**daf-2, daf-16 and daf-23: Genetically Interacting Genes Controlling Dauer Formation in Caenorhabditis elegans**

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**ABSTRACT**

Under conditions of high population density and low food, *Caenorhabditis elegans* forms an alternative third larval stage, called the dauer stage, which is resistant to desiccation and harsh environments. Genetic analysis of some dauer constitutive (Daf-c) and dauer defective (Daf-d) mutants has revealed a complex pathway that is likely to function in particular neurons and/or responding tissues. Here we analyze the genetic interactions between three genes which comprise a branch of the dauer formation pathway that acts in parallel to or downstream of the other branches of the pathway, the Daf-c genes *daf-2* and *daf-23* and the Daf-d gene *daf-16*. Unlike mutations in other Daf-c genes, mutations in both *daf-2* and *daf-23* cause non-conditional arrest at the dauer stage. Our epistasis analysis suggests that *daf-2* and *daf-23* are functioning at a similar point in the dauer pathway. First, mutations in *daf-2* and *daf-23* are epistatic to mutations in the same set of Daf-d genes. Second, *daf-2* and *daf-23* mutants are suppressed by mutations in *daf-16*. Mutations in *daf-16* do not suppress any of the other Daf-c mutants as efficiently as they suppress *daf-2* and *daf-23* mutants. Third, double mutants between either *daf-2* or *daf-23* and several other daf-d mutants exhibit an unusual interaction. Based on these results, we present a model for the function of *daf-2*, *daf-23* and *daf-16* in dauer formation.

**UNDER** optimal growth conditions, *Caenorhabditis elegans* normally develops continuously with no larval arrest through four larval stages (L1-L4) and then becomes a fertile adult hermaphrodite. However, in environments with a high density of animals and a corresponding high level of dauer-inducing pheromone, animals arrest development following the second larval molt as specialized dauer larvae (Cassada and Russell 1975). The formation of a dauer larva involves morphological changes in many tissues of the animal: dauer larvae are thinner than comparable L3 animals, have a specialized cuticle, a pharynx that is remodeled and plugged and intestinal cells that appear dark [see Riddle (1988) for review]. In addition, the molting cycle is suppressed and all feeding and growth are arrested. Because of these adaptations, the animals are more resistant to harsh chemical treatments and desiccation. Following recovery from the dauer stage after exposure to lower levels of pheromone and plentiful food, the animals appear indistinguishable from L4 animals that have not passed through the dauer stage.

The decision to become a dauer larva rather than a non-arrested L3 stage animal is regulated by at least three factors: the degree of overcrowding as determined by the level of a pheromone secreted by animals at all stages, the abundance of food, and temperature (Golden and Riddle 1982). Pheromone is the major determinant of dauer formation but food and temperature modulate the response to pheromone (Golden and Riddle 1984a,b).

Two lines of evidence suggest that sensory neurons are involved in detection of pheromone and thus initiation of dauer formation. First, ultrastructural analysis of one class of mutants that are unable to form dauer larvae in response to pheromone revealed the existence of defects in the endings of ciliated sensory neurons exposed to the environment, suggesting that dauer formation requires intact sensory neurons (Lewis and Hodgkin 1977; Albert et al. 1981; Perkins et al. 1986). Second, ablation of the sensory neuron classes ADF, ASG and ASI induces dauer formation when animals are grown in low pheromone and plentiful food, conditions which normally do not induce dauers (Bargmann and Horvitz 1991). The latter result provided direct evidence for negative regulation of dauer formation by these sensory neurons (Bargmann and Horvitz 1991). Because the absence of these sensory neurons can induce dauer formation in low pheromone conditions, these neurons or other cells signalled by these neurons are likely to secrete a signal to the responding tissues (for example, the pharynx and hypodermis) to induce continuous development when animals are exposed to low pheromone. Pheromone could function to inhibit this signal or cell.

Mutations which prevent or inappropriately induce dauer formation have been isolated [see Riddle (1988) for review]. The mutations define two general classes of genes: dauer defective (Daf-d) and dauer constitutive...
(Daf-c) genes. Animals with a mutation in a Daf-d gene are unable to arrest as dauer larvae when exposed to dauer pheromone while animals with a mutation in a Daf-c gene arrest as dauer larvae when grown in low pheromone conditions. The basis for the Daf-d phenotype may be failure of the sensory neurons to sense pheromone or to transduce this signal to other neurons or to responding tissues, failure in the initiation of dauer differentiation in the responding tissues (such as the hypodermis and pharynx) or inability to maintain the dauer-differentiated state. In contrast, the Daf-c phenotype may be due to inappropriate activation of the pheromone response in any of the cells in the pathway.

Based on genetic interactions, most of the Daf-d and Daf-c genes have been ordered into an epistasis pathway (RIDDLE et al. 1981; VOWELS and THOMAS 1992; THOMAS et al. 1993) (see Figure 1). This genetic pathway may correspond to the process of dauer formation beginning with the response to pheromone through the morphogenesis into a dauer larva. In addition, genetic analysis has suggested that there are parallel pathways for integration of the sensory information controlling dauer formation (THOMAS et al. 1993). This conclusion was based on the synergistic interactions between different classes of Daf-c genes and the observation that mutations in particular sets of Daf-d genes preferentially suppress mutations in one class of Daf-c genes but not the other (THOMAS et al. 1993).

We have been studying the interactions between three genes which comprise another branch of the dauer formation pathway that is acting in parallel or downstream of the other branches of the pathway. These genes are the Daf-c genes daf-2 and daf-23 and the Daf-d gene daf-16. Previous studies had suggested that daf-2 may be functioning on an independent branch of the pathway (RIDDLE et al. 1981; VOWELS and THOMAS 1992); however, there did not appear to be other Daf-c genes that functioned on this branch of the pathway with daf-2. In addition, the position of daf-16 relative to other genes in the pathway was not clear. Our results suggest that daf-2, daf-23 and daf-16 are functioning at a similar point in the pathway for the control of dauer formation that is distinct from the other characterized Daf-c and Daf-d genes. Based on these results, we suggest that when animals are grown in low pheromone conditions, high activity of daf-2 and daf-23 negatively regulate daf-16 activity thereby promoting non-dauer development. Conversely, when animals are exposed to pheromone, activity of daf-2 or daf-23 is down-regulated, leading to up-regulation of daf-16 activity to specify dauer development.

**MATERIALS AND METHODS**

**Methods and strains:** Strains were maintained and handled as described in Brenner (1974) and Sulston and Hodgkin (1988). The mutations used in this study were: LGI: che-3(e1379), daf-16(m26), daf-16(m27), unc-29(e1072); LGII: sqp-1(x13), lin-24(n333), daf-23(m333), daf-5(e1386), unc-52(e444); LGIII: daf-2(e1370), daf-7(e1372), unc-32(e189), dpy-1(e1); LGIV: daf-10(e1387), daf-18(e1375), dpy-9(e12); LGX: daf-6(e1377), daf-12(m20), daf-3(e1376), daf-20(m25), dpy-6(e14), egl-15(n484), unc-1(e719), dpy-3(e27), lin-2(e1309), unc-3(e151). We also used the chromosomal deficiencies mnDf87 and mnDf90 (SICHERSON et al. 1984); the chromosomal duplication sDp3 (KOSLENBTH et al. 1985); and the chromosomal rearrangement mnc1 (Herman 1978). Many of these strains were provided by the Caenorhabditis Genetics Center. daf-23(m333) was provided by PATRICE ALBERT and DON RIDDLE. Jim Thomas provided many of the strains used as markers.

**Isolation of new alleles of daf-16, daf-23 and daf-2:** We isolated two new alleles of daf-16 from different EMS mutants. One allele, mg47, was recovered due to its ability to suppress the dauer arrest phenotype of daf-2(e1370). The other allele, mg11, came from a selection for precocious dauer formation in a daf-2(e1370) background. Both daf-16 mutations were backcrossed twice. In addition, four new alleles of daf-16 were isolated after EMS mutagenesis based on their suppression of the growth arrest phenotype of daf-2(e1370); daf-12(m20) animals (H. TISENSBAUM, personal communication).

