodes a transcription factor (Prasad *et al.* 1998), and *unc-31* and *unc-64*, which encode homologs of CAPS and syntaxin, proteins that regulate secretion and synaptic transmission (Livingstone 1991; Ann *et al.* 1997; Ogawa *et al.* 1998; Saifee *et al.* 1998). We characterize the wild-type response to 27° and show that dauer formation is strongly induced at this temperature in a pheromone-independent manner. We perform epistasis experiments on the new genes to place them in the dauer pathway. This study reveals new branches to the dauer pathway that are not detected at 25° and demonstrates that a number of genes unexpectedly have both positive and negative regulatory influences on dauer formation. Figure 1B illustrates some of the differences in the dauer pathway observed at 27°.

MATERIALS AND METHODS

General growth conditions and strain maintenance: *C. elegans* strains were cultured and manipulated using standard methods (Brenner 1974). All strains were derivatives of the Bristol wild-type strain N2. Worms were grown on *Escherichia coli* strain TJ2, a derivative of OP50. TJ2 has always been used as the standard *E. coli* strain in this lab. TJ2 shows auxotrophic differences from OP50 (data not shown). The possible effect of these differences on *C. elegans* dauer formation was not investigated. TJ2 was cultured by serial passage for up to a few months before returning to the original frozen culture. This article follows the standard *C. elegans* nomenclature (Horvitz *et al.* 1979). The CB246 *unc-64(e246)* strain was found to have an unlinked temperature-sensitive sterile mutation. *unc-64(e246)* was outcrossed to remove this mutation and all assays were performed on the outcrossed strain. A complete list of strains and mutations used is available upon request.

Dauer formation assays: Parents raised continuously on food at 20° were allowed to lay eggs for 3–6 hr at room temperature (~22°) and progeny were incubated at the assay temperature. Dauer and nondauer animals were counted after ~100 hr at 15°, 65 hr at 20°, 54 hr at 22°, 48 hr at 25°, and 44 hr at 27°, which permitted correct scoring of transient dauers

that recover rapidly. Dauer assays have a tendency to show quantitative variability from experiment to experiment (presumably due to the input of multiple environmental conditions that are hard to control rigorously), and this was especially true at 27° due to the particular sensitivity of dauer formation around this temperature. Temperature differences of 0.5° or less can have significant quantitative effects on dauer formation at temperatures near 27°. We found that there was temperature variability of at least 0.5° both at different locations within an incubator and at the same location of an incubator examined at different times. To demonstrate that such variability could have significant effects on dauer formation, we performed an experiment in which we assayed *unc-31(e928)* dauer formation on many plates distributed throughout our incubator. Spatial differences in temperature ranged from 26.5° to 27.1° and *unc-31* ranged from 60 to 100% dauers in agreement with the local temperature. Because of such spatial and temporal variability in dauer formation, each table in this article presents the results from a single experiment in which all strains were assayed in parallel in close proximity in the incubator. In cases where a table is divided by extra space, each section of the table presents the results from a single experiment, but different sections represent different experiments. Experiments were repeated multiple times with quantitative variability in the absolute numbers, but the relative differences between strains were consistent. For assays at 25° and 27°, temperature was measured using a thermometer (ASTM no. 23C from VWR) accurate to 0.1°. This thermometer was placed in close proximity to the assay plates on the same shelf of the incubator. The reported temperature for any given experiment is an average of the temperature measured at the start of the experiment when plates were placed at the assay temperature and the end of the experiment when plates were removed to count dauers. However, since there is temporal variability, this reported temperature might not represent the average temperature of the assay. Temperature in the text is referred to as 25° or 27° for simplicity, but in actuality "25°" was 25.0°–25.6° and "27°" was 26.6°–27.1°. The temperature on the surface of the agar was not measured, so the temperature experienced by the worms may vary slightly from the measured temperature. The primary 27° incubator was a heated incubator placed in a room at 4°. A small fan was placed on the top shelf of the incubator to minimize temperature variability within the incubator. Experiments performed at 27° in a heating/refrigerating incubator at room temperature or in a sealed plastic tupperware container submerged in a 27° water bath gave similar results.

Assays of dauer formation at 27° present technical problems in addition to the variability described above. Some strains (*e.g.*, N2) form dauers at 27°, which recover within a few hours. Tightly synchronized egg lays could not solve this problem completely, since growth of strains at 27° tends to be somewhat asynchronous, even when egg lays were synchronous. This is probably due to the general unhealthiness of worms grown at high temperatures. In all dauer formation assays, animals at the L1 or L2 stage of development were counted, but not included in the presented data.

Pheromone assays: Plates with partially purified dauer pheromone were prepared as described (Vowels and Thomas 1994). Different pheromone preparations were used in different experiments. Dauer formation is induced slightly on pheromone assay media (without pheromone) relative to standard nematode growth plates. Within an experiment, all strains were grown in duplicate at each pheromone concentration and plates were randomly distributed in a sealed plastic tupperware container with a moist paper towel to prevent drying of the small plates. Dauer formation is partially suppressed by drying of the plate (data not shown).

Starvation assays: Dauer formation in response to starvation was assayed by picking two adult animals to plates at 20° and checking to see when the bacterial lawn was completely gone. Four days later the plates were flooded with 1% SDS and scored after 15 min for the presence of dauers (live thrashing animals).

Construction of double and triple mutant strains: Double and triple mutant strains were constructed and confirmed by the methods described previously (Vowels and Thomas 1992; Thomas *et al.* 1993). A detailed description of strain constructions is available upon request.

Dominance tests: Dominance of Daf-c mutants at 27° was assayed by mating wild-type males to marked *daf-c* strains at 20° for 1 day, then performing synchronous egg lays at room temperature and allowing the broods to develop at 27°. Unmarked dauers and nondauers were counted. For *daf-7*, the cross was also performed in the reciprocal direction, mating heterozygous *daf-7/+* males to *unc-33(e204)* hermaphrodites, to control for the possibility of a maternal effect.

Expression of *daf-7::gfp*: Animals carrying the integrated *daf-7::gfp* array *saIs8* were grown at various temperatures to the L2 stage at which maximal expression was observed (Schackwitz *et al.* 1996). Green fluorescent protein (GFP) fluorescence was observed at 1000× magnification using a compound microscope with UV illumination. ASI was identified by cell position viewed with Nomarski optics.

Cell kills: ASI and ADF were identified by cell position and killed by a laser in L1 larvae within 2 hr of hatching as described (Avery and Horvitz 1987; Schackwitz *et al.* 1996), except that parents were grown at 20° rather than being preadapted to the assay temperature.

RESULTS

Synthetic Daf-c genes: Screens for simple loss-of-function mutants with a strong Daf-c phenotype at 25° have probably been saturated (Malone and Thomas 1994). However, a number of mutants with Syn-Daf phenotypes have been described in recent years (Avery 1993; Katsura *et al.* 1994; Iwasaki *et al.* 1997; Take-Uchi *et al.* 1998; Ailion *et al.* 1999; Daniels *et al.* 2000). In this article, we consider three of these genes: *unc-3*, *unc-31*, and *unc-64*. As shown in Table 1, the *unc-64; unc-31*, *unc-64; unc-3*, and *unc-31; unc-3* double mutant strains are strongly Daf-c at 25°, while the three single mutants are either not Daf-c or are only weakly Daf-c (*unc-3* appears to be particularly variable at 25°, possibly due to local starvation of part of a plate). The Syn-Daf phenotypes are less penetrant at 15°, reflecting the intrinsic temperature sensitivity of dauer formation (Golden and Riddle 1984b; Malone and Thomas 1994).

We examined the phenotypes of several triple mutants of two Syn-Daf genes with a Daf-d gene in order to place the Syn-Daf mutant in the dauer pathway (see Figure 1). The Daf-c phenotype of an *unc-64; unc-31* double mutant was not suppressed by either *daf-3* or *daf-5* but was completely suppressed by *daf-12*. The *unc-31; unc-3* double mutant was suppressed by *daf-5* at 15° but not at 25° and was suppressed by *daf-12* at both temperatures. These results suggest that the Syn-Daf combinations act genetically in parallel to or downstream of the group II pathway shown in Figure 1. In support of this

TABLE 1

Dauer formation in synthetic Daf-c mutants

Genotype	Dauer formation (%) 15°	25°
unc-64(e246); unc-3(e151)	6 (236)	100 (272)
unc-64(e246); unc-31(e928)	77 (210)	100 (212)
unc-31(e928); unc-3(e151)	18 (202)	99 (316)
unc-64(e246); unc-31(e928); daf-3(e1376)	87 (176)	100 (204)
daf-5(e1385); unc-64(e246); unc-31(e928)	81 (197)	99 (135)
unc-64(e246); unc-31(e928); daf-12(m20)	0 (177)	0 (173)
daf-5(e1385); unc-31(e928); unc-3(e151)	0 (263)	100 (348)
unc-31(e928); daf-12(m20) unc-3(e151)	0 (155)	0 (204)
unc-64(e246)	1 (177)	1 (178)
unc-31(e928)	0 (228)	0 (271)
unc-3(e151)	0 (237)	33 (325)
daf-3(e1376)	0 (296)	0 (365)
daf-5(e1385)	0 (199)	0 (278)
daf-12(m20)	0 (270)	0 (341)

In all tables with dauer counts, the number in parentheses is the number of animals counted.

idea, the *unc-64; unc-31* double mutant was completely suppressed by mutations in *daf-16* (data not shown). The partial suppression of *unc-31; unc-3* by *daf-5* is consistent with the idea that *unc-3* acts in the group II pathway (see below).

A synthetic Daf-c phenotype could result from true genetic redundancy or from the additive effect of several weak Daf-c phenotypes. To test whether the single mutants are shifted toward forming dauers, we measured dauer formation in response to various amounts of exogenous pheromone. As shown in Figure 2A, *unc-3*, *unc-31*, and *unc-64* mutants are all hypersensitive to dauer pheromone at 25°. The Syn-Daf mutant *aex-3* is not hypersensitive to dauer pheromone (data not shown), indicating that pheromone hypersensitivity is not a property of all Syn-Daf mutants. *unc-3* and *unc-64* mutants remain hypersensitive to dauer pheromone when assayed at 22°, but the *unc-31(e928)* mutant at 22° is actually less sensitive to pheromone than N2 (Figure 2B). To determine whether this surprising phenotype is specific to the *e928* allele (a deletion of most of the *unc-31* gene and expected null; Livingstone 1991), we assayed two other alleles of *unc-31* for pheromone response at 22° and 25°. All three alleles exhibited clear hypersensitivity at 25° (Figure 2C) and reduced sensitivity at 22° (Figure 2D), indicating that this phenotype is not allele specific. While the reversal of the *unc-31* response is not easy to interpret (see discussion), the hypersensitivity of *unc-3*, *unc-31*, and *unc-64* at 25° indicates that some single Syn-Daf mutations do affect dauer formation on their own. This observation is extended in the following section.

Syn-Daf single mutants are Daf-c at 27°: During our study of Syn-Daf mutants, we made a fortuitous discovery while performing experiments in which the incubator temperature was accidentally set slightly high, at approximately 27°. At this temperature, we found that *unc-3*, *unc-31*, and *unc-64* mutants had strong Daf-c phenotypes on their own (Table 2). The Daf-c phenotype of these mutants was clearly weaker at 26°, indicative of the strong temperature dependence. Wild-type N2 worms did not form dauers in initial experiments at 27°. However, during many repetitions of this experiment, we noticed occasional dauers on N2 plates. It is now clear that N2 is weakly Daf-c at 27°, but formation of dauers is variable from experiment to experiment, probably due to slight differences in incubation temperature (see materials and methods). Furthermore, N2 dauers formed at 27° recover rapidly at 27° (data not shown), which can make scoring difficult, even in synchronized broods. The strong 27° Daf-c phenotype is called the *h*igh temperature-*i*nduced *d*auer formation (Hid) phenotype to distinguish it from the weak 27° Daf-c phenotype of wild type. N2 generally has <20% dauers at temperatures around 27°, but on rare occasions was seen to make up to 75% dauers. The Hid phenotype of *unc-3*, *unc-31*, and *unc-64* was not allele specific. *unc-3(e54)*, *unc-3(e95)*, *unc-3(cn4146)*, *unc-31(u280)*, *unc-31(e169)*, *unc-64(md1259)*, and *unc-64(md130)* were all found to have a Hid phenotype (Ailion *et al.* 1999 and data not shown). The Hid phenotype of *unc-3* mutants was confirmed by others subsequent to our finding (Prasad *et al.* 1998).

