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A GENETIC PATHWAY FOR DAUER LARVA FORMATION IN *Caenorhabditis elegans*

(nematode, development, neuron)

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SUMMARY

C. elegans is a roundworm, a free-living soil nematode. The dauer larva is a non-feeding, non-growing larval stage which is formed under conditions of starvation. It possesses a relatively impermeable cuticle and differs from all other larval stages in behavior and morphology. Dauer larva formation is a "developmental switch" in the life cycle which offers special advantages for genetic study. A partial genetic pathway for dauer larva formation has been established. Genetic characterization of additional mutants should reveal more details of this pathway. One class of mutants already characterized exhibits morphological alterations in sensory neurons, as determined by electron microscopy. Such mutants are useful for the study of nerve morphogenesis.

A GENETIC APPROACH TO DEVELOPMENT

The classical approach to the analysis of development has been to perturb a system (either surgically or by the addition of chemical agents) and then to determine the effect of the perturbation on subsequent development. The genetic approach is analogous, except that the perturbations correspond to the activities of single genes. Genetic experiments can, therefore, provide information about the system not obtainable by other methods of analysis.

When considering a genetic approach to developmental control mechanisms in *C. elegans*, I established two criteria to be met by the area of investigation. The developmental process to be examined must (a) lend itself well to methods of genetic selection, and (b) it must be non-essential to survival of the organism in the laboratory. Only if the organism continues to live after the expression of a mutant gene can the effects of the mutation on subsequent development be examined. Also, genetic analysis of developmental mutants is technically more difficult when the mutations lead to death of the organism (HERMAN, ALBERTSON and BRENNER 1976).

With a dispensable developmental pathway it should be possible to detect mutations in every gene which functions specifically in that pathway. This increases the possibility of deducing the logic of the genetic program. In such an undertaking a direct positive selection for mutants offers a significant advantage over the study of developmental mutants which require "brute force" screening for their detection.

Dauer larva formation in *C. elegans* meets the above criteria. The dauer larva is a non-growing, non-feeding stage which is formed under conditions of starvation, possesses a specialized impermeable cuticle, and differs from all other larval stages in behavior and morphology. Dauer larvae survive harsh conditions for long periods of time, but when favorable conditions are encountered, they recover to resume development and produce offspring. Many organisms respond to harsh environmental extremes by producing developmental variants which are specialized for survival. These variants undergo profound changes, in which all organ systems are affected. Hence they constitute a useful means to study control mechanisms governing an organism's development and behavior.

The specific goal is to determine the genetic program for dauer larva formation and recovery and the molecular mechanisms for the implementation of that genetic program. It is hoped that some general principles will be found which are applicable to other developmental control mechanisms in *C. elegans* and ultimately to developmental processes in more complex organisms.

The strategy of analyzing single-step mutants to unravel a developmental pathway is clearly modeled after the strategy of molecular genetics, in which a variety of experimental techniques has been used to characterize biochemical pathways (GOTS and BENSON 1974), ribosome assembly (DAVIES and NOMURA 1972), and bacteriophage morphogenesis (KING and LAEMMLI 1973). These precedents suggest, first, that by genetic analysis of a complete catalogue of single-step mutants one might be able to deduce the structure of the genetic program for development. Secondly, particular mutant phenotypes may provide clues to biochemical defects, so that one can approach the problem of how the genetic program is implemented.

Before discussing the detailed genetic aspects of dauer larva formation, I would like to place this study in the context of what other investigators have learned about the genetics, development and behavior of *C. elegans*. In the following four sections I will briefly review these topics and point out some of the advantages *C. elegans* offers for genetic and microscopic study.

THE EXPERIMENTAL ORGANISM

It is only within the past ten years that Dr. Sydney Brenner of the Medical Research Council Laboratory of Molecular Biology at Cambridge, England, has developed the methods for studying the genetics of *C. elegans* (BRENNER 1974). These methods are being used to study the genetic specification of development and behavior (BRENNER 1973). Dr. Brenner initially chose this organism for study, not only because it is well suited for genetic analysis, but also because the structure of the animal is simple enough to determine its complete neuroanatomy by electron microscopy. Thus behavioral mutants can be carefully analyzed to determine what changes have been produced in nerve or muscle tissue. Since the wild-type organism must be characterized before mutants can be properly studied, much of the published work on *C. elegans* concerns normal development, anatomy and behavior.