The daf-23 allele mg44 was isolated based on its constitutive dauer formation phenotype during an EMS screen, scoring in the F, generation (A. SLUDER, personal communication). Heterozygous mg44/+ animals were recovered from F, siblings. Following mapping (see below) the mutation was maintained by balancing with the chromosomal rearrangement mnc1. mg44 was shown to be an allele of daf-23 by complementation testing with the other existing daf-23 allele, m333 (RIDDLE 1988). The daf-23 allele mg33 was isolated in a y-ray mutagenesis based on its constitutive dauer formation phenotype in the F, generation (A. SLUDER, personal communication) and maintained as described above. It was found to be allelic with mg44.

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**Figure 1.** A genetic pathway for dauer formation as abstracted from VOWELS and THOMAS (1992) and THOMAS et al. (1993).
mg55 is likely to be a translocation based on the following observations. First, mg55/ + hermaphrodites segregate large numbers of dead eggs while mg55 homozygous hermaphrodites segregate only 7% dead eggs. Second, we have been unable to construct strains which are homozygous both for mg55 and several markers on chromosome I. Therefore most of the epistasis analysis described in this study was performed with mg44. daf-23 was mapped between sqt-1 and lin-29 on chromosome II. 86 Sqt non-Lin and 21 Lin non-Sqt recombinants were picked from sqt-1(sc13) + lin-29(n333)/+ daf-23(mg44) + animals. 25 Sqt non-Lin animals segregated Daf progeny, 61 did not; 19 Lin non-Sqt animals segregated Daf progeny and 2 did not. Both daf-23 alleles were backcrossed six times.

To identify mutations which caused non-conditional arrest at the dauer stage we employed two strategies. The first strategy was a selection based on the fact that dauers are resistant to 1% sodium dodecyl sulfate (SDS). The strain used for this selection also had a weak mutation in the Daf-d gene daf-16, mg11 (see results). Animals bearing this mutation are able to form a high percentage of SDS-resistant dauers which then recover from the dauer stage. Therefore, dauers recovered after mutagenesis, would be likely to recover from the dauer stage. Synchronous populations of EMS mutagenized animals were grown at 15° and dauers were isolated by selection in 1% SDS. In addition, the same strain was screened clonally (4980 genomes) at 15° looking for dauer formation in the F1 generation. We recovered one allele of daf-11 and two alleles of daf-8 from the selection and two alleles of daf-7, and three alleles of daf-11 from the screen.

One of the alleles of daf-2 recovered above, mg42, forms 100% dauers at 15°. The daf-2 allele mg43 was isolated independently from an EMS screen based on its constitutive dauer formation phenotype (A. Sluder, personal communication). Both mg42 and mg43 were initially maintained as heterozygotes but, following identification as daf-2 alleles, were subsequently balanced with the chromosome duplication +Dp3. Both daf-2 alleles were backcrossed at least twice.

**Dauer formation in liquid:** To induce synchronous dauer formation in liquid, eggs were isolated by bleach treatment from gravid hermaphrodites and incubated in S Basal (SuSlton and Hodgkin 1988) in the absence of food for 12-16 hr at 25° to synchronize all animals at the L1 stage (SuSlton and Hodgkin 1988). Then, 10,000 synchronized L1 animals were placed into a 50-ml flask containing 10 ml S Medium (SuSlton and Hodgkin 1988), 5 μl of a crude pheromone preparation (prepared as described in Golden and Riddle 1984b) and 600 μl of a 4% solution of streptomycin-treated bacteria. The flask was then placed in a shaking water bath at 25°. One-milliliter aliquots were removed at 50, 55, 60, 72 and 100 hr, and dauer formation was measured as the percentage of animals that survived a 90-min incubation in 1% SDS. Each data point shown in Figure 3A is the average of the percent dauer formation for two independent trials.

**Dauer formation on plates in daf-16; daf-2 experiments:** Synchronized L1 animals were isolated as described above and aliquotted onto plates, eight plates per strain, approximately 600 animals per plate, and incubated at 25°. Animals were washed from the plates at 34, 38, 42, 46, 60, 64 and 84 hr and resuspended in 1% SDS for 30 min. Dauer formation was measured as the percentage of animals that survived. Each data point shown in Figure 3B is the average of the percent dauer formation for two independent trials.

**Double mutants of double mutants between daf-23 and the Daf-d genes were constructed in several different ways. All of these strain constructions utilize the fact that daf-23(mg44) is completely maternally rescued and is also zygotically rescued by mating to males with a wild-type daf-23 gene. In addition, daf-23(mg44) is marked by a mutation in the closely linked gene sqt-1.**

**daf-16 and daf-18:** Wild-type males were mated with maternally rescued sqt-1(sc13) daf-23(mg44) mutant hermaphrodites. These sqt-1(sc13) daf-23(mg44) +/+ males were then mated with daf-16 mutant hermaphrodites. Putative F1 cross progeny were picked to independent plates and Sqt animals were picked from the plates and incubated at 25°. The progeny of the F1 Sqt animals were scored for the suppression of the daf-23 mutant phenotype (all dauer larvae in the F1) or the presence of any novel phenotypes. Since all alleles of daf-16 suppress daf-23(mg44), fertile F3 sqt-1 daf-23; daf-16 adults were obtained and the triple mutant strain could be maintained. The presence of the daf-23 mutation was verified by a complementation test. The results for the daf-16 crosses were as follows: for m26, 9/40 Sqt animals (possible genotypes: sqt-1 daf-23; daf-16, sqt-1 daf-23; daf-16/+ and sqt-1 daf-23; +) segregated F1 fertile adults, 13/40 segregated all dauer larvae, and 18/40 segregated dauer larvae and fertile adults; for m27 8/40 segregated all fertile adults, 11/40 segregated all dauer larvae, 20/40 segregated dauer larvae and fertile adults and 1/40 gave no progeny; for mg11 11/40 segregated all fertile adults, 4/40 segregated all dauer larvae, 24/40 segregated dauer larvae and fertile adults and 1/40 gave no progeny. daf-18(e1375) almost completely suppresses daf-23(mg44). Therefore, the double mutant with mg44 was constructed as described above for doubles with daf-16. The results were as follows: 6/20 Sqt animals segregated all fertile adults, 7/20 segregated all dauer larvae and 7/20 segregated dauer larvae and fertile adults.

daf-6, daf-10 and che-3: The presence of daf-6, daf-10 and che-3 homozygous animals was verified using the phenotype that these mutants are defective in uptake of the fluorescent dye DIO (Perkins et al., 1986; Herman and Hedgecock 1990). Wild-type males were mated with maternally-rescued sqt-1(sc13) daf-23(mg44) mutant hermaphrodites. These sqt-1(sc13) daf-23(mg44) +/+ males were then mated with daf-10(e1387), daf-6(e1377) or che-3(e1379) mutant hermaphrodites. Putative cross progeny were picked to individual plates and six non-Sqt progeny were picked from those plates segregating Sqt animals. Several animals from the broods of these all segregating Sqt animals were tested for their ability to take up DIO. A sqt-1(sc13) daf-23(mg44)/+ + heterozygous strain was maintained from plates homozygous for daf-6, daf-10 or che-3 and Sqt animals were picked for analysis.

daf-3, daf-20 and daf-12: All three of these genes are X-linked, therefore double mutants were constructed in a similar manner as described in Vowels and Thomas (1992). Wild-type males were mated with maternally-rescued sqt-1(sc13) daf-23(mg44) mutant hermaphrodites. These sqt-1(sc13) daf-23(mg44)/+ + males were then mated with Daf-d mutant hermaphrodites. The resulting sqt-1(sc13) daf-23(mg44)/+ + males were then mated with daf-12 ZZ, sqt-1(scl3) daf23(mg44)/+ and daf-d females were then mated with hermaphrodites which contained X chromosome markers flanking the daf-d gene. From the sqt-1(sc13) daf-23(mg44)/+ + females, strains were isolated which no longer segregated m1 or m2, and were heterozygous for sqt-1(sc13). The sqt-1(sc13) daf-23(mg44)/+ + heterozygous strain was maintained and Sqt animals were picked for analysis. The markers were unc-1 and dpy-3 for daf-3; dpy-6 andegl-15 for daf-12; and lin-2 and unc-3 for daf-20.

daf-5: daf-5 maps on chromosome II, 17-26 map units to the right of daf-23, so the strain sqt-1(sc13) daf-23(sc14) daf-5/+ was used as the starting strain (Allan and Riddle 1986). sqt-1(sc13) daf-23(sc14) unc-52/+ + unc-52 was constructed (unc-52 is approximately 9 map units to the right of daf-5). Then sqt-1(sc13) daf-23(sc14) unc-52 males were mated with daf-5 hermaphrodites. 100 non-Sqt progeny from sqt-1(sc13) daf-23(sc14) unc-52/+ + unc-52 females were tested for their ability to take up DIO. A sqt-1(sc13) daf-23(sc14)/+ + heterozygous strain was maintained from plates homozygous for daf-5, daf-10 or che-3 and Sqt animals were picked for analysis.