Dauers formed at 27° in mutant or wild-type strains are often paler than dauers of the same strains formed at 25°. To assess whether 27° dauers are true dauers (as opposed to partial dauers such as those made by *daf-16* mutants), we scored several dauer-specific features that can be visualized by Nomarski microscopy: presence of dauer alae, remodeling of the pharynx, presence of hypodermal bodies, and the presence of highly refractile material in the gut (Vowels and Thomas 1992). We also scored another dauer feature, resistance to 1% SDS. N2, *unc-3*, *unc-31*, and *unc-64* dauers formed at 27° had all the characteristic features of dauers formed at lower temperatures and thus are indeed true dauers (though there were often fewer hypodermal bodies in 27° dauers). The amount of refractile material in the gut correlated with the darkness of a dauer seen using a dissecting microscope.

Temperature sensitivity of pheromone response: The *unc-3*, *unc-31*, and *unc-64* mutants are clearly sensitive to small temperature differences in the narrow range from 25° to 27°. To see if this sensitivity is specific to these mutants or is a wild-type phenomenon, we assayed N2 dauer formation in response to exogenous pheromone at various temperatures. As shown in Figure 3A,

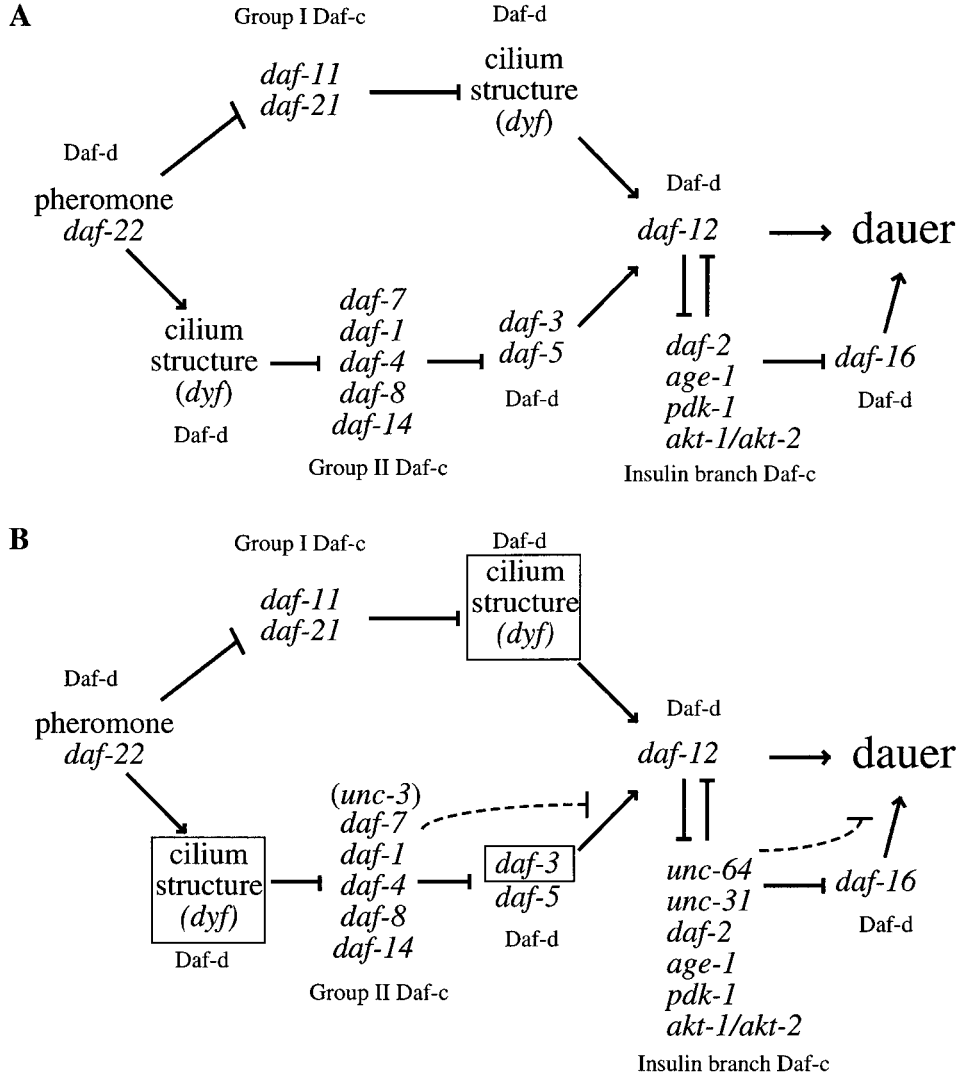

FIGURE 1.—Genetic pathways that regulate dauer formation. (A) The pathway as determined at 25°. (B) Additions to the pathway as observed at 27°. Genes with different phenotypes at 27° (*dyf* and *daf-3*) are boxed and additional branches to the pathway are drawn with dashed lines. *unc-64*, *unc-31*, and *unc-3* are added to the pathway. Since *unc-3* acts partially in parallel to the group II Daf-c genes, it is drawn in parentheses. See text for detailed explanations.

temperature had a modest effect on wild-type pheromone response from 15° to 25° as shown previously (GOLDEN and RIDDLE 1984a,b). However, N2 responded much more strongly to pheromone at 27° than at 25°, suggesting that wild-type dauer formation is highly sensitive to this temperature difference and that the mutant phenotypes are likely to reflect an underlying wild-type sensitivity.

We also performed similar pheromone response assays on the mutant *ttx-1(p767)*, which has defects in the morphology of the candidate thermosensory cell AFD and defects in thermotaxis behavior (HEDGECOCK and RUSSELL 1975; PERKINS *et al.* 1986; MORI and OHSHIMA 1995). As shown in Figure 3B, the *ttx-1* mutant formed more dauers as the temperature was increased from 15° to 25° and like N2 showed an extremely strong response at 27°, including a low frequency of dauer formation in the absence of exogenous pheromone. Thus, it seems unlikely that AFD is solely responsible for the temperature input to dauer formation. As demonstrated before (GOLDEN and RIDDLE 1984b), the *ttx-1* mutant is hypersensitive to the dauer pheromone, suggesting that AFD plays some role in dauer formation, albeit not an essential one.

Dauer formation at high temperatures can occur independently of pheromone: As noted earlier, N2 dauer formation at 27° is much more sensitive to pheromone than at 25°. However, N2 also forms a low frequency of dauers at 27° on plates with ample food and no exogenously added pheromone, which does not happen at 25°. Two possibilities could account for this phenomenon. Dauer formation by N2 at 27° could result from endogenous pheromone made by the tested larvae, but present at a level insufficient to induce dauer formation at 25°. Alternatively, dauer formation at 27° could occur independently of pheromone. To distinguish between these possibilities, we assayed dauer formation of *daf-22(m130)* mutant animals at 27°. The *daf-22* mutant does not produce pheromone and has a Daf-d phenotype at lower temperatures that can be rescued by exogenously supplied pheromone (GOLDEN and RIDDLE 1985). When grown at 27°, *daf-22* mutants formed dauers at a fre-

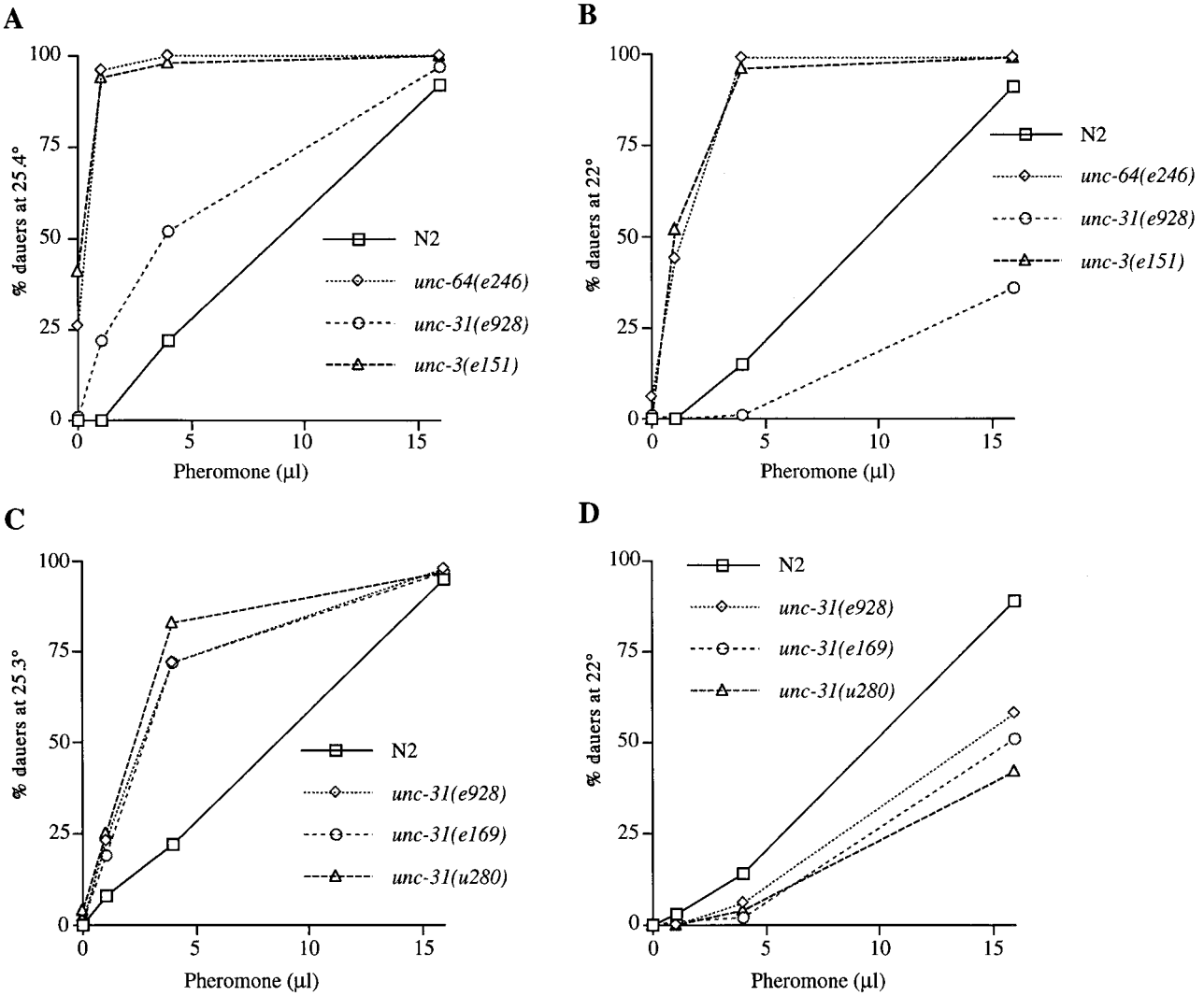

FIGURE 2.—Dauer formation of *unc-64*, *unc-31*, and *unc-3* mutants in response to exogenous pheromone. Each graph plots the percentage of animals that formed dauers in response to different concentrations of pheromone at the given temperature. Approximately 100–200 animals were counted at each concentration of pheromone.

quency similar to N2. *daf-22* dauers formed at 27° were examined by Nomarski microscopy and had all the features typical of dauers. Thus, it appears that in addition to a highly sensitized pheromone response, dauer formation can also occur independently of pheromone at 27°, although the possibility that *daf-22* animals make pheromone only at 27° is not excluded.

Dauer formation at 27° in Daf-d mutants: The finding that *daf-22*, a Daf-d mutant, behaves similarly to N2 at 27° in producing dauers led us to examine other Daf-d mutants at 27°. Daf-d mutants are characterized by several phenotypes at 25° or lower temperatures: inability to form dauers following starvation, inability to form dauers in response to exogenously added pheromone, and suppression of Daf-c mutants upstream in the dauer pathway. Since dauer formation at 27° can occur independently of pheromone, these phenotypes of Daf-d mutants are not necessarily predicted to be the same at 27°.