C. elegans is grown in the laboratory in liquid medium or on the surface of agar-filled petri dishes, feeding on *Escherichia coli*. Adults are about 1 millimeter in length, with a tubular body consisting of a hypodermal wall and an underlying musculature which encloses the digestive and reproductive organs. Although *C. elegans* has most major types of differentiated tissue (nerve, muscle, hypodermis, intestine and gonad), an adult contains only about 800 somatic cells (SULSTON and HORVITZ 1977).

The hypodermal body wall is covered by an external cuticle composed primarily of modified forms of collagen (BIRD 1971). The hypodermis consists of four longitudinal ridges (dorsal, ventral, left and right lateral) joined circumferentially by thin sheets of cytoplasm which separate the muscle cells from the cuticle. The hypodermal cells secrete cuticular components and display a periodic activity associated with molting (SULSTON and HORVITZ 1977). The body muscles are organized into four longitudinal rows of twenty-four cells each, located between the hypodermal ridges. When the animal moves, the two subventral muscle strips are coordinated together as are the two subdorsal strips, producing a sinusoidal wave motion in the dorsal-ventral plane. The alimentary canal includes a muscular pharynx (ALBERTSON and THOMSON 1976) which takes up food, grinds it and pumps it into the intestine. The intestine consists of thirty to thirty-four cells and is lined with microvilli leading to an anal sphincter valve which is operated by a pair of muscles.

The nervous system of *C. elegans* consists of approximately 300 neurons, including a circumpharyngeal nerve ring, dorsal and ventral nerve cords, and a variety of sensory receptors and ganglia (WHITE 1974). About 70% of the neurons are located in the head. Sense organs (sensilla) are arranged in two concentric rings around the mouth and include both chemoreceptors and mechanoreceptors (WARD, THOMSON, WHITE and BRENNER 1975; WARE, CLARK, CROSSLAND and RUSSELL 1975). *C. elegans* exhibits chemotaxis to a number of compounds (WARD 1973). It also responds to a tap on the head by moving backward and then turning to move forward in a different direction. The nerve ring contains the endings of the sensory receptors in the head plus interneurons, motor neurons and processes which come from the tail and form axonal connections (WARE, et al 1975). The ventral nerve cord, posterior to the pharynx, is made up of interneurons from the ring and tail as well as motor neurons whose cell bodies are linearly positioned along the cord (WHITE, SOUTHGATE, THOMSON and BRENNER 1976). The dorsal nerve cord drives the sub-dorsal muscles and is made up of cell processes but no cell bodies. These processes reach the dorsal cord via commissures from their cell bodies in the ventral cord.

MODES OF REPRODUCTION

C. elegans generally reproduces as a self-fertilizing hermaphrodite, with each animal producing both sperm and eggs. Eggs are fertilized by endogenous sperm, and zygotes undergo some of their development inside the parental hermaphrodite. Self-fertilization drives populations to homozygosity so that it is easy to isolate isogenic clones of animals. This greatly facilitates the detection of mutants in a diploid organism. Many individual hermaphrodites may be grown together and screened for mutants together, since there is no danger of losing the homozygous form of a mutant by cross-fertilization. Since self-fertilization does not require copulation, severely uncoordinated or deformed mutants can be propagated as homozygotes. Thus many mutants which would be lethal in Drosophila or the mouse are viable in *C. elegans*. This not only simplifies maintenance of genetic stocks, but it also makes possible the growth of large populations of homozygous mutants for biochemical analysis.