These mutants were then crossed to a daf-23 strain, so the strains were suitably mutagenized. Then, the strain (daf-23) daf-23(mg44) unc-52/+ + unc-52 was constructed. Both daf-23(mg44) and daf-5 were mapped using the translocation 1(sc13) daf-23(mg44) unc-52/+ + unc-52. In addition, daf-23(mg44) was marked by a mutation in the closely linked gene sqt-1.
daf-23 (mg44) + unc-52/2 + daf-5 + parents were picked and their progeny scored for the presence of only Sqt non-Unc and wild-type animals. It is expected that ~85% of such animals would have the genotype sqt-1 (scl3) daf-23 (mg44) daf-5/+ + daf-5. To verify that the strain was actually homozygous for daf-5, we tested whether the strain contained a suppressor of the Daf-c mutant daf-7 (e1372), since a mutation in daf-7 suppresses a mutation in daf-7, daf-7 (e1372); him-8 (e1489) males were mated with both Sqt and non-Sqt segregants of the strain, F2 non-Sqt dauer larvae were picked at 25°C (the non-permissive temperature for daf-7), allowed to recover from the dauer stage at 15°C and shifted back to 25°C. If daf-5 was present, then some of the daf-7 (e1372) dauer larvae that were isolated would now produce progeny that bypass the dauer stage. All isolates tested segregated suppressors of daf-7 (e1372). The sqt-1 (scl3) daf-23 (mg44) daf-5/+ + daf-5 strain was maintained and Sqt animals were picked for analysis.

daf-2: daf-2(e1370) males were mated with maternally rescued sqt-1 (scl3) daf-23 (mg44) hermaphrodites. Non-Sqt F1 cross progeny were incubated at 25°C, the non-permissive temperature for daf-2. Non-Sqt F2 dauer larvae were picked to 15°C to recover from the dauer stage. Non-Sqt animals were picked from plates segregating Sqt animals to maintain the daf-23 heterozygous strain. Subsequently, Sqt animals were picked to 25°C and 15°C and the phenotype of their progeny examined. In all cases, Sqt animals gave no fertile progeny.

All double mutants between alleles of daf-16 and daf-2 (e1370) were isolated by first picking F2 dauer larvae at 25°C from a cross between homozygous daf-16 and daf-2 strains, downshifting to 15°C, the daf-2 permissive temperature, and then picking from among the broods of the homozygous daf-2 mutant animals for segregants that now bypass the dauer stage at 25°C. In all cases, strains were rechecked for the presence of the daf-2 mutation by complementation tests. The daf-16 double mutants with daf-2 (mg43), a nonconditional allele of daf-2, were constructed by crossing daf-16 males with dpy-1 (e1) daf-2 (mg43); sdp3. sdp3 is a chromosomal duplication that covers both daf-2 and dpy-1 (ROSENBLUTH et al. 1985). Dpy non-Daf animals were picked in the next generation. Those animals which did not segregate dauer larvae in subsequent generations were then tested for the presence of the daf-2 mutation.

The strain unc-29(e1072) daf-16 (m27); daf-12 (m20) was constructed as follows: unc-29(e1072) daf-16 (m27) +/- males were mated with daf-12 hermaphrodites. The cross-progeny males were then mated with dpy-6 egl-15 hermaphrodites (dpy-6 and egl-15 flank daf-12). The resulting non-Unc non-Dpy non-Egl cross progeny were then allowed to self, and Unc non-Dpy non-Egl progeny were picked. Isolates were kept from those plates which segregated no Dpy Egl animals (genotype unc-29 daf-16; daf-12). To test for the presence of the daf-16 mutation, daf-2 (e1370) males were mated with the presumed unc-29(e1072) daf-16 (m27); daf-12 (m20) strain and after subsequent selection for homozygous daf-2 (e1370) animals, it was demonstrated that a suppressor of daf-2 (e1370) was segregating as expected in the strains.

Construction of triple mutants: The strain daf-16 (m27); sqt-1 (scl3) daf-23 (mg44); daf-2 (e1370) unc-32 (e189) was constructed by crossing daf-16; daf-2/+ daf-2 unc-32 males with daf-16; sqt-1 daf-23(+) +; daf-2/2+ and ½ daf-16; sqt-1 daf-23/+ +; daf-2 unc-32(+) +, Unc Sqt F1 animals were picked. These animals were then tested for the presence of both daf-23 and daf-2 by complementation tests. The strain daf-16 (mg11); sqt-1 (scl3) daf-23 (mg44); daf-2 (e1370) was constructed by mating daf-16; daf-2/+ males with daf-16; sqt-1 daf-23 hermaphrodites. Ten non-Sqt F1 (½ daf-16; sqt-1 daf-23/+ +; daf-2/2+) and ½ daf-16; sqt-1 daf-23/+ +; daf-2 unc-32(+/+) animals were picked and incubated at 25°C. The F2 generation was then scored for the presence of transient dauer larvae as would be expected since daf-16 (mg11) is a weak suppressor of mutations in daf-2. Non-Sqt dauer larvae (% sqt-1 daf-23/+ +) were picked from the one F2 brood which was segregating dauer larvae and allowed to recover at 15°C. F2 Sqt animals were then selected and tested for the presence of both daf-23 and daf-2. The strain daf-16 (m27); sqt-1 (scl3) daf-23 (mg44); dpy-1 (e1) daf-2 (mg43) was constructed by crossing daf-16 (m27); sqt-1 (scl3) daf-23 (mg44)/+ males with daf-16 (m27); dpy-1 (e1) daf-2 (mg43) hermaphrodites. The resultants non-Dpy cross progeny were then allowed to self and Sqt non-Dpy progeny were picked. From those, Dpy segregants were picked. The resulting strain was then checked for the presence of the daf-2 and daf-23 mutations.

The daf-16 (m27); daf-2 (e1370); daf-12 (m20) strain was constructed as follows: daf-16 (m27); daf-2 (e1370) males were mated with daf-2 (e1370); daf-12 (mg55) hermaphrodites (grown at 15°C). The daf-16 (m27) +/-; daf-2 (e1370); daf-12 (m20) males were then crossed with dpy-6 egl-15 hermaphrodites. The non-Dpy non-Egl cross progeny hermaphrodites were then incubated at 25°C, the nonpermissive temperature for daf-2(e1370), and non-Dpy dauer larvae of genotype daf-16/+ or +; daf-2; daf-12 or daf-12/dpy-6 egl-15 were picked. The dauer larvae were allowed to recover at 15°C and then shifted back to 25°C as L4s or early adults and allowed to self. Non-Dpy non-Egl animals which did not arrest as dauer larvae (genotype: daf-16; daf-2; daf-12 or daf-12/dpy-6 egl-15) were picked from their progeny. Animals which did not segregate Dpy Egl progeny were kept and retested for the presence of daf-2 (e1370) and daf-12 (m20).

The daf-16 (m27); sqt-1 (scl3) daf-23 (mg44); daf-12 (m20) strain was constructed in a similar manner as described above for the triple mutant with daf-2 (e1370) except that daf-23 was selected by picking the linked marker sqt-1 (scl3). The resulting strain was rechecked for the presence of daf-23 (mg44) and daf-12 (m20).

Scoring dauer bypass: In those cases where suppression of the daf-23 or daf-2 mutant phenotype was observed, synchronized populations of animals were analyzed to monitor transient dauer formation. Gravid hermaphrodites were picked to plates at the appropriate temperature and allowed to lay eggs for 6 hr. The hermaphrodites were then removed from the plates. The plates were scored two days later to determine if any of the eggs had failed to hatch or if L1 animals were not developing. Final scoring of the plates was done after 3 days for plates incubated at 25°C, and 6 days for plates incubated at 15°C. Examination of plates at these times allowed the identification of animals that transiently arrested as dauer larvae.