As shown in Table 3, Daf-d mutants show several unexpected phenotypes at 27°. Mutations in the Dyf genes such as *daf-10* and *osm-6*, which affect the structure of the ciliated endings of the amphid sensory neurons, lead to a Daf-c phenotype at 27°. This varies in strength from gene to gene but, in the strongest (*e.g.*, *osm-6*, *osm-5*, *che-11*), is almost completely penetrant and is always significantly stronger than N2. This Hid phenotype was seen in all 16 Dyf mutants that we tested (Table 4) and was confirmed by others subsequent to our finding (APFELD and KENYON 1999). The *daf-6(e1377)* mutation, which affects the structure of the amphid sheath cell (ALBERT *et al.* 1981), did not affect dauer formation as strongly. In multiple assays of the *daf-6* mutant, a weak Daf-c phenotype at 27° was occasionally seen but usually it formed dauers at a level similar to N2. However, unlike N2 and like the other Dyf mutants, *daf-6* dauers failed to recover at 27°, consistent with a defect in responding

TABLE 2

Dauer formation of Syn-Daf mutants at 27°

Genotype	Dauer formation (%)		
	25°	26°	27°
N2	ND[a]	0 (446)	0 (76)
unc-64(e246)	0 (106)	54 (161)	99 (164)
unc-31(e928)	0 (68)	25 (203)	99 (87)
unc-3(e151)	0 (71)	12 (274)	97 (187)
unc-64(e246); unc-3(e151)	81 (95)	ND	100 (174)
unc-31(e928); unc-3(e151)	85 (106)	ND	100 (238)

[a] ND, not determined. N2 was not assayed in this experiment, but of the thousands of N2 animals grown at 25° at multiple other times, <0.1% formed dauers.

TABLE 3

Dauer formation of Daf-d mutants at 27°

Genotype	Dauer formation (%)	
	26.6°	27°
N2	1 (77)	20 (83)
unc-31(e928)	100 (58)	100 (46)
daf-22(m130)	2 (52)	7 (57)
daf-6(e1377)	1 (69)	43 (83)
che-12(e1812)[a]	1 (73)	30 (44)
daf-10(e1387)	49 (79)	72 (101)
osm-6(p811)	54 (89)	99 (91)
daf-3(sa213)	94 (63)	96 (53)

Genotype	Dauer formation at 26.9° (%)
N2	5 (181)
osm-6(p811)	68 (170)
daf-3(e1376)	74 (193)
daf-3(mgDf90)	95 (126)
daf-5(e1385)	1 (137)
daf-16(m27)	0 (177)[b]
daf-12(m20)	0 (130)[c]

[a] *che-12* is not Daf-d but is included in this table because of its amphid structure defects.

[b] Although *daf-16* did not form dauers in this particular assay, it generally formed a low percentage of dauers at 27°, similar to the level of N2 dauer formation (*e.g.*, see Tables 5 and 11).

[c] In many assays of *daf-12* at 27°, no dauer has ever been observed among >1000 animals scored.

to dauer recovery conditions. Another mutant of interest is *che-12(e1812)*, which has defects in secretion of matrix material by the amphid sheath cell but is not strongly defective in dye filling by the amphid sensory neurons (Perkins *et al.* 1986). At 27°, *che-12* mutants formed dauers at a level similar to N2 (Table 3) and the dauers recovered efficiently. Similarly, the *mec-1(e1066)* and *mec-8(e398)* mutants, which have defects in the fasciculation of the amphid cilia (Lewis and Hodgkin 1977; Perkins *et al.* 1986), are not Daf-d at lower temperatures and are not Hid (data not shown). Thus, the Hid phenotype appears to be specific to mutants with defects in the structure of the ciliated neurons themselves. Mutants that affect the structure of the amphid pore in other ways are not Hid.

Mutations in either *daf-3* or *daf-5* exhibit a strong Daf-d phenotype at 25° or lower temperatures and strongly suppress the Daf-c phenotype of group II Daf-c mutations. Surprisingly, *daf-3* mutants were strongly Daf-c at 27° while *daf-5* mutants behaved similarly to N2, forming dauers at a low percentage (Table 3). Since *daf-3* and *daf-5* had indistinguishable phenotypes in other assays, we tested whether these 27° phenotypes were allele specific. Eleven alleles of *daf-3*, including *mgDf90*, a deletion of the entire *daf-3* coding sequence (Patterson *et al.* 1997), exhibited the Hid phenotype while all four *daf-5* alleles tested behaved like N2 (Table 4), indicating that these phenotypes are not allele specific. Thus, although *daf-3* and *daf-5* have indistinguishable phenotypes at 25°, they have strikingly different phenotypes at 27°.

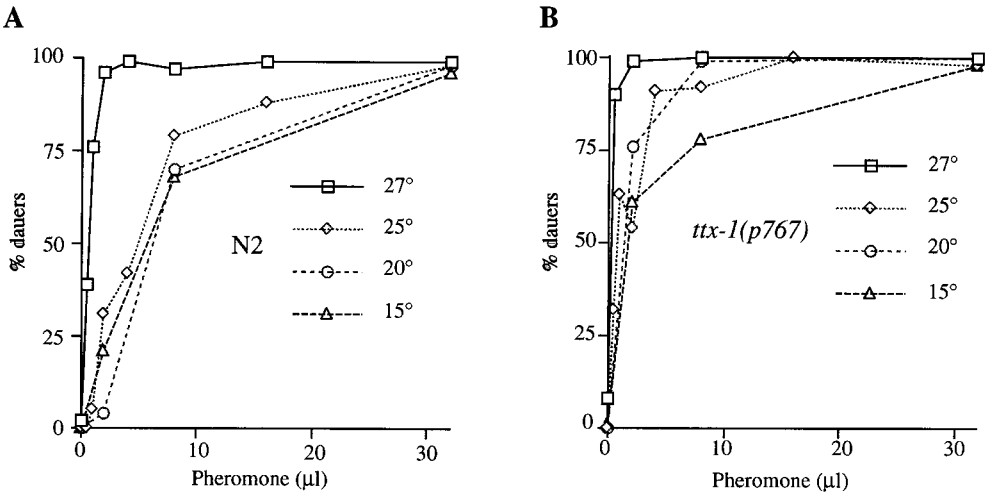

Figure 3.—Dauer formation of N2 and the *ttx-1* mutant in response to exogenous pheromone at different temperatures. Approximately 100–200 animals were counted at each concentration of pheromone.

TABLE 4

Summary of Daf-d mutants at 25° and 27°

Genotype	25°	27°
N2	+	+
daf-22	Daf-d	+
dyf (16 genes)	Daf-d	Daf-c
daf-3 (11 alleles)	Daf-d	Daf-c
daf-5 (4 alleles)	Daf-d	+
daf-16	Daf-d	+
daf-12	Daf-d	Daf-d

dyf mutants tested were *che-2(e1033)*, *che-3(e1124)*, *che-10(e1809)*, *che-11(e1810)*, *che-13(e1805)*, *daf-10(e1387)*, *dyf-1(mn335)*, *dyf-4(m158)*, *dyf-6(m175)*, *dyf-9(n1513)*, *dyf-11(mn392)*, *dyf-12(sa127)*, *osm-1(p808)*, *osm-3(e1806)*, *osm-5(p813)*, and *osm-6(p811)*. *daf-3* alleles tested were *e1376*, *mgDf90*, *mg125*, *mg132*, *sa206*, *sa207*, *sa208*, *sa209*, *sa213*, *sa214*, and *sa216*. *daf-5* alleles tested were *e1385*, *e1386*, *sa205*, and *sa244*.

Mutations in the *daf-16* gene suppress the Daf-c phenotype of mutants in the insulin branch of the dauer pathway. At 27°, *daf-16(m27)* mutants formed partial dauers at a low frequency similar to that of N2 dauer formation (Tables 3, 5, and 11). This result was seen in several other *daf-16* alleles, including *m26* and *mgDf50*, a deletion of almost all of the *daf-16* coding sequence (Ogg *et al.* 1997). Thus, dauer formation at 27° can occur independently of the insulin pathway or of the group I or group II Daf-c signaling pathways. However, dauer formation at 27° depends absolutely on the *daf-12* gene (Table 3), indicating that dauer induction at 27° shares a common output with dauer induction by other stimuli. The phenotypes of the tested Daf-d mutants at 25° and 27° are summarized in Table 4.

Response of Daf-d mutants to pheromone at 27°: Daf-d mutants do not respond to pheromone or respond only very weakly at temperatures at or below 25°. The observation that all Daf-d mutants except *daf-12* were capable of dauer formation at 27° led us to examine whether these mutants responded to pheromone at 27°. As shown in Table 5, N2 responded strongly to pheromone at a temperature near 25° while the *osm-6* and *daf-12* mutants did not respond at all and *daf-3* and *daf-5* mutants responded only very weakly. *daf-16* responded to a lesser degree than N2 and made partial dauers. At 27°, N2 still responded strongly and *daf-3* and *daf-5* mutants responded strongly as well. *osm-6* and *daf-12* still failed to respond and *daf-16* continued to respond to a lesser extent. Assaying the pheromone responsiveness of *daf-3* and *osm-6* at 27° was complicated by the fact that these mutants are Daf-c without pheromone at 27°. To circumvent this problem, we assayed pheromone responsiveness at a slightly lower temperature at which the Daf-c phenotypes of *daf-3* and *osm-6* were only partially penetrant. Under these conditions, *daf-3* responded strongly to pheromone while *osm-6* (and several other Dyf mutants) did not respond at all (data not shown). Thus, pheromone-induced dauer formation at 27° depends on the ciliated endings of sensory neurons, as at lower temperatures, but does not depend on the activities of the *daf-3* and *daf-5* genes.

Dauer formation at 25° and 27° in *tax-4* and *tax-2* mutants: To continue our characterization of dauer mutants at 27°, we examined mutants in the genes *tax-4* and *tax-2*. *tax-4* and *tax-2* encode α- and β-subunits of a cyclic nucleotide-gated (CNG) ion channel that appears to be part of the signal transduction machinery in the amphid cilia (Coburn and Bargmann 1996; Komatsu *et al.* 1996). *tax-4* and *tax-2* mutants have interesting dauer phenotypes at 25° that appear to be a combination of dauer-promoting and dauer-repressing activities; *i.e.*, mutations in *tax-4* or *tax-2* suppress the Daf-c phenotype of mutants in the group I pathway but enhance the Daf-c phenotype of mutants in the group II pathway (Coburn *et al.* 1998). This is suggestive of a *tax-4*/*tax-2* site of action in the group I pathway. To investigate this further, we examined the phenotype of *tax-4(ks11)* double mutants with Daf-d genes. *tax-4(ks11)* has a strong Daf-c phenotype at 25° (Table 6) so epistasis could be performed at this temperature. Other *tax-4* mutants including a putative null (*p678*) are only weakly Daf-c at 25°. Mutations in the group I cilium-

TABLE 5

Pheromone responses of Daf-d mutants at 25° and 27°

	Dauer formation at 25.7° (%)		Dauer formation at 26.9° (%)	
Genotype	− pheromone	+ pheromone[a]	− pheromone	+ pheromone[a]
N2	0 (177)	91 (173)	6 (190)	99 (166)
osm-6(p811)	0 (149)	0 (224)	94 (144)	91 (136)
daf-3(e1376)	0 (212)	2 (224)	84 (202)	91 (266)
daf-3(mg90)	0 (198)	18 (192)	91 (195)	100 (244)
daf-5(e1385)	0 (182)	2 (167)	0 (161)	95 (183)
daf-16(m27)	0 (220)	36 (224)[b]	5 (172)[b]	33 (215)[b]
daf-12(m20)	0 (194)	0 (229)	0 (89)	0 (186)

[a] Plates with 16 μl pheromone.
[b] Partial dauers as described (Vowels and Thomas 1992).

TABLE 6

Dauer formation in *tax-4* double mutants at 25°

Genotype	Dauer formation (%)
tax-4(ks11)	97 (421)
daf-11(sa195)	100 (210)
osm-3(e1806)	0 (>200)
daf-12(m20)	0 (>200)
tax-4(ks11); daf-11(sa195)	89 (426)
tax-4(ks11); osm-3(e1806)	0 (>200)
daf-5(e1385); tax-4(ks11)	19 (413)
daf-16(m27); tax-4(ks11)	34 (478)[a]
tax-4(ks11); daf-12(m20)	0 (>200)

[a] This count was performed in a different experiment from the rest of the counts. *tax-4(ks11)* formed 93% dauers in this experiment.