Reproduction by self-fertilization, though convenient for mutant isolation and maintenance, does not provide a means to recombine independently isolated mutations. Genetic analysis, therefore, depends on the existence of males, which are produced spontaneously in hermaphrodite populations by meiotic non-disjunction at a frequency of 0.1% (HODGKIN 1974). Males possess 5 pairs of autosomes and one x chromosome, while hermaphrodites possess two x chromosomes in addition to the autosomal complement (HERMAN, ALBERTSON and BRENNER 1976); NIGON 1949). Mutants have been found which have an increased frequency of non-disjunction. These mutants, called *him*, produce a "high incidence of males" (HODGKIN 1974). Males are distinguished from hermaphrodites by their smaller size and by the fan-like copulatory bursa at the tip of the male tail. A male culture can be propagated by mating males with hermaphrodites. Half of the progeny produced by such cross-fertilization are male. In practice, a culture of wild-type males is constantly maintained, and these males are used for mating with mutant hermaphrodites. Such crosses produce heterozygous males which are then used for mating with mutant hermaphrodites, so that genetic mapping and complementation tests are possible.

NEMATODE GENETICS

The small size of the animal (1 mm), its short generation time (3 1/2 days at 20°C), and the large number of progeny produced per animal (250-300) are important factors in genetic analysis. A petri plate seeded with a single hermaphrodite will contain nearly 10^5 individuals after one week. Confining the animals to an agar surface permits the observation of rare individuals in a large population with the aid of a dissecting microscope. Individual animals can be transferred from plate to plate by picking them up on the tip of a platinum needle.

The methods for isolation, complementation and mapping of mutants affected in morphology or behavior have been described by Brenner (1974). Mutations are induced by ethyl methanesulfonate (EMS) at a high frequency, averaging 5×10^{-4} per gene (BRENNER

1974). This means that, on the average, one in every 2000 F_1 progeny of mutagenized animals will be heterozygous for a mutation in a given gene. Nearly 200 loci have now been placed on the genetic map. Most genes are represented by more than one independently isolated allele.

The majority of the mutants characterized thus far are nonlethal and display a clearly visible phenotype. A large number of mutants are "uncoordinated" (now representing nearly 100 genes). Uncoordinated phenotypes range from small aberrations in movement to nearly complete paralysis. Morphological mutants include dumpy, small, long and blistered animals. Dumpy mutants are shorter than wild-type animals and correspond to twenty different genes dispersed over the six linkage groups. Dumpy mutations are particularly useful markers for mapping most other classes of mutants, since the double mutants are easily distinguished. Both morphological and uncoordinated mutants can be used for mapping many types of developmental mutants.

An increasing variety of mutations is now being studied, including those affecting drug resistance (BRENNER 1974), sensory behavior such as chemotaxis (DUSENBERY, SHERIDAN and RUSSELL 1975), thermotaxis (HEDGECOCK and RUSSELL 1975) and male sexual behavior (HODGKIN 1974), catabolic pathways (BABU 1974), dopamine biosynthesis (SULSTON, DEW and BRENNER 1975), muscle assembly (EPSTEIN, WATERSTON and BRENNER 1974), sex determination (KLASS, WOLF and HIRSH 1976), development of the ventral nerve cord (SULSTON 1976), and temperature-sensitive lethal mutants affecting embryogenesis (VANDERSLICE and HIRSH 1976) and gonadogenesis (HIRSH, OPPENHEIM and KLASS 1976). A series of translocations and duplications has recently been characterized as a first step in assembling a collection of "balancers" for recessive lethal mutations (HERMAN, ALBERTSON and BRENNER 1976).

C. elegans possesses few cells and few genes. Based on the frequency of recessive lethal mutations, Brenner has estimated that *C. elegans* has only about 2000 genes with indispensable functions (BRENNER 1974). The haploid DNA content is 8 X 10^7 base pairs (about 20 times the genome of *E. coli*), and 83% of the DNA consists of unique sequences (SULSTON and BRENNER 1974). This is the smallest DNA content reported for an animal. Nevertheless, only 1/25 of the DNA would be required to code for 2000 average polypeptide chains. This same dilemma exists in *Drosophila melanogaster*. Genetic experiments (JUDD, SHEN and KAUFMAN 1972) estimate the total number of genes at 5000,while the haploid DNA content is about 2 X 10^8 base pairs (LAIRD 1973). This corresponds to about 40,000 base pairs per gene. Various theories have been advanced to explain why the genetic units in eukaryotic cells are so large (PAUL 1974). Regardless of that problem, the point to be emphasized here is that the total number of genes in *C. elegans* is small. The genetic and anatomical simplicity of *C. elegans* increases the possibility of understanding the development of the whole organism.