Measuring dominant suppression by daf-16 alleles: To measure the dominant suppression of dauer formation by daf-16 alleles, a heterozygous (daf-16 +) parent was picked to a plate at 25°C at the L4 stage. The parent was transferred to a fresh plate every day until it stopped laying eggs. All of the progeny were picked to individual plates and their progeny (or lack of progeny) were scored.

RESULTS

A general screen for non-temperature-sensitive Daf-c mutations identified daf-23 and daf-2. In a genetic screen for mutations which cause a non-temperature-sensitive Daf-C phenotype, we have isolated two mutations, mg44 and mg55, that are allelic and cause a maternal effect Daf-C phenotype. We mapped mg44
The dauer constitutive phenotype is maternally rescued for both \( \text{daf-23} \) alleles. Homozygous progeny from heterozygous parents do not arrest at the dauer stage. In addition, the mutant phenotype can be rescued zygotically by crossing wild-type males to homozygous mutant hermaphrodites. The phenotype of \( \text{daf-23} \left( \text{mg44} \right) / \text{mnD}[87] \) was examined and the results are shown in Table 1: approximately 30% of the progeny are dead eggs (homozygous deficiency), approximately 70% arrest at the dauer stage (\( \text{daf-23/Df} \) or \( \text{daf-23} \)) and less than 1% arrest development as newly hatched L1 animals. The L1 animals are developmentally arrested but appear normal when examined by Nomarski microscopy. These data suggest that \( \text{mg44} \) is a reduction-of-function allele of \( \text{daf-23} \).

In addition to \( \text{daf-23} \), we identified non-temperature-sensitive \( \text{Daf-c} \) mutations in one other gene, \( \text{daf-2} \). While the majority of mutant alleles of the \( \text{Daf-c} \) gene \( \text{daf-2} \) are temperature sensitive, we isolated two non-conditional alleles of \( \text{daf-2} \) from EMS screens for dauer formation at 15°. Both mutations cause 100% dauer arrest at 15°. The dauer larvae that are formed are indistinguishable from pheromone-induced wild-type dauers. This has been reported previously that non-conditional alleles of \( \text{daf-2} \) can be recovered (Riddle 1988; Malone and Thomas 1994). These non-temperature-sensitive \( \text{daf-2} \) alleles behave similarly to conditional alleles in epistasis tests (see below). We assume that the non-temperature-

<table>
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<th>Genotype</th>
<th>Dead egg</th>
<th>Dauer*</th>
<th>L1°</th>
<th>Adult</th>
<th>N°</th>
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<tr>
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<td>93</td>
<td>0.5</td>
<td>0</td>
<td>365</td>
</tr>
</tbody>
</table>

* This class includes animals that arrest as either dauers or in the specialized stage preceding dauers, the L2d stage.
° These animals never develop beyond the L1 stage.
† This number represents the entire broods of three hermaphrodites for each strain.
* Since this strain is likely to be a complex rearrangement (see MATERIALS AND METHODS), the presence of dead eggs may not be due to the \( \text{daf-23} \) mutant phenotype but rather to lack of backcrossing.
sensitive daf-2 alleles are more null-like, but without analysis of these alleles in trans to deficiencies, such an assumption is provisional.

In this screen for non-conditional Daf-c mutants, we also isolated alleles of several other previously identified Daf-c genes: two alleles of daf-7, two alleles of daf-8 and four alleles of daf-11. Although these mutations were isolated because they form dauer larvae at 15°, the Daf-c phenotype was less severe at 15° than at 25°. Thus, similar to alleles isolated in high temperature screens for Daf-c mutants, these new daf-7, daf-8 and daf-11 alleles show temperature sensitivity (Swanson and Riddle 1981; Malone and Thomas 1994).

daf-23 epistasis analysis: Extensive genetic epistasis analysis between mutations in Daf-d genes and daf-2 has already been done (Riddle et al. 1981; Vowels and Thomas 1992). However, the position of daf-23 relative to the Daf-d genes was not known. To determine where daf-23 fits into the existing genetic pathway for dauer formation, we constructed double mutants between daf-23(mg44) and mutations in nine of the existing Daf-d genes: daf-3, daf-5, daf-6, daf-10, daf-12, daf-16, daf-18 and daf-20 and che-3 (see MATERIALS AND METHODS). These nine Daf-d genes appear to affect several distinct steps in the pathway for dauer formation (see Figure 1) based on their dauer defective phenotype, genetic interactions with Daf-c genes and known ultrastructural defects. Three mutants, daf-6, daf-10 and che-3 are thought to function in the sensory portion of the pathway for dauer formation since mutations in these genes cause ultrastructural defects in the chemosensory sensilla, the amphid, as well as in other chemosensory neurons. daf-6 mutants have a defect in sheath cell formation which causes the amphid opening to form improperly such that the endings of the sensory neurons are no longer exposed to the environment (Albert et al. 1981; Herman 1984). The sensory neurons of both che-3 and daf-10 mutants have defective ciliated endings (Lewis and Hodgkin 1977; Albert et al. 1981). daf-23 is epistatic to all three of these genes (Table 2) and therefore is unlikely to be functioning in the sensory endings. However, daf-23 exhibits an additional interaction with both che-3 and daf-10 that is not observed in double mutants with daf-6. 27% of daf-23; daf-10 animals and 67% of che-3; daf-23 animals arrest as dead eggs or unhatched L1 animals (Table 2). The arrested L1 animals are not dead; they move (although somewhat sluggishly) and pharyngeal pumping occurs occasionally. This suggests that daf-23 has a function in addition to dauer formation and that while daf-23 is epistatic to daf-10 and che-3 for dauer formation there is a synergistic interaction between the two genes in a different, essential pathway. This phenotype was observed previously in double mutants between the Daf-c gene daf-2 and the ciliom-structure mutants (Vowels and Thomas 1992).

The Daf-d genes daf-3 and daf-5 function further downstream in the pathway. Mutations in these genes do not appear to affect the sensory endings and are able to suppress to varying degrees mutations in a large group of Daf-c genes. Because mutations in daf-3 and daf-5 suppress the dauer formation normally induced by laser ablation of the sensory neurons ASI, ADF and ASG, daf-3 and daf-5 are unlikely to be functioning exclusively in those neurons (Bargmann and Horvitz 1991). Mutations in daf-3 and daf-5 do not suppress daf-23(mg44) (Table 2), suggesting that daf-23 either functions downstream of daf-3 and daf-5 or on a different pathway.

The Daf-d gene daf-12 also functions downstream of most Daf-c genes but displays an unusual interaction with daf-23. daf-12 mutations suppress mutations in every other Daf-c gene except daf-2. The double mutant between daf-12(m20) and daf-23(mg44) exhibits a novel phenotype unlike either single mutant (Table 2). Double mutant animals initially arrest development during the L2 stage although after several days ~30% of the animals continue to develop into late larvae or sterile adults. Unlike daf-23 mutant larvae and adults, these animals do not have a dark intestine. Therefore a mutation in daf-12 is able to suppress some of the daf-23 dauer-like phenotypes. However, because a mutation in daf-12 does not suppress all daf-23 phenotypes, it is not possible to order the genes relative to one another; rather, it is more likely that these genes function in independent pathways. Interestingly, the phenotype of this double mutant is similar to the phenotype of a daf-2; daf-12 double mutant (Vowels and Thomas 1992). The

<table>
<thead>
<tr>
<th>daf-d</th>
<th>Fertile adults</th>
<th>Dauer*</th>
<th>Other</th>
<th>N*</th>
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<td>507</td>
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<tr>
<td>daf-16(m27)</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>2014</td>
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<tr>
<td>daf-18(e1375)</td>
<td>99</td>
<td>0</td>
<td>1'</td>
<td>903</td>
</tr>
</tbody>
</table>

* This class includes animals that arrest as either dauers or in the specialized stage preceding dauers, the L2d stage.
* N = the total number of animals scored.
* Twenty-seven percent of progeny arrest development at the L1 stage or die as embryos.
* Sixty-seven percent of progeny arrest development at the L1 stage or die as embryos.
* Animals initially stop development at the L2 stage but, after several days, many continue developing into late larvae or sterile adults.
* One percent of progeny become sterile adults.
similarity between daf-2 and daf-23 will be discussed further below.