Partial dauers are as described (Vowels and Thomas 1992).

TABLE 7

Dauer formation in *tax-4* and *tax-2* mutants at 27°

Genotype	Site of mutation in the CNG channel[a]	Dauer formation at 27.0° (%)
N2	NA[b]	9 (251)
tax-4(ks11)	P to L missense in pore[c]	100 (230)
tax-4(ks28)	D to V missense in S2[d]	100 (284)
tax-4(p678)	Early stop (putative null)	100 (258)
tax-2(p671)	C to R missense in S1[d]	53 (266)[e]
tax-2(p691)	P to S missense in pore[c]	100 (233)
tax-2(p694)	Promoter deletion[f]	7 (250)

[a] Data are from Komatsu *et al.* (1996) and Coburn and Bargmann (1996).

[b] NA, not applicable.

[c] *ks11* and *p691* mutate the identical proline in the two subunits. This proline is found at the extracellular face of the pore.

[d] S1 and S2 are the first and second transmembrane domains of the channel subunits.

[e] Almost all *tax-2(p671)* dauers recovered in 24 hr.

[f] This deletion probably leads to a loss of *tax-2* expression in the AFD, ASE, ADE, and BAG neurons while not affecting channel function in other cells (Coburn and Bargmann 1996).

structure Daf-d gene *osm-3* or in *daf-12* completely suppressed the Daf-c phenotype of *tax-4(ks11)*, while mutations in *daf-5* and *daf-16* only partially suppressed it (Table 6). This is very similar to epistasis data seen with the group I Daf-c gene *daf-11* (Vowels and Thomas 1992), providing further evidence that *tax-4* functions in the group I pathway.

At 27°, all *tax-4* alleles exhibited a strong Daf-c phenotype and the dauers did not recover (Table 7). The *tax-2(p691)* mutant was also strongly Daf-c at 27° and failed to recover. The *p691* mutation affects the same proline residue in the channel pore as the strongest Daf-c *tax-4* allele *ks11* (Coburn and Bargmann 1996; Komatsu *et al.* 1996). The *tax-2(p671)* mutant was moderately Daf-c at 27°, but the dauers recovered efficiently. The *p671* mutation may simply be a weaker mutation of *tax-2* than *p691*, with a smaller defect in both dauer formation and dauer recovery. The *tax-2(p694)* mutant was not Daf-c at 27°. As inferred from an analogous GFP expression construct, this mutation eliminates expression of the channel in the AFD, ASE, ADE, and BAG neurons but does not affect expression or function of the channel in seven other cells (Coburn and Bargmann 1996), suggesting that the Hid phenotype of the other alleles results from loss or impaired channel function in other cells.

The native CNG channel is likely to be a heteromer formed of both TAX-4 α-subunits and TAX-2 β-subunits. However, the TAX-4 protein may be able to form a functional homomeric channel in the absence of TAX-2 although the reverse is unlikely (Komatsu *et al.* 1996, 1999). To determine whether *tax-4* phenotypes depended on *tax-2* or vice versa, we examined dauer formation in *tax-2; tax-4* double mutants. As shown in Table 8, all three *tax-2* mutations suppressed the Daf-c phenotype of *tax-4(ks11)* at 25°, with suppression stronger by the *p691* and *p694* mutations, consistent with the idea that *p671* is a weaker mutation. The suppression by *tax-2(p694)* implies that the 25° Daf-c phenotype of *tax-4(ks11)* depends on TAX-2 function in AFD, ASE, ADE, or BAG. However, neither *tax-4(ks11)* nor *tax-4(p678)* was suppressed by any of the *tax-2* mutations at 27°, suggesting that the 27° Daf-c phenotype of *tax-4* mutations does not depend on TAX-2 function in AFD, ASE, ADE, or BAG. The dauer recovery defect of *tax-4* mutants at 27° was partially suppressed by the *tax-2* mutations *p691* and *p694*, implying that the dauer recovery phenotype of *tax-4* depends on TAX-2 function in AFD, ASE, ADE, or BAG. The fact that *tax-2* mutations have

TABLE 8

Dauer formation of *tax-2; tax-4* double mutants

Genotype	Dauer formation (%)	
	25.2°	26.8°
N2	ND	14 (241)
tax-2(p671)	0 (349)	61 (174)[a]
tax-2(p691)	0 (201)	98 (119)
tax-2(p694)	0 (285)	0 (180)
tax-4(ks11)	78 (162)	100 (105)
tax-2(p671); tax-4(ks11)	28 (190)	99 (145)
tax-2(p691); tax-4(ks11)	9 (208)	100 (124)[b]
tax-2(p694); tax-4(ks11)	5 (176)	99 (116)[b]
tax-4(p678)	3 (156)	100 (172)
tax-2(p671); tax-4(p678)	4 (231)	100 (135)
tax-2(p691); tax-4(p678)	0 (186)	99 (112)[b]
tax-2(p694); tax-4(p678)	11 (312)	99 (151)[b]

ND, not determined.

[a] Almost all dauers recovered in 24 hr.

[b] Some dauers recovered in 24 hr.

TABLE 9

Pheromone responses of *tax-4* and *tax-2* mutants at 25° and 22°

Genotype	Dauer formation at 25.2° (%)		Dauer formation at 22° (%)	
	− pheromone	+ pheromone[a]	− pheromone	+ pheromone[a]
N2	0 (185)	92 (191)	0 (172)	86 (168)
tax-4(ks11)	92 (78)	98 (83)	5 (58)	16 (58)
tax-4(ks28)	12 (107)	33 (159)	12 (104)	31 (124)
tax-4(p678)	16 (124)	53 (97)	0 (76)	1 (124)
tax-2(p671)	0 (162)	0 (174)	0 (160)	0 (202)
tax-2(p691)	0 (75)	0 (118)	0 (60)	0 (88)
tax-2(p694)	0 (174)	0 (179)	0 (156)	0 (161)
N2	0 (243)	95 (245)	0 (260)	93 (269)
tax-2(p671)	0 (64)	0 (91)	0 (50)	0 (119)
tax-2(p691)	0 (66)	3 (66)	0 (77)	0 (73)
tax-2(p694)	0 (140)	2 (130)	0 (165)	1 (125)
tax-4(ks11)	ND	ND	7 (108)	19 (104)
tax-2(p671); tax-4(ks11)	ND	ND	4 (144)	13 (169)
tax-2(p691); tax-4(ks11)	ND	ND	4 (115)	2 (126)
tax-2(p694); tax-4(ks11)	ND	ND	0 (107)	1 (120)
tax-4(p678)	69 (143)	78 (176)	ND	ND
tax-2(p671); tax-4(p678)	67 (89)	79 (148)	ND	ND
tax-2(p691); tax-4(p678)	0 (176)	0 (161)	ND	ND
tax-2(p694); tax-4(p678)	30 (125)	50 (132)	ND	ND

ND, not determined.

[a] Plates with 14 μl pheromone in the top 7 lines and 13.3 μl of a different pheromone preparation in the bottom 12 lines.

effects in a putative *tax-4* null background also suggests that the TAX-2 protein can function in the absence of TAX-4, possibly as the partner of other α-subunits.

Responses of *tax-4* and *tax-2* mutants to pheromone: To further examine the role of *tax-4* and *tax-2* in dauer formation, we assayed dauer formation of *tax-4* and *tax-2* single and double mutants in response to exogenous pheromone. We performed these assays at both 25° and 22° since the *tax-4(ks11)* mutant is strongly Daf-c at 25° without pheromone and because there was a precedent for opposite pheromone responses at these two temperatures (*unc-31*, see above). As shown in Table 9, the three *tax-4* mutants have a weak pheromone response and the three *tax-2* mutants do not respond to pheromone at all. The complete pheromone insensitivity of the *tax-2(p694)* mutant is particularly notable as it suggests that this defect is due to a site of action in one or more of the AFD, ASE, ADE, or BAG neurons, none of which have been implicated previously in regulating the response to pheromone. The pheromone responsiveness of *tax-4* mutants appears to be suppressed by *tax-2(p691)* but not by *tax-2(p671)*, though the weakness of pheromone induction of dauer formation in *tax-4* single mutants makes this somewhat difficult to interpret. Dauer formation of *tax-4(p678)* in the absence of pheromone was strongly suppressed by *tax-2(p691)* and partially suppressed by *tax-2(p694)*, again suggesting that the TAX-2 protein may function in the absence of TAX-4.

The lack of pheromone responsiveness of *tax-2* mutants and reduced response of *tax-4* led us to examine these mutants for defects in dye-filling of the amphid sensory neurons, a phenotype characteristic of cilium-structure mutants (Perkins *et al.* 1986; Starich *et al.* 1995) that also fail to respond to pheromone. Six amphid neuron pairs (ASJ, ADF, ASH, ASI, ADL, and ASK) fill with the fluorescent dye FITC (Hedgecock *et al.* 1985). *tax-4* and *tax-2* mutants were capable of FITC dye-filling by all six cells, though filling of ASJ and ASI was often weaker or not detectable (data not shown). This could indicate a weak dye-filling defect specific to these cells, but since ASJ and ASI fill more weakly in wild type this could also simply reflect a general weak defect that is only detectable in these cells. Coburn and Bargmann (1996) showed that both ASI and ASJ fill relatively normally with the dye DiO in *tax-2* and *tax-4* mutants. Thus, *tax-4* and *tax-2* mutants do not appear to have strong defects in the structure of the amphid cilia.

Epistasis based on the Hid phenotype: At least three parallel pathways regulate dauer formation (Figure 1). These pathways were inferred by examining epistatic interactions among Daf-c and Daf-d genes at temperatures ranging from 15° to 25° (Vowels and Thomas 1992; Thomas *et al.* 1993; Gottlieb and Ruvkun 1994). To determine the pathway in which the Hid mutants function, we built double mutants between Hid mutants and Daf-d mutants in each branch of the pathway. We also reexamined epistasis of the previously characterized

Daf-c genes to test whether the same epistatic relationships hold at 27° as at lower temperatures.

Double mutants with daf-22: As shown above, *C. elegans* is capable of weak pheromone-independent dauer formation at 27° but is also highly sensitized to pheromone at 27°. Since several Hid mutants are hypersensitive to pheromone, it was possible that the Hid phenotype was caused by an increased response to low levels of endogenous pheromone that only weakly induced dauer formation of wild type. To determine whether any Hid phenotypes depend on pheromone, we built double mutants of Hid mutants with *daf-22*, which does not make pheromone. *daf-22* double mutants with *unc-3(e151)*, *unc-31(e928)*, *unc-64(e246)*, *osm-6(p811)*, and *daf-3(sa213)* formed 100% dauers at 27°, indicating that the Hid phenotype does not depend on endogenous pheromone production.

Double mutants with dyf genes: Mutations in many Dyf genes suppress the Daf-c phenotype of group I Daf-c mutants at 25° (Vowels and Thomas 1992; Starich *et al.* 1995). Epistasis with the Dyf mutants at 27° is complicated by the fact that Dyf mutants are Daf-c on their own at 27°. Nevertheless, we built a number of double mutants of *unc-3*, *unc-31*, and *unc-64* with mutations in the Dyf genes *osm-1*, *osm-3*, *osm-5*, *osm-6*, *che-3*, *che-11*, and *daf-10*. Surprisingly, all such double mutants had a Syn-Daf phenotype at temperatures from 15° to 25° (data not shown). This interaction suggests that *unc-3*, *unc-31*, and *unc-64* act in parallel to the group I pathway. This also suggests that Dyf mutations have both positive and negative effects on dauer formation between 15° and 25°.

Double mutants with daf-3 and daf-5: Mutations in *daf-3* and *daf-5* completely suppress the Daf-c phenotype of group II Daf-c mutants at 25° and partially suppress the Daf-c phenotype of group I Daf-c mutants (Vowels and Thomas 1992; Thomas *et al.* 1993). Epistasis with *daf-3* at 27° is complicated by the Hid phenotype of *daf-3* mutants so we concentrated on epistasis with *daf-5*. Double mutants of *unc-3*, *unc-31*, and *unc-64* with *daf-3* were not Syn-Daf and exhibited the same epistasis relationships as the double mutants with *daf-5* under conditions that permitted scoring of suppression (data not shown).