DEVELOPMENT

Nematode development is characterized by the early determination of cell fates. This simplifies the interpretation of the effects of genetic alteration upon development, since tissue in which the mutant gene is not expressed is likely to develop normally and independently of neighboring tissue expressing a mutant phenotype. In a highly regulative organism, tissue in which the mutant gene is not expressed may be altered secondarily by the attempt of the organism to compensate for its deviation from normal development.

It is important to note that in nematodes the cell number and the relative position of cells with respect to one another are essentially invariant among isogenic wild-type individuals (SULSTON and HORVITZ 1977). This simplifies the comparison of a mutant's anatomy with wild type. The phenotypes of isogenic mutant animals are generally quite uniform.

A newly hatched animal resembles an adult in general proportions and body movement, but is only about one-sixth the length of an adult. During growth, developing larvae undergo four molts in which the old cuticle is shed and replaced by a new underlying cuticle. The intermolt stages are designated L1 through L4 followed by adulthood. Worms stop pharyngeal pumping and become lethargic while molting. During growth at 20°C, molting occurs at approximately 14, 22, 30, and 40 hours after hatching, with egg laying commencing at about 50 hours (CASSADA and RUSSELL 1975).

Observation of living animals with the aid of Normarski differential interference contrast optics in the light microscope allows one to directly follow cell divisions, migrations, and cell deaths. During larval development the number of non-gonadal nuclei increases from about 550 in the newly hatched L1 to about 810 in the mature hermaphrodite and to about 970 in the mature male. These lineages range in length from 1 to 8 sequential divisions and lead to significant developmental changes in neuronal, muscular, hypodermal and digestive systems (SULSTON and HORVITZ 1977). In the case of ventral cord neurons, the correlation of cell type with lineage history has been directly determined using serial section electron micrographs of individuals of known lineage (SULSTON 1976).

At hatching the L1 has only 4 gonadal cells. These cells proliferate to form the adult reproductive system of approximately 2500 nuclei. Most of these nuclei are contained in the adult ovaries in a common core of cytoplasm, and some are packaged into oocytes during the reproductive period (HIRSH, OPPENHEIM and KLASS 1976). Divergence of the sperm and egg lines apparently occurs after the L2 stage, since it is possible to isolate homozygous recessive mutants among F_1 progeny of L1 and L2 hermaphrodites (HODGKIN 1974).

At the second molt, *C. elegans* can either continue development on to sexual maturity or, if environmental conditions are unsuitable, arrest development and enter the dauer larva state (Fig. 1.) The dauer larva may survive for months in the absence of food (KLASS and HIRSH 1976), but if it does encounter food, it begins to eat, then molts to resume normal development. Dauer larvae will begin pharyngeal pumping within 3 hours after being placed in food.

Recovering animals will then molt to shed the dauer-specific cuticle at 12-15 hours, molt again at 30-35 hours, and begin laying eggs at 40 hours after being placed in food at 20°C. The duration of the dauer state has no effect on post-dauer life span (KLASS and HIRSH 1976).

The following sections are specifically concerned with the dauer larva state and the genetic characterization of mutants affected in dauer larva formation.