The three remaining Daf-d genes, daf-16, daf-18 and daf-20, were not clearly placed in the previously published versions of the dauer pathway (Riddle et al. 1981; Vowels and Thomas 1992). Both daf-16 and daf-18 mutants inefficiently form dauer larvae in pheromone and the dauer larvae then recover inappropriately [Vowels and Thomas (1992); this study (see below)]. daf-20 mutant animals are almost completely defective in dauer formation in response to pheromone (Vowels and Thomas 1992). However, all three mutations cause formation of partial dauers when in double mutant combination with several of the Daf-c mutants, such as daf-7 and daf-11 (Vowels and Thomas 1992). These double mutants arrest development at the dauer stage but the dauers that are formed are incomplete, indicating that these Daf-d genes are necessary for complete dauer formation in these mutant backgrounds but do not completely suppress. Because of this partial dauer phenotype, it is not possible to position these Daf-d genes with respect to the other genes in the pathway shown in Figure 1.

The epistasis pathway is clearer with daf-23. Mutations in daf-16 completely suppress daf-23 mutants (Table 2 and Figure 2C). Double mutant animals form fertile adults. Synchronized populations of animals were followed to monitor transient dauer formation and none was observed. In addition, even the phenotypically weakest allele of daf-16, mg11 (see below), completely suppresses daf-23(mg44). The only other Daf-c mutant that is suppressed in this way by mutations in daf-16 is daf-2 (Vowels and Thomas 1992). Therefore, daf-16 functions downstream of both daf-2 and daf-23.

A mutation in one other Daf-d gene, daf-18, exhibits suppression of daf-23 mutants: 99% of daf-23(mg44); daf-18(e1375) animals formed fertile adults (Table 2). However, the dauer constitutive phenotype of daf-23(mg55) was only partially suppressed by daf-18(e1375): 4% of the progeny of daf-23(mg55); daf-18(e1375) animals became fertile adults, 8% became sterile adults and the remainder arrested as dauer larvae (n = 543). As mentioned above, it is likely that mg55 is a stronger allele of daf-23 than mg44. Since it is possible that the single existing allele of daf-18 is a weak allele, a stronger allele of daf-18 may suppress mg55 more completely. Therefore, based on these results daf-18 may also function downstream of daf-23; however, more alleles of daf-18 need to be identified and tested. A mutation in daf-20 does not suppress daf-23(mg44) (Table 2).

don-23 and daf-2 exhibit similar epistasis patterns: It is clear from the results of the epistasis analysis that daf-23 is functioning at a different point in the pathway or on an independent pathway from the Daf-c genes daf-1, daf-4, daf-7, daf-8, daf-11, daf-14 and daf-21 (Riddle et al. 1981; Vowels and Thomas 1992; Thomas et al. 1993). However, one other Daf-c gene, daf-2, shows a genetic epistasis pattern nearly equivalent to daf-23 (Riddle et al. 1981; Vowels and Thomas 1992). Mutations in either gene are epistatic to mutations in the Daf-d genes daf-6, daf-10, daf-3, daf-5 and daf-20 and, for both daf-2 and daf-23, double mutants with mutations in the Daf-d gene daf-12 exhibit a similar L2 arrest phenotype (Riddle et al. 1981; Vowels and Thomas 1992; this study). In addition, double mutants with either daf-2 or daf-23 and Daf-d mutations that cause defects in cilium structure exhibit an incompletely penetrant L1 arrest phenotype (Vowels and Thomas 1992; this study). The constitutive dauer formation phenotype of both daf-2 and daf-23 is suppressed by mutations in daf-16 (this study; Riddle et al. 1981; Vowels and Thomas 1992). In addition, although the single mutant allele of daf-18 does not suppress daf-2 mutants as strongly as daf-23 mutants, 10% of daf-2(mg44); daf-18(e1375) animals bypass dauer arrest and the dauers that form are defective and recover from the dauer stage within 24 hr (data not shown). Finally, we note that unlike mutations in the other Daf-c genes mentioned above, there are non-temperature-sensitive alleles of both daf-23 and daf-2 which exhibit dauer arrest at all temperatures (this study; Riddle 1988; Malone and Thomas 1994).

Most previous epistasis analysis utilized temperature-sensitive alleles of daf-2 (Riddle et al. 1981; Vowels and Thomas 1992). To investigate the possibility that a non-temperature-sensitive daf-2 allele might not be suppressed by mutations in daf-16, we constructed double mutants between the non-temperature-sensitive daf-2 allele, mg43, and two daf-16 alleles, mg11 and m27. In both cases, mutations in daf-16 suppressed the daf-2 mutant phenotype (Table 3). In addition, daf-2(mg43); daf-12(m20) double mutants display the same growth arrest observed in double mutants between daf-12(m20) and a temperature-sensitive allele of daf-2 (data not shown). Therefore, based on these criteria, the non-temperature-sensitive and temperature-sensitive alleles of daf-2 behave similarly.

We also constructed daf-2; daf-23 double mutants and the phenotype of the double mutants is dauer constitutive (Table 3). The dauer constitutive phenotype of these double mutants also is suppressed by mutations in daf-16 (Table 3). These results suggest that daf-2 and daf-23 act on the same pathway that also includes daf-16 and that daf-16 acts downstream of both daf-2 and daf-23.

Dauer formation in daf-16 mutants: The original alleles of daf-16 were isolated as suppressors of mutations in the Daf-c gene daf-2 (Riddle et al. 1981). Alone, mutations in the daf-16 gene cause a dauer defective phenotype. To examine more closely the basis of the defect, we monitored the development of synchronous populations of daf-16 mutant animals when exposed to high pheromone/low food conditions in liquid media (see
The number in parentheses is the total number of animals scored, so, not determined.

The percent fertile adults was scored after 3 days; over time, all dauers recover.

Although it is somewhat surprising that daf-16(mg11) suppresses mg43, the nonconditional allele of daf-2, more strongly than it suppresses e1370, the temperature-sensitive allele, it is possible that this difference is due to the presence of the dpy-1 mutation in the mg43 strain.

Dpy worms scored as segregants from the strain dpy-1(e1) daf-2(e1370); dpy-3.

This strain is also unc-32(e189). The presence of this mutation does not affect the Daf-c phenotype of daf-2.

We also monitored dauer formation in synchronized populations of daf-16 mutants which were induced to form dauer larvae using a temperature-sensitive allele of the Daf-c gene daf-2, e1370, at the nonpermissive temperature. For this experiment, we tested four different alleles of daf-16, of which were isolated in this study (see MATERIALS AND METHODS), and two of which were isolated previously (Riddle et al. 1981). The relative effects of the different daf-16 alleles on dauer formation in this assay were similar to that observed with pheromone-induced dauer larvae (Figure 3B). Specifically, daf-16(mg11) formed dauer larvae at a high frequency while the other daf-16 mutants formed dauer larvae inefficiently [daf-16(mg26)] or not at all [daf-16(m27) and daf-16(mg47)]. Again, the pharaynxes of many of the daf-16 mutant animals that survived the SDS treatment were incompletely constricted. Other daf-16 alleles in a daf-2 genetic background behaved similarly. Synchronized broods of four other independent daf-16 mutations isolated based on their suppression of the daf-2(e1370); daf-12(m20) growth arrest phenotype (H. Tissenbaum and G. Ruvkun, personal communication) behaved similarly to the previously isolated mutants: two alleles formed no dauer larvae in combination with daf-2; one allele formed 23% transient dauer larvae and one allele formed 86% transient dauer larvae.

One difference between daf-2-induced and pheromone-induced dauer larvae is that strong daf-16 alleles completely suppress dauer formation induced by mutations in daf-2 but still form a low percentage of transient dauer larvae with pheromone. A simple explanation for this observation is that pheromone downregulates both the daf-2/daf-23 pathway and the other

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**TABLE 3**

Suppression of the daf-2, daf-23 and daf-2; daf-2 dauer constitutive phenotype by mutations in daf-16

<table>
<thead>
<tr>
<th>Genotype</th>
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<th>daf-16(mg11)*</th>
<th>daf-16(m27)</th>
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<tr>
<td>sqt-1(sci3) daf-23(mg44)</td>
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<td>100 (973)</td>
<td>100 (2014)</td>
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<td>100 (618)</td>
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<tr>
<td>daf-16(mg47)</td>
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<td>76 (459)</td>
<td>100 (290)</td>
</tr>
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<td>daf-16(mg43)</td>
<td>0 (212)</td>
<td>100 (655)</td>
<td>100 (583)</td>
</tr>
<tr>
<td>daf-16(m27)</td>
<td>ND</td>
<td>ND</td>
<td>100 (509)</td>
</tr>
</tbody>
</table>

The percent fertile adults was scored after 3 days; over time, all dauers recover.