As shown in Table 10, mutations in *daf-5* did not suppress the Hid phenotype of *unc-64* or *unc-31*, suggesting that these genes act in parallel to the group II pathway. Mutations in *daf-5* partially suppressed *unc-3* or *daf-7* at 26.6° but showed little suppression at a higher temperature. The lack of suppression seen at the highest temperatures may be due to inability to detect partial suppression when dauer formation is maximally induced. The similarity of *unc-3* and *daf-7* suppression by *daf-5* suggests that *unc-3* and *daf-7* act at a similar position in the group II branch of the dauer pathway. The fact that *daf-5* only partially suppresses the Daf-c phenotype of *daf-7* at 27° while it completely suppresses the Daf-c phenotype at 25° suggests that there are outputs of the group II pathway at 27° that either do not exist at 25° or are not detectable. *daf-5* mutations also only partially suppress *daf-1* and *daf-14* mutants at 27° (data not shown), consistent with the *daf-7* results. Mutations in *daf-5* showed no suppression of the group I Daf-c gene *daf-11* at 27°.

TABLE 10

Dauer formation of *daf-5* double mutants at 27°

	Dauer formation (%)	
Genotype	26.6°	27.1°
N2	5 (119)	11 (128)
daf-5(e1385)	0 (91)	3 (72)
unc-64(e246)	95 (55)	100 (77)
daf-5(e1385); unc-64(e246)	98 (103)	100 (116)
unc-31(e928)	84 (74)	100 (85)
daf-5(e1385); unc-31(e928)	100 (85)	100 (94)
unc-3(e151)	92 (118)	100 (133)
daf-5(e1385); unc-3(e151)	33 (132)	95 (113)
daf-5(e1385); daf-7(e1372)[a]	26 (134)	97 (121)
daf-5(e1385); daf-11(sa195)[a]	99 (73)	100 (53)

Genotype	Dauer formation at 27.0° (%)
N2	1 (482)
daf-5(e1385)	0 (304)
daf-3(mgDf90)	55 (280)
daf-5(e1385); daf-3(mgDf90)	51 (203)
daf-3(sa213)	93 (122)
daf-5(e1385); daf-3(sa213)	89 (342)
daf-3(sa206)	98 (393)
daf-5(e1385); daf-3(sa206)	92 (477)

	Dauer formation (%)	
Genotype	26.7°	27.1°
N2	0 (114)	2 (112)
daf-5(e1385)	2 (155)	2 (140)
osm-6(p811)	86 (139)	99 (172)
daf-5(e1385); osm-6(p811)	23 (168)	72 (176)

[a] The *daf-7* and *daf-11* single mutants were not assayed in this experiment, but in other experiments always formed 100% dauers at temperatures >25°.

The opposing phenotypes of *daf-3* and *daf-5* at 27° permitted us to perform epistasis on these two genes for the first time. We built double mutants of three different *daf-3* alleles with mutations in *daf-5*. As shown in Table 10, mutations in *daf-5* did not suppress the Daf-c phenotype of any of the *daf-3* mutants, suggesting that *daf-3* acts downstream of *daf-5* in the group II pathway. This is consistent with the fact that *daf-3* encodes a SMAD protein that may act in the nucleus as a transcription factor to directly regulate genes involved in dauer development (Patterson *et al.* 1997; Thatcher *et al.* 1999). Similar results were seen with the *sa205* allele of *daf-5* (data not shown). Finally, we observed partial suppression by *daf-5* of the Hid phenotype of

TABLE 11

Dauer formation of *daf-16* double mutants at 27°

Genotype	Dauer formation (%) 26.8°	26.7°
N2	4 (251)	2 (231)
daf-16(m27)	2 (258)	7 (298)
unc-64(e246)	99 (177)	98 (156)
daf-16(m27); unc-64(e246)	3 (227)	2 (226)
unc-31(e928)	73 (104)	95 (134)
daf-16(m27); unc-31(e928)	3 (177)	3 (130)
unc-3(e151)	64 (306)	92 (291)
daf-16(m27); unc-3(e151)	19 (252)	28 (183)
daf-2(e1370)	100 (46)	ND
daf-16(m27); daf-2(e1370)	33 (339)	ND

Genotype	Dauer formation at 27.2° (%)
N2	4 (166)
daf-16(m27)	7 (205)
osm-6(p811)	89 (190)
daf-16(m27); osm-6(p811)	83 (157)
daf-3(sa213)	95 (78)
daf-16(m27); daf-3(sa213)	31 (221)

Genotype	Dauer formation (%) 26.6°	27.0°
N2	2 (205)	11 (202)
daf-16(m27)	5 (245)	17 (206)
osm-6(p811)	82 (261)	95 (140)
daf-16(m27); osm-6(p811)	37 (166)	64 (89)
daf-10(e1387)	12 (240)	77 (146)
daf-16(m27); daf-10(e1387)	29 (249)	46 (140)
osm-5(p813)	88 (242)	99 (139)
daf-16(m27); osm-5(p813)	25 (296)	52 (152)

ND, not determined. All dauers in strains carrying *daf-16* are partial dauers as described (Vowels and Thomas 1992).

the Dyf mutant *osm-6*, consistent with *osm-6* functioning in parallel to the group II branch of the dauer pathway.

Double mutants with daf-16: Mutations in *daf-16* completely suppress the Daf-c phenotype at 25° of Daf-c mutants in the insulin branch of the dauer pathway (Vowels and Thomas 1992; Gottlieb and Ruvkun 1994; Larsen *et al.* 1995; Paradis *et al.* 1999). Mutations in *daf-16* partially suppress group I Daf-c mutants and only very weakly suppress group II Daf-c mutants at 25° (Vowels and Thomas 1992). As shown in Table 11, mutations in *daf-16* completely suppressed the Hid phenotype of *unc-64* and *unc-31* but only partially suppressed the Hid phenotype of *unc-3*. This suggests that *unc-64* and *unc-31* function in the insulin branch of the dauer pathway, while *unc-3* probably functions in parallel, consistent with the *daf-5* epistasis results. Mutations in *daf-2* were only partially suppressed by mutations in *daf-16* at 27°, suggesting that there are *daf-16*-independent outputs of the insulin signaling pathway at 27° that either do not exist at 25° or are not detectable. Mutations in *daf-16* partially suppressed the Hid phenotype of *daf-3* and three different Dyf mutants, *osm-6*, *daf-10*, and *osm-5*, consistent with these genes functioning in parallel to the insulin branch of the dauer pathway.

Double mutants with pdk-1(gf) and akt-1(gf): The *pdk-1* and *akt-1* genes function downstream of *daf-2* and *age-1* in the insulin branch of the dauer pathway, but upstream of *daf-16* (Figure 1). Dominant gain-of-function mutations in either *pdk-1* or *akt-1* suppress the Daf-c phenotype of *age-1* mutants at 25° but do not suppress *daf-2* (Paradis and Ruvkun 1998; Paradis *et al.* 1999), suggesting that there is a bifurcation of the insulin signaling pathway downstream of *daf-2*. Since *unc-64* and *unc-31* appear to act in the insulin pathway, we built double mutants of *unc-64(e246)* and *unc-31(e928)* with the *pdk-1(mg142)* and *akt-1(mg144)* gain-of-function mutations. *unc-64* and *unc-31* double mutants with either *pdk-1(mg142)* or *akt-1(mg144)* formed 100% dauers at 27°, suggesting that *unc-64* and *unc-31* act upstream of the bifurcation in the pathway, downstream of *pdk-1* and *akt-1*, or in the branch that does not consist of *age-1*, *pdk-1*, and *akt-1*. Alternatively, the gain-of-function mutations may not activate the pathway enough to suppress upstream Daf-c mutations at 27°.

Epistasis based on pheromone response at 25°: As another method of positioning *unc-64*, *unc-31*, and *unc-3* in the dauer pathway, we examined whether *daf-5* could suppress dauer formation induced by a high level of pheromone at 25° in these mutants. As shown in Table 12, mutations in *daf-5* completely suppressed the pheromone response of *unc-3* and *daf-7* but did not suppress the pheromone response of either *unc-64* or *unc-31*. Similar results were seen with *daf-3* in place of *daf-5* (data not shown). This provides further evidence that *unc-3* acts in the group II pathway and that *unc-64* and *unc-31* act in parallel. A *daf-5; daf-11* double mutant also did not respond to pheromone. Since *unc-64* and *unc-31* double mutants with *daf-5* responded normally to pheromone, this suggests that *unc-64* and *unc-31* do not act in the group I pathway.

One possible explanation for the failure to see suppression of the *unc-64* or *unc-31* pheromone responses by *daf-5* is that dauer formation was so strongly induced by the high level of pheromone in this experiment that partial suppression could not be detected. To investigate this possibility, we assayed the *daf-5; unc-64* and *daf-5; unc-31* double mutants at a range of pheromone concentrations. As shown in Figure 4, at pheromone concentrations that induced an intermediate level of dauer formation, the *daf-5; unc-64* and *daf-5; unc-31* double mutants responded almost identically to the *unc-64* and *unc-31* single mutants. Thus, the lack of *unc-64* and *unc-31* suppression by *daf-5* cannot be accounted for by mere quantitative differences between these genes and *unc-3*.

As a final method of assessing epistatic interactions,

TABLE 12

Pheromone responses of *daf-5* double mutants at 25°

Genotype	Dauer formation at 25° (%)	
	− pheromone	+ pheromone[a]
N2	0 (180)	90 (189)
daf-5(e1385)	0 (155)	1 (143)
unc-64(e246)	27 (124)	100 (117)
daf-5(e1385); unc-64(e246)	7 (107)	99 (94)
unc-31(e928)	2 (107)	98 (85)
daf-5(e1385); unc-31(e928)	1 (129)	95 (122)
unc-3(e151)	19 (134)	100 (100)
daf-5(e1385); unc-3(e151)	0 (186)	0 (173)
daf-5(e1385); daf-7(e1372)[b]	0 (76)	0 (175)
daf-5(e1385); daf-11(sa195)[b]	60 (121)	51 (81)

[a] Plates with 50 μl pheromone.

[b] The *daf-7* and *daf-11* single mutants were not assayed in this experiment but in other experiments formed 100% dauers at 25° with or without pheromone.

we assayed the Daf-d phenotype of double mutants of *unc-64*, *unc-31*, and *unc-3* with either *daf-3* or *daf-5*. The *daf-3(e1376)* and *daf-5(e1385)* mutants have a strong Daf-d phenotype at 20°, including a failure to form dauers in response to starvation. *unc-64*, *unc-31*, and *unc-3* mutants form dauers readily when starved, at levels comparable to or greater than wild-type N2. Mutations in *daf-3* and *daf-5* completely abolished starvation-induced dauer formation of *daf-7* or *unc-3* mutants but had no discernible effect on starvation-induced dauer formation of *unc-64* or *unc-31* mutants (data not shown). This provides further evidence that *unc-3* acts in the group II pathway and that *unc-64* and *unc-31* act in parallel.

Double mutants of *unc-64*, *unc-31*, and *unc-3* with other Daf-c genes: Double mutants of Daf-c genes in different branches of the dauer pathway have a stronger Daf-c phenotype than either single mutant, while double mutants of Daf-c genes in the same branch do not have an enhanced Daf-c phenotype (Thomas *et al.* 1993; Ogg *et al.* 1997). As a complementary approach to epistasis with Daf-d mutants, we built double mutants of *unc-64*, *unc-31*, and *unc-3* with Daf-c mutants in each branch of the dauer pathway. Many of these double mutants formed 100% nonrecovering dauers at all temperatures, preventing the establishment of a strain. The incompletely penetrant Daf-c phenotype of *daf-7* at 15° was enhanced to 100% by mutation of *unc-3*, *unc-31*, or *unc-64*, suggesting that these genes act in parallel to *daf-7*. This was expected for *unc-64* and *unc-31*, which appear to act in the insulin branch of the pathway, but was unexpected for *unc-3*, which appeared to act in the group II pathway on the basis of the epistasis results presented above. Double mutants of *unc-3* with the other group II Daf-c genes *daf-1* and *daf-14* also exhibited 100% dauer formation at 15°. Thus, although *unc-3* may function upstream of *daf-5* in the group II pathway, it must act at least partially in parallel to the group II Daf-c genes. *unc-31* and *unc-64* did not enhance the Daf-c phenotype of a *daf-2* mutant at 15° (Ailion *et al.* 1999), supporting the idea that these genes function in the insulin branch of the pathway.