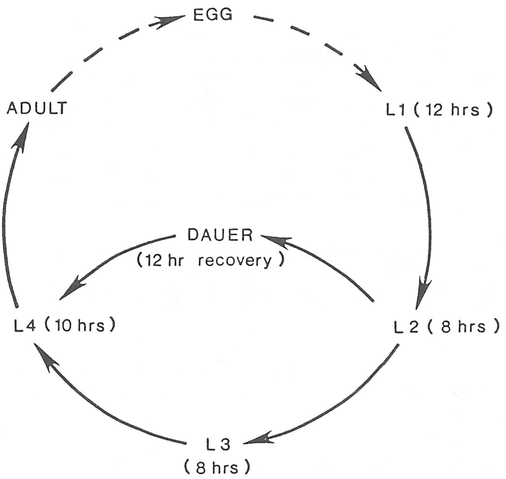

Figure 1. The life cycle of *C. elegans*. The solid arrows represent molts. The duration of each intermolt period is given in parentheses. [These data are taken from Cassada and Russell (1975) and from J. A. Lewis, personal communication.] Recovery from the dauer state involves the initiation of pharyngeal pumping, followed some 12 hours later by shedding the dauer-specific cuticle.

DAUER LARVAE

Dauer larvae were first identified as a special larval stage of insect-parasitic nematodes. These larvae, which differ structurally from all other stages of the same species, were termed "dauerlarven" by Fuchs (1915). This German term may be translated into English as "everlasting larvae." Stages analogous to dauer larvae are characteristic of many nematode species and represent an obligate stage in the life cycle of some parasites. This stage also has been called the "infective form" or the "dauer modification." In *C. elegans* dauer formation is facultative. A similar stage has been observed for the closely related nematode *Caenorhabditis briggsae* (YARWOOD and HANSEN 1969).

In *C. elegans* the dauer larvae have several properties which distinguish them from other larval stages (CASSADA and RUSSELL 1975). Dauer larvae are relatively thin with an axial ratio (length:width) nearly double that of other larvae. They have an increased specific gravity as measured in density gradients. They have an altered cuticle as seen in electron micrographs. The outer cortex of the cuticle is thicker, and there is an additional striated underlayer not seen in other juvenile stages. It has not been determined if the dauer cuticle contains proteins not found in the other larval cuticles or if it contains the same proteins but in different arrangements or different states of modification. As a consequence of their unique cuticular structure, dauer larvae are relatively resistant to inactivation by detergents, anesthetics, and a variety of other chemical agents.

Dauer larvae exhibit characteristic behavior patterns. Pharyngeal pumping is completely suppressed. Much of the time they lie motionless, but they respond to mechanical stimuli by moving away quite rapidly. If they encounter projections on the agar surface, dauers may stand on their tails and wave their heads in the air. In the soil this behavior may allow them to attach to passing vectors so that they may be carried to new locations. Dauers are thermotactically opposite to non-dauer larvae (HEDGECOCK and RUSSELL 1975).

DEVELOPMENTAL CONSEQUENCES OF DAUER LARVA FORMATION

Many post-embryonic cell divisions occur just prior to the L1 and L2 molts and thus are completed prior to dauer formation. Some blast cells, however, are destined to divide after the L2 molt, and these lineages are arrested upon dauer formation. Primarily, these lineages involve proliferation and differentiation of gonadal tissues and formation of sexual organs. For example, while dauers invariably contain about 30 gonadal nuceli, the number of these nuclei increases from 30 to more than 120 during the L3 stage (HIRSH, OPPENHEIM and KLASS 1976). Development of the male ventral nerve cord and pre-anal ganglion also involves cell divisions during the L3 stage. These divisions do not occur during the dauer stage.

Dauer formation does not introduce additional molts into the animal's life cycle (CASSADA and RUSSELL 1975). In this sense, the dauer is an alternate state to the L3. However, recovering dauer larvae must "catch up" with the L3 developmental program, and cell divisions otherwise correlated with the L3 stage occur during the premolt recovery period following the dauer. Recovered dauer larvae reach sexual maturity just after the final molt, as do animals which have never entered the dauer state.

DAUER LARVA GENETICS

The initial characterization of the dauer larva state in wild-type *C. elegans* by Cassada and Russell (CASSADA and RUSSELL 1975) provided the background for a genetic approach to the developmental processes involved in dauer larva formation and recovery. The unique resistance of dauer larvae to detergents like sodium dodecyl sulfate (CASSADA and RUSSELL 1974) provides an effective means of selecting mutants which form dauers abnormally in the presence of food. Detergent resistance also permits selection of mutants which recover slowly from the dauer state or mutants whose recovery is temperature-dependent.