Although it is somewhat surprising that daf-16(mg11) suppresses mg43, the nonconditional allele of daf-2, more strongly than it suppresses e1370, the temperature-sensitive allele, it is possible that this difference is due to the presence of the dpy-1 mutation in the mg43 strain.

This strain is also unc-32(e189). The presence of this mutation does not affect the Daf-c phenotype of daf-2.

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MATERIALS AND METHODS. Using these conditions, we induced greater than 95% dauer formation in wild type (see Figure 3A). We tested three different alleles of daf-16, one of which was isolated in this study (see MATERIALS AND METHODS) and two of which were isolated previously (Riddle et al. 1981). The efficiency of dauer formation in pheromone was monitored by determining the percentage of animals which acquire resistance to SDS. We note, however, that animals which are not completely differentiated into dauers can survive an SDS test. For example, some of the daf-16 mutant animals which form SDS-resistant dauers do not have the constricted pharynx typical of wild-type dauers. However, these animals are dauers by all other criteria. The results from this experiment are shown in Figure 3A. Several conclusions can be drawn from this experiment. First, the three daf-16 alleles differ in the severity of their dauer defective phenotype. mg11, which was isolated in a selection for precocious dauer formation (see below), can form high levels (almost 90%) of transient, SDS-resistant dauer larvae and is therefore presumed to be the weakest allele; m26 is intermediate and m27 appears to be the strongest allele, forming only about 5% transient dauer larvae in pheromone. Yet, despite the difference in the ability to form SDS-resistant dauer larvae, all three mutant populations are unable to maintain the dauer differentiated state as indicated by the fact that there are no SDS-resistant animals at 100 hours in any of the daf-16 mutant cultures. Wild-type animals in pheromone maintain SDS-resistant dauer larvae over this period. This suggests that daf-16 is required both to initiate dauer formation as well as to maintain the dauer differentiated state. Alternatively, it is possible that the dauer larvae formed in weak daf-16 mutants are somehow defective and this is the cause of their inappropriate recovery.

When we induced dauer formation on plates containing a high level of pheromone and little food, we obtained similar results. More than 50% of both mg11 and m27 animals formed dauers or partial dauers after two days. The dauers were indistinguishable from wild-type dauers except that the pharynx was not constricted. The partial dauers had unconstricted pharynxes that pumped occasionally, the dauer alae were indistinct and the dauers were not as thin as wild-type dauers. One day later, all of the dauers had recovered and there were 10-15% partial dauers. Surprisingly, m26 formed no dauers or partial dauers on plates. Similar results were obtained by Vowels and Thomas (1992).
Dauer Formation in *C. elegans*

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**major Daf-C pathways** (which include, for example, *daf-7* and *daf-11*) and mutations in *daf-16* can only efficiently suppress the *daf-2* pathway so that in pheromone the other pathway induces transient and partial dauer formation. Such an explanation is consistent with the observation that mutations in *daf-16* do not efficiently suppress Daf-C mutants other than *daf-2* or *daf-23*. An alternative explanation is that the alleles of *daf-2* which were tested do not cause as severe a decrease in *daf-2* gene activity as does pheromone treatment in which case *daf-2* (null); *daf-16* mutants would form transient dauers like pheromone treated *daf-16* mutants.

*daf-16* does not suppress dauer formation induced by other Daf-c mutations as efficiently as it suppresses *daf-2* and *daf-23*. For comparison, we monitored the effect of *daf-16(m27)* on dauer formation in another Daf-c mutant *daf-7(e1372)*. Observation of synchronized broods of *daf-16(m27); daf-7(e1372)* double mutants indicated that 2% of animals completely bypassed the dauer stage while the remaining animals arrested as partial dauers. Therefore, as shown previously by VOWELS and THOMAS (1992), mutations in *daf-16* strongly suppress mutations in *daf-2* but not other Daf-c genes. In addition, we note that many of the partial dauers formed in *daf-16(m27); daf-7(e1372)* double mutants are SDS-resistant although the percentage of SDS-resistant dauers decreases over time (data not shown).

**Precocious dauer formation in *daf-16(mg11)*:** Our results also demonstrate that, of the animals that enter the dauer stage, both pheromone-induced dauer larvae and *daf-2*-induced dauer larvae in a *daf-16* mutant background enter the dauer stage about six hours early (Figure 3). To determine more precisely the basis of this precocious phenotype, we followed the development at 25° of synchronous populations of wild type, *daf-2(e1370)*, *daf-16(mg11); daf-2(e1370)* and *daf-16(mg11)* animals. Synchronous populations of starved L1 animals were plated on food to initiate development. After 16.5 hr, the stage of the animals was determined based on two criteria: the presence or absence of L1 alae and the size of the gonad. More than 90% of wild type animals were already at the L2 stage. In contrast, more than 90% of *daf-2(e1370)* animals were still at the L1 stage. Therefore, animals that are going to differentiate into dauer larvae have a longer L1 stage (as observed previously by SWANSON and RIDDLE 1981). At 16.5 hr, the population of *daf-16(mg11); daf-2(e1370)* animals was at the L2 or predauer L2d stage. Therefore, the precocious of animals were aliquotted onto plates with food at 25°, the nonpermissive temperature for *daf-2(e1370)*. Dauer formation was monitored by washing worms off of plates at the indicated time and incubating them in 1% SDS for 30 min. The animals were then washed and moved to a plate with food. The percentage of animals that survived the SDS treatment and continued development was calculated.

**Figure 3.**—Each data point in both graphs represents the average of two independent trials for each strain. (A) Dauer formation in wild-type and *daf-16* mutant animals in liquid culture in the presence of added pheromone. Synchronous cultures of animals were grown under dauer-inducing conditions (see MATERIALS AND METHODS). Dauer formation was monitored by removing aliquots at the time points indicated and incubating animals in 1% SDS for 30 min. The animals were then washed and moved to a plate with food. The percentage of animals that survived the SDS treatment and continued development was calculated. (B) Dauer formation in *daf-2* and *daf-16; daf-2* strains on plates at 25°. Synchronous populations of animals were aliquotted onto plates with food at 25°, the nonpermissive temperature for *daf-2(e1370)*. Dauer formation was monitored by washing worms off of plates at the indicated time and incubating them in 1% SDS for 30 min. The animals were then washed and moved to a plate with food. The percentage of animals that survived the SDS treatment and continued development was calculated.
whether mutations in both genes. In order to further position downstream of both growth phenotype of mutations in contrast, if should have a addition, this result suggests that has a role in the early stages of dauer differentiation. does not accelerate all development because this strain did not reach sexual maturity and begin laying eggs faster than wild type (data not shown).

daf-16 suppresses the growth arrest phenotype of daf-23; daf-12 and daf-2; daf-12 double mutants: The results presented thus far suggest that daf-16 functions downstream of both daf-2 and daf-23 because mutations in daf-16 completely suppress the mutant phenotype of both genes. In order to further position daf-16, daf-12, daf-2 and daf-23 in the dauer pathway, we determined whether mutations in daf-16 suppress the abnormal growth phenotype of daf-23; daf-12 and daf-2; daf-12 double mutants. If daf-16 functions downstream of daf-12, then daf-16; daf-23; daf-12 and daf-16; daf-2; daf-12 triple mutants would arrest growth with a daf-2; daf-12 mutant phenotype. This result would suggest that the suppression of daf-23 and daf-2 mutants by mutations in daf-16 is independent on daf-12 gene activity. In contrast, if daf-16 functions downstream of daf-2 and daf-23 and independently of daf-12, the triple mutants should have a daf-12 phenotype and bypass dauer arrest. To distinguish between these models, we constructed the triple mutants and monitored development in synchronized populations of animals. As is shown in Table 4, the triple mutants exhibit neither dauer arrest nor the daf-2; daf-12 or daf-23; daf-12 growth arrest phenotype. The daf-2 and daf-23 and daf-2; daf-12 and daf-23; daf-12 mutant phenotypes are completely suppressed. These results are consistent with the model that daf-16 is functioning downstream of daf-2 and daf-23 and that the novel arrest phenotype of a daf-2; daf-12 or daf-23; daf-12 double mutant depends on daf-16 gene activity.