Dominance of Daf-c genes at 27°: Daf-c mutants with a strong Daf-c phenotype at 25° are recessive at this temperature, with the exception of the semidominant mutant *daf-28* (Malone and Thomas 1994). *daf-28* does not appear to act in any of the three branches of the dauer pathway depicted in Figure 1 (Malone *et al.* 1996). We tested whether any of the 25° Daf-c mutants were dominant at the more strongly dauer-inducing temperature of 27°. As shown in Table 13, the *daf-7(e1372)* mutant was moderately dominant at 27°, while no other Daf-c mutants tested exhibited any dominance. The *unc-64*, *unc-31*, and *unc-3* mutants also did not exhibit any dominance at 27° (Ailion *et al.* 1999 and data not shown). To verify that *daf-7* dominance was not allele specific, we tested the *daf-7(m62)* mutant and found that it also was partially dominant at 27° (data not shown). *daf-7/+* heterozygotes exhibited a Daf-c phenotype at 27° regardless of whether the *daf-7* mutant gene came from the male or hermaphrodite parent, indicating that this phenotype could not be accounted for by a maternal effect of *daf-7*. *daf-7* encodes a TGF-β-like protein that acts as a secreted ligand (Ren *et al.* 1996). *e1372* is a missense mutation and *m62* is a nonsense mutation (Ren *et al.* 1996), indicating that these are loss-of-function mutations and that dominance is caused by haploinsufficiency. Reducing the gene dosage of *daf-7(+)* would be expected to decrease the concentration of DAF-7 ligand. We hypothesize that at 27°, this decrease is below the threshold needed for nondauer signaling.

Expression of *daf-7::gfp* at 27°: One possible explanation for the partial dominance of *daf-7* at 27° is the fact that *daf-7* expression is reduced by increased temperature (Schackwitz *et al.* 1996). Perhaps, downregulation of *daf-7* at 27° is significantly greater than at 25°, resulting in a Daf-c phenotype when *daf-7* gene dosage is reduced. Previously, Schackwitz *et al.* (1996) examined expression of the integrated *daf-7::gfp* array *saIs7* and found that GFP was undetectable at 25°. To examine *daf-7::gfp* expression at 27°, we made use of the integrated array *saIs8*, which expresses GFP at considerably higher levels than *saIs7*. As shown in Table 14, the percentage of ASI neurons expressing *daf-7::gfp* remains roughly the same from 15° to 27°, but the percentage strongly expressing GFP drops considerably, particularly at 27°. This is consistent with the idea that the dominance of *daf-7* mutants at 27° results from the greater reduction in *daf-7* expression. Some differences in *daf-7::gfp* expression were seen between the left and right

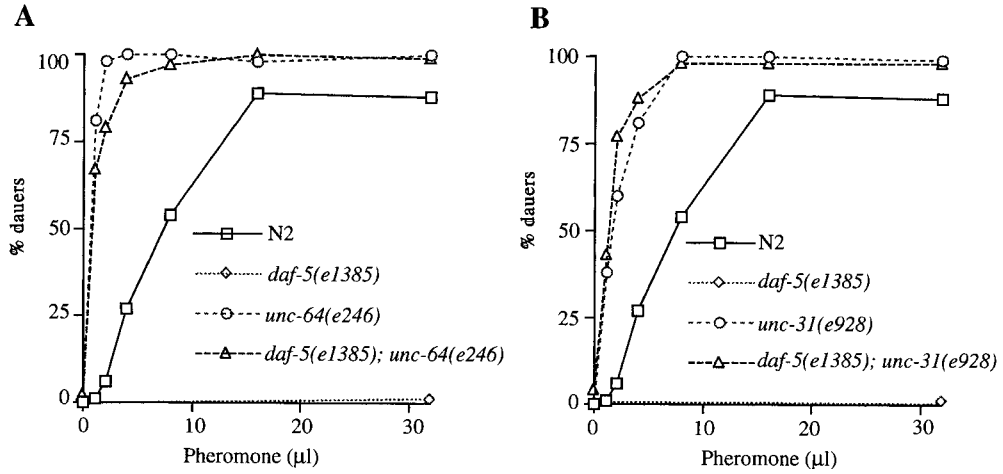

FIGURE 4.—Dauer formation of *daf-5; unc-64* and *daf-5; unc-31* double mutants in response to exogenous pheromone at 25°. Approximately 50–100 animals were counted at each concentration of pheromone. Both graphs use data from the same experiment but are plotted separately to facilitate comparison of the *unc-64* and *unc-31* single mutants with the *daf-5* double mutants.

ASI neurons, but it is not clear whether these differences are significant. At temperatures >15°, there was a significant percentage of animals that expressed *daf-7::gfp* in cells other than ASI. The possible significance of this is also unclear.

Cell kills: The identification of particular neurons involved in regulating dauer formation has been accomplished by killing identified neurons with a laser microbeam (BARGMANN and HORVITZ 1991; SCHACKWITZ *et al.* 1996). From these studies, the ASI and ADF neurons were shown to function as redundant dauer-repressing neurons. Killing both these neurons results in a Daf-c phenotype at 20°, but killing either ASI or ADF alone in a wild-type background does not lead to a Daf-c phenotype (BARGMANN and HORVITZ 1991). Killing ASI (but not ADF) alone in an *unc-31* mutant results in a Daf-c phenotype (AVERY *et al.* 1993). To determine the involvement of ASI in dauer formation at 27°, we killed this cell in the wild-type N2 and assayed dauer formation at 27°. As shown in Table 15, killing ASI alone was sufficient to result in a Daf-c phenotype at 27°. This is similar to the genetics of the Syn-Daf phenotype where apparent redundancies at lower temperatures evaporate at 27°. We confirmed that killing ASI alone in an *unc-31* mutant at 25° was sufficient to cause dauer formation and also showed that killing ASI alone was sufficient to cause dauer formation at 25° in an *unc-64* mutant.

unc-3 encodes a transcription factor expressed only in ASI and ventral cord motor neurons (PRASAD *et al.* 1998). Thus, the dauer phenotype of *unc-3* mutants is expected to have a site of action in ASI and it was possible that the *unc-3* mutation leads to misfunction of ASI equivalent to an ASI cell kill. Evidence in support of this idea is that (1) *unc-3* is Daf-c at 27° and killing ASI leads to a Daf-c phenotype at 27°; (2) *unc-31* and *unc-64* are Syn-Daf with *unc-3* or with an ASI cell kill; and (3) *unc-3* appears to function with the group II pathway, which is thought to act through ASI (SCHACKWITZ *et al.* 1996). Killing ASI did not cause dauer formation in an *unc-3* mutant (Table 15), consistent with the idea that *unc-3* perturbs ASI function. However, killing ADF did not cause dauer formation in the *unc-3* mutant either, as would be expected if the *unc-3* mutant was equivalent to an ASI cell kill. Since ADF was killed in only three animals, this result should be interpreted cautiously. Thus, while it is probable that ASI does not function properly in an *unc-3* mutant, it likely retains some of its ability to regulate dauer formation.

Male dauer formation: In the course of performing crosses with *unc-3* mutants at 20°, we observed dauers after mating wild-type males to *unc-3* hermaphrodites. Since *unc-3* maps to the X chromosome, we hypothesized that these might be *unc-3* male dauers. To test this idea, we picked these dauers and allowed them to recover to score their sex. All such dauers were male, confirming our hypothesis. At 20°, 38% of the *unc-3(e151)* males formed dauers and 0% of the *unc-3* hermaphrodites formed dauers. Thus, there is differential regulation of dauer formation in *unc-3* males and hermaphrodites.

To investigate whether the increased dauer formation of males was specific to *unc-3*, we assayed dauer formation of N2 wild-type males and hermaphrodites in response to pheromone at 25°. As shown in Figure 5, males showed much stronger dauer formation in response to pheromone than hermaphrodites. Thus, males appear to be generally more sensitized to dauer-inducing conditions. The increased frequency of male dauer formation in several Daf-c mutants has been noted previously (VOWELS and THOMAS 1992). Males carry one X chromosome while hermaphrodites carry two X chromosomes. To determine if the number of X chromosomes was responsible for the dauer formation differences observed between males and hermaphrodites, we examined *tra-2(q276)* males, which are phenotypically male, but carry two X chromosomes. *tra-2(q276)* XX males exhibited a hypersensitive response to pheromone similar to N2 XO males (data not shown), indicating that the increased male response comes as a result of being

TABLE 13

Dominance of Daf-c genes at 27°

Genotype	Dauer formation at 27.0° (%)
+/+	2 (131)
+/*daf-7(e1372) dpy-1(e1)*	52 (84)[a]
+/*daf-1(sa184);* +/*dpy-11(e224)*	0 (63)
+/*dpy-5(e61) daf-8(e1393)*	0 (37)
+/*unc-24(e138) daf-14(m77)*	0 (34)
+/*unc-42(e270) daf-11(sa195)*	5 (39)
+/*daf-2(e1370) dpy-17(e164)*	0 (24)

[a] A total of 58% of males and 48% of hermaphrodites formed dauers.

phenotypically male and not from the number of X chromosomes. We examined male dauer formation of *unc-64* and *unc-31* in double mutants with *him-5*. Unlike *unc-3*, *unc-31* and *unc-64* males did not form dauers at 20°. Since the *unc-64* and *unc-31* hermaphrodite Daf-c phenotype appears to be at least as strong as that of *unc-3*, this difference is unlikely to be merely quantitative. The increased sensitivity of males to dauer-inducing conditions suggests that males have either additional dauer-inducing pathways not present in hermaphrodites or an enhanced response by the same pathways.

DISCUSSION

Dauer formation is strongly induced at 27°: Previous work showed that dauer formation is induced more at 25° than at 15° (Golden and Riddle 1984a,b). Here we show that 27° is a much more strongly inducing dauer stimulus than 25°. Wild-type worms show a stronger increase in dauer induction by pheromone over the 2° temperature change from 25° to 27° than they do over the 10° temperature change from 15° to 25°. Thus, induction of dauer formation at 27° is likely a specific nonlinear response to this temperature and not due to general thermodynamic effects on biological processes, which should increase linearly with increasing temperature (in degrees Kelvin). Since wild-type worms are highly sensitive to pheromone at 27°, the thermal input to dauer formation must be acting at least partially in parallel to the pheromone response pathways. The thermal input to dauer formation at 27° could either be a more extreme version of the thermal input received at lower temperatures or it could be a distinct input. Over the temperature range from 15° to 25°, dauer phenotypes exhibit quantitative differences, with dauer formation becoming stronger as the temperature is increased. Since dauer phenotypes at 27° are not merely quantitatively stronger than phenotypes at 25° but show qualitative differences (see below), we favor the hypothesis that 27° provides a dauer-inducing input partially distinct from the input received at 25° or lower temperatures. Thus, there may be sensory pathways and cells dedicated to detecting a 27° stimulus. This of course does not preclude that a 27° stimulus would also affect pathways that receive temperature input at lower temperatures. Many organisms have distinct thermoreceptors that are activated at different temperatures, rather than a single thermoreceptor whose activity is modulated over a wide temperature range (Spray 1986). Thus, perception of any given temperature occurs by a combinatorial mechanism of activating multiple thermoreceptors. In vertebrates, sensation of noxiously high temperatures is mediated by the capsaicin receptor (Caterina *et al.* 1997). *osm-9* encodes the closest *C. elegans* homolog of the capsaicin receptor (Colbert *et al.* 1997). The *osm-9(n1601)* null mutant had the same phenotype as N2 at 27° (data not shown), suggesting that sensation of the 27° stimulus occurs by a different mechanism.

A cellular pathway that responds to temperature in *C. elegans* has been defined for thermotaxis behavior (Mori and Ohshima 1995). In this pathway, AFD appears to be a primary thermosensory cell. Mutations in the *ttx-1* gene cause defects in the structure of AFD (specific to the proposed thermosensory structures) and severe defects in thermotaxis behavior. We show here that *ttx-1* mutants induce dauer formation normally in response to temperature over the entire range from 15° to 27°, but are hypersensitive to dauer pheromone at all temperatures. Mutations in the *ttx-3* gene affect the function of AIY, an interneuron in the thermotaxis pathway (Mori and Ohshima 1995). *ttx-3* mutations cause severe defects in thermotaxis but affect dauer formation only mildly; additionally, the dauer effect appears to be specific to recovery, not formation of dauers (Hobert *et al.* 1997). Thus, thermal inputs that regulate dauer formation appear to be at least in part distinct from thermal inputs that regulate thermotaxis. *C. elegans* also exhibits a thermal avoidance behavior at even higher temperatures (Wittenburg and Baumeister 1999). Like dauer formation, thermal avoidance behavior appears to have thermal inputs distinct from the thermotaxis circuit. Thus, *C. elegans* appears to resemble other organisms in having multiple thermoreceptors that respond to different temperatures.

In addition to strongly inducing dauer formation in wild-type animals, growth at 27° leads to a strongly penetrant Daf-c phenotype in several mutants (*unc-64*, *unc-31*, and *unc-3*) that do not exhibit any Daf-c phenotype on their own at 25°. Double mutants of these genes exhibit a Syn-Daf phenotype at 25°. Such a synthetic phenotype could have indicated full genetic redundancy, but in this case the synthetic phenotype appears to result from a combination of weak phenotypes that are not detectable at 25°. These mutants would have been difficult to isolate in screens for Daf-c mutants at 25° since they would require the simultaneous occurrence of two mutations. The finding that these mutants have strong single mutant phenotypes at 27° allows for

TABLE 14

Expression of *daf-7::gfp* in L2 animals at various temperatures

Temperature	Expression in ASIL (%)		Expression in ASIR (%)		Expression in ASI combined (%)		Expression in cells other than ASI[a] (% of animals)
	Any[b]	Strong[c]	Any	Strong	Any	Strong	
15°	70 (30)	70 (30)	90 (30)	87 (30)	80 (60)	78 (60)	0 (30)
20°	73 (26)	38 (26)	81 (26)	54 (26)	77 (52)	46 (52)	27 (26)
25.2°	86 (29)	52 (29)	72 (29)	24 (29)	79 (58)	38 (58)	21 (29)
27.0°	75 (24)	13 (24)	50 (24)	0 (24)	63 (48)	6 (48)	21 (24)

[a] In all cases, the other cells expressing *daf-7::gfp* were located in the lateral ganglion and were probably amphid interneurons. In any animal, only one cell other than ASI expressed *daf-7::gfp* but no effort was made to identify the cell. Based on position, it appeared that the identity of this cell varied from animal to animal.

[b] Expression was scored as "Any" if there was detectable fluorescence.

[c] Expression was scored as "Strong" if it was of comparable intensity to the normally bright expression seen at 15°.

an efficient way to identify new dauer genes that could not have been isolated in screens performed at 25°. We have performed such a screen for Daf-c mutants at 27°, and we isolated several new alleles of *unc-31* and *unc-3* as well as a number of new dauer genes (M. Ailion and J. H. Thomas, unpublished results).

High temperature is sufficient to induce dauer formation: At temperatures of 25° or lower, pheromone is both necessary and sufficient to induce dauer formation. At 27°, wild-type and pheromone-deficient *daf-22* animals are capable of forming dauers in the absence of exogenous pheromone when food is plentiful, a phenotype not seen at lower temperatures. This implies that the more extreme temperature is a sufficient stimulus to induce dauer formation, unless there is a novel pheromone production pathway operative at 27° that does not depend on *daf-22* gene activity. The fact that Dyf mutants, which block pheromone detection, are Daf-c at 27° is consistent with the possibility that dauer formation at 27° is pheromone independent.

Pheromone is likely to act as a measure of population density. Since high temperatures appear sufficient to induce dauer formation on their own, worms in the wild probably encounter hot temperatures at low population densities, where dauer formation is dictated by the stressful thermal stimulus rather than a lack of resources, as occurs with overcrowding. We note that N2 wild-type animals form dauers only transiently at 27°. Why induce dauer formation if only to recover immediately? There are several possible explanations that are not mutually exclusive. One possibility is that pheromone concentrations are artificially low in the lab growth conditions of high food concentrations and relatively few animals on a naive plate that had no time to accumulate endogenously produced pheromone by earlier generations of animals. Since very low concentrations of pheromone are effective at inducing nontransient dauer formation at 27°, it is easy to imagine that such low levels of pheromone might usually be present in natural environments. A second possibility similar to the first is that recovery of dauers at 27° is especially sensitive to decreased amounts of food. The concentration of food present in our laboratory assays is probably rarely achieved in nature. Perhaps with reduced amounts of food more likely to mimic natural conditions, high temperature may be sufficient to induce dauer formation and inhibit recovery. A third possibility is that temperatures >27° may be sufficient to induce dauer formation and inhibit recovery. A fourth possibility is that dauer formation really is more sensitive to temperature than dauer recovery and that this has biological significance. Inducing dauer formation transiently may be advantageous in highly variable environments that change rapidly. By inducing dauer formation at temperatures that are dangerous but not lethal, the animals may be "hedging their bets" against future expectations. If conditions continue to worsen, animals can inhibit recovery once they have achieved the dauer stage, but since the decision to form a dauer must be made beginning at the L1 molt (Swanson and Riddle 1981; Golden and Riddle 1984a), it would be too late to induce dauer formation if they had not already done so. On the other hand, if conditions stay the same or improve, animals that only transiently induced dauer formation will have a "head start" over animals that arrested at the dauer stage. Such a head start would lead to earlier reproduction and could be essential for progeny survival in a highly competitive resource-limited environment. While many genes that regulate dauer formation also regulate dauer recovery, there is evidence for some differential regulation of the two processes (Malone *et al.* 1996; Hobert *et al.* 1997; Sze *et al.* 2000; Tissenbaum *et al.* 2000).

Genes with positive and negative influences on dauer formation: One of the unexpected findings of this study was that many genes have opposing positive and negative influences on regulation of dauer formation, as revealed by their 27° phenotypes. Mutations in any of

TABLE 15

Dauer formation after killing ASI or ADF neurons

Genotype	Temperature	Cells killed		
		Mock[a]	ASI	ADF
N2	27°	0/16	3/4	ND
N2	25°	0/15	0/3	ND
unc-31(e928)	25°	0/9	3/3	ND
unc-64(e246)	25°	1/9	7/8	ND
unc-3(e151)	25°	0/2	0/5	0/3

For each type of kill, the number of animals that formed dauers is given as a fraction of the total number of animals operated. ND, not determined.

[a] Mock kills were treated the same as the real kills, but no cell was killed.

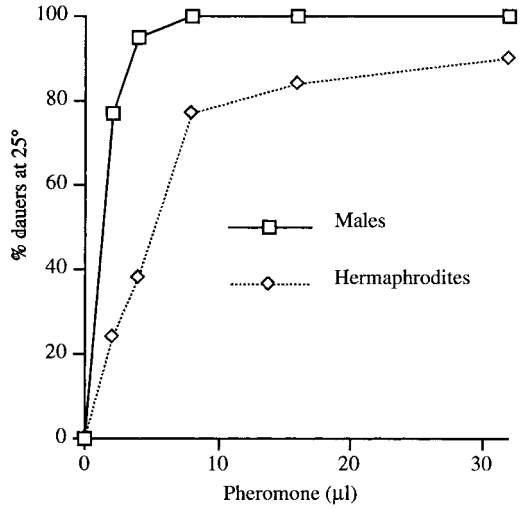

FIGURE 5.—Males are hypersensitive to pheromone. The graph plots dauer formation of wild-type males and hermaphrodites in response to exogenous pheromone at 25°. The progeny of mated N2 hermaphrodites were grown on different concentrations of pheromone. The number of dauers and male and hermaphrodite nondauers was counted. Dauers were then picked to plates without pheromone at 15°. After dauers recovered, they were scored as either male or hermaphrodite. Approximately 50–100 animals of each sex were counted at each concentration of pheromone.

the large group of Dyf genes, which affect the structure of the ciliated endings of sensory neurons, result in a Daf-d phenotype and nonresponsiveness to pheromone at 25°, but a strong Daf-c phenotype at 27°. This reversal of a Daf-d phenotype is also seen for mutants in the *daf-3* gene, which occupies a distinct position in the pathway. Here we discuss several different possibilities that could account for such reversals of phenotype and suggest that different mechanisms may be operating in the different cases observed.

How do genes that are Daf-d at 25° become Daf-c with only a 2° increase in temperature? For the Dyf genes, we favor the following hypothesis. At temperatures of 25° or lower, pheromone is necessary to induce dauer formation. The Dyf mutants have defects in the structure of the amphid neuron endings exposed to the environment where pheromone detection occurs. These structural defects prevent pheromone detection and hence lead to a Daf-d phenotype. However, at 27°, detection of pheromone is no longer necessary to induce dauer formation. Perhaps the basal activity state of the amphid neurons is different in the Dyf mutants. This altered basal activity may be insufficient to induce dauer formation at 25°, but it may be above the threshold for constitutive dauer formation at 27°. Support for this idea comes from analysis of mutants with amphid structural defects, but which affect cells other than the neurons themselves. For example, the *daf-6* mutant has defects in the amphid sheath cell that lead to a Dyf phenotype and the inability to respond to dauer pheromone (ALBERT *et al.* 1981; HERMAN 1984, 1987; PERKINS *et al.* 1986). *daf-6* mutants are not Daf-c at 27°, suggesting that the inability to sense the environment *per se* does not lead to an altered basal neuronal activity. Since Dyf mutants remain pheromone insensitive at 27°, it is unlikely that there are additional pheromone-sensing pathways at 27° that do not operate at 25°. Since there are both dauer-repressing and dauer-promoting neurons, the Daf phenotype of Dyf mutants at any given temperature could depend on a balance of the activities of different cells. This model could also help explain the Daf-c phenotype of *daf-19* mutants. *daf-19* mutants completely lack sensory cilia, but unlike all other Dyf mutants, *daf-19* mutants are Daf-c at all temperatures (PERKINS *et al.* 1986; SWOBODA *et al.* 2000). Perhaps *daf-19* mutations more severely alter the basal activity of the amphid neurons so that it is above the threshold to induce dauer formation independently of the pheromone input at all temperatures.

Does a similar explanation account for the reversal of the *daf-3* mutant phenotype? Mutations in *daf-3* could have both positive and negative influences on the activity of some neurons, perhaps altering the basal activity but preventing inducibility by pheromone. However, we think that this is unlikely in the case of *daf-3* for two reasons. First, *daf-3* mutants are Daf-c at 27° while *daf-5* mutants are not. If the 27° Daf-c phenotype of *daf-3* were a nonspecific characteristic of mutants in this part of the pathway (as is the case for the Dyf mutants), we would expect *daf-3* and *daf-5* to behave identically at 27°, as they behave identically in regulating dauer formation at lower temperatures. Second, *daf-3* and *daf-5* mutants have normal pheromone sensitivity at 27°. Thus, pheromone response pathways that act in parallel to *daf-3* and *daf-5* must be sufficient for dauer formation at 27°. The different 27° phenotypes of *daf-3* and *daf-5* suggest that there may be specific regulation of *daf-3* activity at 27°. *daf-5* has not yet been cloned, but the molecular identification of the DAF-3 gene product as a SMAD transcription factor (PATTERSON *et al.* 1997)

suggests a few specific possibilities. DAF-3 is hypothesized to act in the nucleus as a transcription factor either to activate genes involved in dauer development or to repress genes involved in nondauer development. Its ability to translocate to the nucleus and bind DNA may depend on positive or negative regulation by partner SMAD proteins or other transcription factors (Attisano and Wrana 2000). One of several ways to explain the reversal of the *daf-3* mutant phenotype posits that DAF-3 binds to the same target genes at 25° and 27° but forms different partnerships that make it an activator of transcription at one temperature and a repressor at the other. For example, mutations in *daf-3* could lead to excessive transcription of dauer-promoting genes at 27° and reduced transcription of the same target genes at 25°.