The number in parentheses is the total number of animals scored. * This strain is also unc-29(e1072). The presence of this mutation does not affect the Daf-d phenotypes of daf-16 and daf-12.

These animals stop development at the L2 stage.

### DISCUSSION

We have shown here that the daf-2, daf-23 and daf-16 dauer regulatory pathway defines a separate branch of the genetic pathway which transduces the pheromone signal for dauer formation. Unlike mutations in most Daf-c genes, mutations in both daf-2 and daf-23 can cause non-conditional arrest at the dauer stage. This suggests that in contrast to other Daf-c genes, daf-2 and daf-23 function at a point in the pathway that is not subject to modulation by temperature. In addition, our epistasis analysis suggests that daf-2 and daf-23 are functioning at a similar point in the dauer pathway, upstream of daf-16 but downstream or parallel to all other Daf-c and Daf-d genes shown in Figure 1 except daf-12.

A combination of neuronal laser ablations in some Daf-d mutants, detection of ciliated neuron structural defects in particular Daf-d mutants and genetic mosaic analysis of one Daf-d gene has allowed a partial alignment between the genetic epistasis pathway and the sensory neurons which signal dauer formation (Lewis and Hodgkin 1977; Albert et al. 1981; Herman 1984; Perks et al. 1986; Bargmann and Horvitz 1991). For example, a number of Daf-d mutations cause defects in the ciliated neurons which are normally open to the environment. But animals with this class of mutations can form dauers if they also have a mutation in one of the Daf-c genes.

### TABLE 4

<table>
<thead>
<tr>
<th>Genotype</th>
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</tr>
<tr>
<td>daf-2(e1370); daf-12(m20)</td>
<td>0 (742)*</td>
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The presence of this mutation does not affect the Daf-d phenotypes of daf-16 and daf-12.

These animals stop development at the L2 stage.

The results from this analysis are shown in Table 5. As expected, approximately ¼ of the progeny from daf-16/+ heterozygous parents became fertile adults whose progeny all bypass dauer arrest indicating that they were homozygous for daf-16. However, 5% of the progeny from the daf-2 strain and 18–35% of the progeny from daf-23 strains were fertile adults of the genotype daf-16/+, based on progeny tests (see Table 5). Examination of synchronized populations indicated that many of the daf-16/+ animals initially arrest at the dauer stage, but then recover over 1–3 days (data not shown). The dominant suppression is neither allele-specific for daf-16 nor for daf-23 (data not shown). The suppression is not complete and daf-23 mutants are suppressed to a greater degree than daf-2 mutants. In addition, the daf-16/+; daf-2 or daf-16/++; daf-23 fertile hermaphrodites have low brood sizes (usually less than 50) relative to daf-16; daf-2 or daf-16/++; daf-23 homozygotes (brood sizes around 250) again suggesting that suppression is not complete. A mutation in the Daf-d gene daf-12 enhances the dominant suppression of daf-2(e1370) (Table 5). Since there is no deficiency covering daf-16 we cannot determine whether the dominant suppression is due to haploinsufficiency of the daf-16 gene or to dominant function of the mutant alleles. However, the fact that two different alleles of daf-16, which themselves differ in their ability to suppress dauer formation, both exhibit dominant suppression, suggests that this effect is due to haploinsufficiency at the locus.
Thus, these genes are likely to function downstream in those ciliated neurons, including those we and others have isolated in screens for non-conditional alleles (RIDDLE et al. 1981; SWANSON and RIDDLE 1981; MALONE and THOMAS 1994; this study). This suggests that temperature modulation of dauer formation occurs in these Daf-c mutants. In contrast, alleles of daf-2 and daf-23 form 100% dauers at all temperatures. It is possible that temperature input to the dauer pathway occurs downstream or in parallel to the daf-1, -4, -7, -8, -11, -14, and -21 pathway but upstream of or on the daf-2 and -23 pathway. Alternatively, daf-2 and daf-23 may be more pleiotropic and affect temperature perception itself or any temperature sensitive step in dauer formation.

Based on the data presented here we propose a model to explain the functions of daf-2, daf-23 and daf-16 in the regulation of dauer formation and continuous development (Figure 4). The consequences of exposure to either dauer-inducing (high pheromone) or non-dauer-inducing (low pheromone) conditions are illustrated. The primary environmental inputs of pheromone, food and temperature are through the sensory neurons where at least some of the Daf-c and Daf-d genes, such as daf-11 and daf-10, are presumed to function. When animals are exposed to growth-promoting conditions, defined as high food and low pheromone, the sensory neurons send a signal which prevents dauer formation through the inactivation, either directly or indirectly, of the Daf-d gene daf-12 and activation of the Daf-c genes daf-2 and daf-23. Under these conditions, daf-2 and daf-23 would function to prevent dauer formation by the inactivation of the Daf-d gene daf-16 as well as by negatively regulating the activity of daf-12. The secondary inactivation of daf-12 by daf-2 and daf-23 is presented to explain two observations: first, daf-2 and daf-23 mutants form dauer larvae rather than simply arresting development at the L2 stage and second, the formation of dauer larvae in daf-2 and daf-23 mutants is dependent on daf-12 gene activity. When exposed to high pheromone dauer-inducing conditions, the sensory neurons no longer send a growth-promoting signal. This leads to the activation of daf-12 and the inactivation of daf-2 and daf-23. The absence of daf-2 and daf-23 function relieves repression of daf-16. The resulting

### Table 5

Partial dominant suppression of daf-2 and daf-23 mutants by mutations in daf-16

<table>
<thead>
<tr>
<th>Genotype of parent strain</th>
<th>Dauer (+/+ and daf-16/+)</th>
<th>Sterile adult (daf-16/+)</th>
<th>Fertile adult (daf-16/+)</th>
<th>Fertile adult (daf-16)</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>daf-16(m27)/+; daf-2(e1770)</td>
<td>64</td>
<td>8</td>
<td>3</td>
<td>25</td>
<td>145</td>
</tr>
<tr>
<td>daf-16(m27)/+; daf-2(e370); daf-12(m20)</td>
<td>32</td>
<td>2</td>
<td>36</td>
<td>30</td>
<td>97</td>
</tr>
<tr>
<td>unc-29(e1072); daf-16(m27)/+; sqt-1(cx3) daf-23(mg44)</td>
<td>26</td>
<td>22</td>
<td>22</td>
<td>30</td>
<td>156</td>
</tr>
<tr>
<td>daf-16(mg11)/+; sqt-1(cx3); daf-23(mg44)</td>
<td>27</td>
<td>9</td>
<td>35</td>
<td>30</td>
<td>101</td>
</tr>
</tbody>
</table>

- These animals were presumed to be of genotype +/+ or daf-16/+ because greater than 25% of the animals arrested as dauers indicating that some of the daf-16/+ animals are arresting as dauers.
- Although the genotype of these animals could not be determined by examining their progeny, they were presumed to be of genotype daf-16/+ because +/+ animals arrest at the dauer stage.
- These animals were daf-16/+ because they segregated progeny that both arrested as dauers and bypassed the dauer stage.
- These animals were homozygous daf-16 because all their progeny bypassed dauer.
- This number is all of the progeny from a single hermaphrodite.
- These animals did not arrest as dauers but exhibited the L2 arrest phenotype characteristic of daf-2; daf-12 double mutants.
**Non-Dauer-Inducing Conditions**

- Low pheromone
- High food
- Low temperature

**Sensory Neurons**

**Dauer-Inducing Conditions**

- High pheromone
- Low food
- High temperature

**Figure 4.**—Model for the functions of *daf-2*, *daf-23*, and *daf-16* in dauer formation and continuous development. The primary inputs to the sensory neurons are pheromone, food and temperature and the relative contribution of each is represented by the relative size of the lettering and the thickness of the arrow. In the rest of the figure, the arrows and bars are meant to reflect proposed positive and negative interactions, respectively, although they do not imply a direct interaction. Under both dauer-inducing and non-dauer-inducing conditions, the arrows and bars that are in bold represent active regulatory events. Those arrows or bars which have an X through them represent regulatory events that are not occurring. The output from non-dauer-inducing conditions is progression to the L3 and the output from dauer-inducing conditions is to arrest as a dauer. An alternate version of this model would remove the arrow from sensory neurons to *duf-2* and *duf-23* and rather have all input mediated through *daf-12*. See text for discussion.