The *unc-31* gene was also shown to have both positive and negative effects on dauer formation. Unlike the Dyf and *daf-3* mutants, *unc-31* mutants are not Daf-d at any temperature and the reversal of phenotype is seen at lower temperatures. *unc-31* mutants have a reduced sensitivity to dauer pheromone at 22° and an increased sensitivity at 25°. *unc-31* clearly has both dauer-promoting and dauer-repressing effects simultaneously, since at 22° it exhibits a reduced response to dauer pheromone but also strongly enhances the Daf-c phenotype of other Syn-Daf genes. The simplest explanation for these effects is that *unc-31* functions in different cells to promote or inhibit dauer formation, and that the balance of opposing inputs can be tilted in either direction depending on environmental stimuli or mutation of other genes in parallel pathways. This hypothesis is similar to the hypothesis presented above for the Dyf genes, except in that case the opposing forces were proposed to act on the activity of the same cell, but in different ways. Support for the idea that *unc-31* functions in multiple cells comes from the observation that an *unc-31::lacZ* reporter is expressed throughout the nervous system (Livingstone 1991). Furthermore, *unc-31* encodes a homolog of Ca^{2+}-dependent activator protein for secretion (CAPS), a protein that regulates exocytosis of dense-core vesicles and likely affects signaling throughout the nervous system (Livingstone 1991; Walent *et al.* 1992; Ann *et al.* 1997).

The *tax-4* and *tax-2* genes, which encode subunits of a CNG ion channel (Coburn and Bargmann 1996; Komatsu *et al.* 1996), have both dauer-promoting and dauer-repressing effects that have been noted previously (Coburn *et al.* 1998). Interpretation of the *tax-4*/*tax-2* results is complicated by the fact that several of the alleles are clearly not null and may have gain-of-function phenotypes. Additionally, it is not clear what happens *in vivo* to channel activity in the absence of one subunit, especially given the presence of other potential channel subunits encoded by the genome (Bargmann 1998). Nevertheless, from the previous data and the results presented here, we can draw several striking conclusions.

At 25°, *tax-4* and *tax-2* mutations suppress the dauer phenotype of group I Daf-c mutants and enhance the dauer phenotype of group II Daf-c mutants (Coburn *et al.* 1998). Thus, *tax-4* and *tax-2* have properties of both group I Daf-d and Daf-c genes. The *tax-4(ks11)* mutant has a Daf-c phenotype on its own at 25° and resembles the group I Daf-c genes in its genetic interactions at this temperature. This suggests that it may act in ASJ as a target of cGMP made by the DAF-11 guanylyl cyclase. However, ASJ is a dauer-promoting neuron, and the loss of a nonspecific cation channel such as *tax-4* would be expected to hyperpolarize the neuron, thereby inhibiting it and reducing dauer formation, the opposite of the phenotype observed for the *tax-4(ks11)* mutant. This highlights the problem of explaining the *daf-11* mutant phenotype in ASJ as the result of effects on a cyclic nucleotide-gated channel. The *daf-11* mutant should have reduced levels of cGMP, which in any scenario with a wild-type CNG channel as the target would lead to hyperpolarization of the cell. In a dauer-promoting cell, this would lead to reduced dauer formation, the opposite of the *daf-11* Daf-c phenotype, unless the dauer-promoting cell is an unusual neuron that transmits signals when hyperpolarized. Clearly, something is missing from the picture. One obvious possibility is that *daf-11* functions in dauer-repressing neurons in addition to ASJ.

At 27°, all *tax-4* mutants appear to be strongly Daf-c. Of *tax-2* mutants, only *p691* has an equally strong Daf-c phenotype. Interestingly, this allele mutates the identical proline residue in the channel pore mutated in the strongest Daf-c allele of *tax-4*, *ks11*. The *tax-2(p694)* mutation eliminates expression of the TAX-2 subunit from four neurons (AFD, ASE, ADE, and BAG) but has normal expression and function in the other seven neurons that express the channel (Coburn and Bargmann 1996). Since this mutant is not Daf-c at 27°, it suggests that the *tax-2(p691)* 27° Daf-c phenotype has a site of action in one or more of the seven expressing neurons (which include ASJ and ASI). However, the *tax-2(p694)* mutation suppresses the 25° Daf-c phenotypes of *tax-4(ks11)* and *daf-11* (Coburn *et al.* 1998), suggesting that the group I Daf-d phenotype has a site of action in one of the cells AFD, ASE, ADE, or BAG, none of which have been previously implicated in regulating dauer formation. Thus, there is evidence that the Daf-c and Daf-d phenotypes of *tax-2* have different cellular sites of action. *tax-2(p694)* does not suppress *tax-4* Daf-c phenotypes at 27°, suggesting that there may be different cellular sites of action for *tax-4* Daf-c phenotypes at 25° and 27° or different dependence on TAX-2 subunits.

Since *tax-4* encodes an α-subunit that can form homomeric ion channels in the absence of β-subunits (Komatsu *et al.* 1996, 1999), *tax-2* phenotypes are expected to be weaker than *tax-4* phenotypes. For the Daf-c phenotype, *tax-4* mutants clearly appear to be stronger than *tax-2* mutants as expected. However, *tax-2* mutants show an unexpectedly stronger defect in re-

sponse to dauer pheromone. This defect is seen in all *tax-2* alleles, including *p694*, suggesting that one or more of the cells AFD, ASE, ADE, or BAG may be involved in pheromone sensation. Since *tax-2* mutants show a stronger defect than *tax-4* mutants, *tax-2* may function in the absence of *tax-4*. Other evidence to suggest that TAX-2 has functions in the absence of TAX-4 is that *tax-2* mutations can suppress either the dauer formation or recovery defects of *tax-4* mutants, including the putative *tax-4* null allele *p678*. If *tax-2* mutations only eliminated the function of the TAX-4/TAX-2 channel, there should be no effect of *tax-2* mutations in a *tax-4* null background. This suggests that TAX-2 has functions independently of TAX-4, either by itself or in partnership with other CNG channel subunits. *C. elegans* appears to have four other CNG channel subunits encoded in its genome, all of which appear to be α-subunits, supporting this idea (Bargmann 1998; data not shown).

Implications for the dauer genetic pathway: We placed *unc-64*, *unc-31*, and *unc-3* in the dauer pathway by performing epistasis with Daf-d genes under several different conditions. The 27° Daf-c phenotype of *unc-64* and *unc-31* mutants was completely suppressed by mutations in *daf-16* but neither the Daf-c nor pheromone response of these mutants was suppressed at all by mutations in *daf-5*. These data support the conclusion that *unc-64* and *unc-31* act in the insulin branch of the dauer pathway as suggested previously (Ailion *et al.* 1999). In addition to regulating dauer formation, the insulin branch regulates adult longevity (Kenyon *et al.* 1993; Dorman *et al.* 1995; Larsen *et al.* 1995). Consistent with a function in the insulin branch, *unc-64* and *unc-31* mutants have extended life spans (Ailion *et al.* 1999). *unc-64* and *unc-31* encode homologs of syntaxin and CAPS, proteins that mediate Ca^{2+}-regulated secretion (Livingstone 1991; Ann *et al.* 1997; Ogawa *et al.* 1998; Saifee *et al.* 1998), and they have been proposed to function in the regulation of insulin secretion (Ailion *et al.* 1999). *unc-64* and *unc-31* are not suppressed by gain-of-function mutations in *pdk-1* or *akt-1*, which function in one branch of a divergent pathway downstream of the DAF-2 insulin receptor. This is consistent with *unc-64* and *unc-31* functioning upstream of *daf-2* or downstream of *daf-2* in the other branch.

Unlike *unc-64* and *unc-31*, the 27° Daf-c phenotype of *unc-3* is partially suppressed by mutations in *daf-5* while the pheromone response at 25° is completely suppressed. This suggests that *unc-3* acts in the group II Daf-c pathway that consists of a TGF-β signaling cascade. Consistent with this, *unc-3* encodes a transcription factor expressed in the sensory neuron ASI (Prasad *et al.* 1998). Killing ASI is not sufficient to cause a Daf-c phenotype in *unc-3* mutants at 25°, while it is sufficient to cause a Daf-c phenotype in *unc-64* and *unc-31* mutants at 25°, providing further evidence that *unc-3* functions in ASI but that *unc-64* and *unc-31* act in a parallel pathway. *unc-3* probably also acts partly in parallel to the group II pathway since *unc-3* mutations enhance the Daf-c phenotype of group II mutants at 15°.

unc-3 mutants are partially suppressed by *daf-16* at 27°. *daf-16* mutations also partially suppress the 27° Daf-c phenotype of *daf-3* and Dyf mutants. Thus, partial suppression by *daf-16* at 27° appears to be a nonspecific phenomenon that probably results from effects on a parallel pathway that converges further downstream. This is similar to the partial suppression of group I Daf-c mutants by *daf-3* and *daf-5* at 25°. The partial suppression of Dyf, *daf-3*, and *unc-3* mutants by *daf-16* at 27° contrasts with the complete suppression of *unc-64* and *unc-31*. Since *unc-64* and *unc-31* mutants have stronger Daf-c phenotypes at 27° than *unc-3*, *daf-3*, and Dyf mutants, the partial suppression by *daf-16* of these latter mutants cannot be explained by mere quantitative differences. It was reported by others that *daf-16* mutations completely suppress the Daf-c phenotype of Dyf mutants at 27° (Apfeld and Kenyon 1999). Several possibilities could account for the discrepancy between their results and ours. First, they use a different allele of *daf-16* in their experiment that could give stronger suppression. While we have not performed epistasis with the *daf-16* null allele they used, we have performed epistasis of *unc-64* and *unc-31* with both *m27* (the allele used in our Dyf epistasis experiments) and *mgDf50*, a different null allele, and both behave identically. Thus, while we have not ruled out the possibility that *m27* is a weaker allele for suppression of Dyf mutants, it behaves like a null allele in suppression of the stronger Daf-c phenotypes of *unc-64* and *unc-31*. Two more likely possibilities for the discrepancy are that their assays were performed at a slightly lower temperature at which partial suppression appears complete or that they failed to detect *daf-16* partial dauers, which recover more rapidly. Our results do not support the model proposed by Apfeld and Kenyon (1999) that the Dyf mutants block specific sensory input to the insulin branch of the pathway. It seems more likely that the Dyf mutants act largely in parallel to the insulin branch, though it is possible that there is a direct effect of Dyf mutations on the activity of the insulin branch in addition to their effects on parallel pathways.

Reexamining the epistatic interactions of strong Daf-c genes at 27° leads to several new findings. First, mutations in *daf-16* completely suppress *daf-2* dauer formation at 25°, but only partially suppress it at 27°. This suggests that *daf-2* has *daf-16*-independent outputs at 27°. This additional branch of the pathway downstream of *daf-2* may either not exist at 25° or may not be stimulated enough to be detected. Similarly, mutants in the group II Daf-c genes are completely suppressed by *daf-5* mutations at 25° but only partially suppressed at 27°. This suggests that there may also be an additional branch of the group II Daf-c pathway detected at 27° that acts in parallel to *daf-5*. Suppression of group II Daf-c phenotypes by *daf-5* mutations illustrates several

interesting points on interpreting epistasis results. At 25°, *daf-5* completely suppresses *daf-7*. At temperatures near 27°, *daf-5* partially suppresses *daf-7*. At slightly higher temperatures, *daf-5* shows almost no suppression of *daf-7*. Thus, depending on the temperature of the assay, *daf-5* could be interpreted as completely suppressing or not suppressing at all. Is this simply a quantitative difference in suppression at different temperatures? Epistasis of dauer formation induced by pheromone at 25° suggests not. The wild-type strain N2 forms almost 100% dauers on high levels of pheromone at 25°, but forms <20% dauers at 27° without pheromone. Thus, in a wild-type background, pheromone at 25° is a stronger dauer-inducing stimulus than 27° alone. If *daf-5* mutations failed to suppress group II Daf-c mutants at 27° because the dauer-inducing stimulus was too strong, one would predict that *daf-5* mutations would be even less effective at suppressing group II Daf-c mutants on pheromone at 25°. This is not the case; mutations in *daf-5* completely suppress dauer formation in group II mutants on pheromone at 25°. Thus, although pheromone is a stronger dauer-inducing stimulus for wild type, 27° is a stronger dauer-inducing stimulus for *daf-5* mutants. The epistasis result does not correlate to intrinsic strength of the stimulus but shows qualitative differences depending on the environmental conditions of the assay and the genetic mutations present in the strains. Gene interactions inferred from epistasis experiments performed under one set of conditions may not be the same as those under a different set of environmental conditions. In complex regulatory pathways responding to multiple inputs, such differences are likely to be common.

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