*daf-16* gene activity represses non-dauer development and/or activates dauer entry and prevents dauer recovery.

A slight variant of this model, which removes the arrow from the sensory neurons to *daf-2* and *daf-23*, is also consistent with the data. In such a model, sensory input is received only through *daf-12* which then regulates *daf-2* and *daf-23*. Based on our data, we cannot yet distinguish whether the *daf-2*, *daf-23*, *daf-16* pathway receives sensory input independently of *daf-12* or whether *daf-2* and *daf-23* are functioning downstream of the main pathway, with all input mediated through *daf-12*. One constraint on possible mechanisms for regulation of *daf-23* activity derives from the fact that the *daf-23* mutant phenotype is maternally rescued suggesting that the down-regulation of *daf-23* by *daf-12* or by the upstream pathway is not likely to be transcriptional.

This model assumes that the Daf-d genes are up-regulated in high pheromone and down-regulated in low pheromone and that the Daf-c genes are down-regulated in high pheromone and up-regulated in low pheromone. However, only reduction-of-function (and not necessarily null) alleles of each gene have been tested thus far. In addition, these genes could be necessary for the development or functioning of particular neurons or cells in the pathway but not regulate the pathway directly as shown. If this were true, then excess or ectopic activity of these genes would not be sufficient to impose the opposite phenotype from reduction of activity. However for any genes whose activity is regul-
lated as we depict, dominant hypermorphic mutations would allow tests of this model.

This model suggests that both \textit{daf-12} and \textit{daf-16} activity are required for dauer formation. \textit{daf-12} may have a more central role since mutations in \textit{daf-12} completely suppress dauer formation induced by pheromone as well as dauer formation induced by the presence of mutations in most of the Daf-c mutants, while \textit{daf-16} mutants form partial dauers on pheromone and in combination with many of the Daf-c mutants. Together, \textit{daf-12} and \textit{daf-16} may function to activate dauer-specific genes and repress genes required for continuous development. However, under those conditions where \textit{daf-16} activity is high and \textit{daf-12} activity is low, such as in a \textit{daf-2}; \textit{daf-12} double mutant, animals neither arrest as dauers nor continue development to fertile adults. One possible explanation for this phenotype first suggested for \textit{daf-2} by Vowels and Thomas (1992), is that \textit{daf-2} and \textit{daf-23} are required for progression to the L3 stage while \textit{daf-12} is required for progression to the dauer stage. Therefore, \textit{daf-2}; \textit{daf-12} and \textit{daf-23}; \textit{daf-12} double mutants cannot progress past the L2 stage. The requirement for \textit{daf-12} and either \textit{daf-2} or \textit{daf-23} for progression past the L2 stage is relieved in \textit{daf-16} mutants suggesting that the inability to progress to the L3 stage is due to the high activity of \textit{daf-16} that occurs in \textit{daf-2}; \textit{daf-12} or \textit{daf-23}; \textit{daf-12} double mutants. An alternative explanation is that \textit{daf-12} and \textit{daf-23} have distinct but overlapping roles. This would suggest that either \textit{daf-2}/\textit{daf-23} or \textit{daf-12} alone can function in preventing or promoting dauer formation, respectively, while both play a minor role in both processes or that \textit{daf-2}/\textit{daf-23} and \textit{daf-12} may have redundant functions in an essential process which takes place during the L2 stage unrelated to dauer formation. This redundant function would no longer be essential in a \textit{daf-16} mutant.

In addition to the dauer constitutive phenotype, there are other phenotypes associated with mutations in \textit{daf-23} and \textit{daf-2}. Both \textit{daf-2} and \textit{daf-23} double mutants with two Daf-c mutants that have defects in cilia structure exhibit a high penetrance of L1 arrest (Vowels and Thomas 1992; this study). The arrest is not due to lethality since \textit{daf-2}(e1370); \textit{daf-10}(e1387) animals that are arrested at 25° can recover and continue to grow when shifted to 15°, the permissive temperature for \textit{daf-2}(e1370) (H. Tissenbaum and G. Ruvkun, personal communication). This result suggests that \textit{daf-2} and \textit{daf-23} have an essential function at or before the late embryo/early L1 stage that is revealed in these strains with abnormal ciliated sensory neurons. However, it is also possible that defects in other cells in these mutants are the basis for the synergistic interaction. Another phenotype of both \textit{daf-2} and \textit{daf-23} mutant animals is that the brood sizes are reduced at least twofold relative to wild type (S. Gottlieb, H. Tissenbaum and G. Ruvkun, unpublished observations). Significantly, mutations in \textit{daf-16} suppress both the L1 arrest and the reduced brood size phenotypes (S. Gottlieb, H. Tissenbaum and G. Ruvkun, unpublished observations). This suggests that \textit{daf-2} and \textit{daf-23} have additional functions independent of the regulation of dauer formation and that \textit{daf-16} similarly is involved in those processes, acting antagonistically to \textit{daf-2} and \textit{daf-23}.

The temperature-sensitive period for dauer formation begins during the L1 stage and both the L1 and L2 stages of animals that are going to become dauer larvae, the L1d and L2d stages, are lengthened relative to those animals that are not going to form dauer larvae (this study; Swanson and Riddle 1981; Golden and Riddle 1984a). A mutation in \textit{daf-16} suppresses the lengthened L1 stage characteristic of \textit{daf-2} mutants. This result indicates that \textit{daf-16} has a role in dauer formation beginning during the L1 stage. In addition, experiments with a phenotypically weak \textit{daf-16} allele, \textit{mg11}, suggest that the \textit{daf-16} gene product is required continuously to maintain the dauer-differentiated state since mutant animals efficiently form SDS-resistant dauer larvae which then recover. In addition, \textit{daf-16}(\textit{mg11}) animals form precocious dauers in pheromone or in double mutants with \textit{daf-2}. One possible explanation for both these phenotypes is that in addition to repressing continuous development, \textit{daf-16} is required for preventing recovery of L1d, L2d and dauer stage animals exposed to dauer-inducing conditions. It is known that during the stages preceding the L2d molt, if animals are removed from pheromone and exposed to food, they exit from the dauer program and undergo continuous development (Golden and Riddle 1984a). This recovery may involve down-regulation of \textit{daf-16}. The precocious dauers formed in \textit{daf-16}(\textit{mg11}) may be due to insufficient \textit{daf-16} gene activity to maintain the longer L1d and L2d stages but sufficient activity to molt into L2d and dauer, respectively.

Consistent with this view that a high level of \textit{daf-16} activity is needed for dauer maintenance, animals heterozygous for mutations in \textit{daf-16} exhibit partial suppression of the dauer constitutive phenotype of \textit{daf-2} and \textit{daf-23} mutants. Most animals enter the dauer stage but fail to maintain. \textit{daf-23} mutants are suppressed more completely than \textit{daf-2} mutants, but the \textit{daf-23} alleles may be weaker (see Table 3). Interestingly, a mutation in \textit{daf-12} enhances the dominant suppression of \textit{daf-2} mutants. This latter effect may be due to the fact that \textit{daf-2}; \textit{daf-12} animals are missing important regulators of the dauer/continuous development decision and hence are more sensitive to slight changes in another regulator, \textit{daf-16}. In the absence of a deficiency of the locus it is not possible to prove that the effect is due to haploinsufficiency of \textit{daf-16} rather than a dominant effect of the mutant alleles but mutations in \textit{daf-16}
behave as simple recessive alleles when daf-16/+ animals are induced to form dauer larvae by pheromone (S. Gottlieb and G. Ruvkun, unpublished observation).

The process of dauer formation involves sensing pheromone, presumably through a pheromone receptor in the sensory neurons, the transduction of the pheromone signal within the sensory neurons and perhaps to secretory cells and the transmission of that signal to the responding tissues. The known Daf-c and Daf-d genes appear to function at different steps in this pathway. Our data suggest that daf-2, daf-23 and daf-16 are functioning at a similar point in the pathway. Our epistasis analysis suggests that these genes may act in either downstream secretory cells or in the responding tissues. daf-16 cannot be functioning solely in ADF, ASI and ASC, the sensory neurons thought to be involved in the regulation of dauer formation, because daf-16 is required for dauer formation in animals in which those cells have been ablated with a laser (Bargmann and Horvitz 1991). Future analyses will be directed toward revealing where and how these genes function.

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LITERATURE CITED