When first considering the possible mechanisms for dauer larva formation, it is clear that a wide variety of mutational defects potentially could be represented in any collection of mutants. Some mutants might be defective in receiving and transducing an environmental signal. Mutants unable to form dauers might be unable to undergo the special molt or unable to synthesize the dauer-specific cuticle. In addition to genes specifically involved in the process of dauer formation, one would expect to detect genes involved indirectly. Some mutants might be sensory mutants unable to detect the presence or absence of food, or pharyngeal mutants unable to feed efficiently, or even digestive mutants unable to utilize food properly. Such mutants would provide clues about the mechanism of dauer formation, and they would be of interest in their own right since they could be useful for studying the development and function of the affected organs. However, the existence of a large number of indirect effects on dauer formation would make the system genetically complex, and the goal of focusing on a small, discrete subset of genes would be made more difficult. Therefore the first question I addressed was: how many genes are involved in dauer larva formation? To determine this, I have isolated two classes of mutants: (a) a class which forms dauers in the presence of bacterial food ("constitutive" mutants), and (b) a class which is unable to form dauers when starved ("defective" mutants).

DAUER-CONSTITUTIVE MUTANTS

Eighteen independent mutants which form dauers in the presence of bacteria were collected. While the frequency of dauers in well-fed, wild-type populations is less than 10^{-6} (I have never found a dauer larva in such a culture), the frequency of dauer formation in mutant populations can be as high as 100%. Dauer formation itself is a dispensable function, but mutants which always enter the dauer state are lethal if they are unable to recover because they will not reproduce. Such lethal mutants are common. However, the problem of lethality has been circumvented by the isolation of temperature-sensitive (*ts*) mutants. The best of the *ts* mutants grow normally at 15°C, but larvae grown at 25°C become dauers. The dauers are unable to recover until the incubation temperature is shifted down to 15°C. Therefore these mutants are *ts* lethals (with the advantage that the "lethality" is reversible).

Mutants were obtained in the following ways. (a) Three mutants were held in the Cambridge mutant collection and were obtained from Dr. Sydney Brenner. These mutations were originally detected as secondary mutations in strains isolated for other phenotypes. (b) Ten mutants were selected by treating F_2 descendants of EMS-mutagenized worms with sodium dodecyl sulfate (SDS). The animals were grown at 25° prior to SDS selection, and survivors of the treatment (which involves suspension in 1% SDS for 20 minutes) were placed on fresh plates at 15° for recovery. Seven of the ten mutants isolated in this way are *ts*, while three are "leaky" at all growth temperatures. The leaky mutants either do not always enter the dauer state, or if they do become dauers at least some of them are able to recover. Since only about 4% of mutants with other visible phenotypes (morphological or uncoordinated) are *ts*, the fraction of dauer-constitutives which is temperature-sensitive is surprisingly high. This may reflect a bias toward leaky mutants which is inherent in the SDS selection. (Dauers unable to recover do not form clones.) (c) Five mutants were obtained by visually screening F_1 clones after EMS mutagenesis. Out of 2000 F_1's tested, five segregated homozygous recessive dauer-constitutive mutants. Two of the mutants are *ts*, while three are absolute lethals and were recovered by picking heterozygotes from the clone. Again, the fraction of mutants which is temperature-sensitive is surprisingly high. In this experiment, dauer-constitutive mutants occurred at a frequency of 0.25% or about five times the average frequency per gene under our conditions of mutagenesis. This suggests that there are relatively few genes which can produce this phenotype.

The eighteen mutants fell into six complementation groups, and each of the six genes (*daf*1, 2, 4, 7, 8 and 9) has been located on the genetic map (Figure 2). Thirteen of the eighteen mutants comprise a single complementation group, *daf*2, while the other five genes are represented by a single mutant allele. Thus, with the exception of one frequently-mutated gene, constitutive

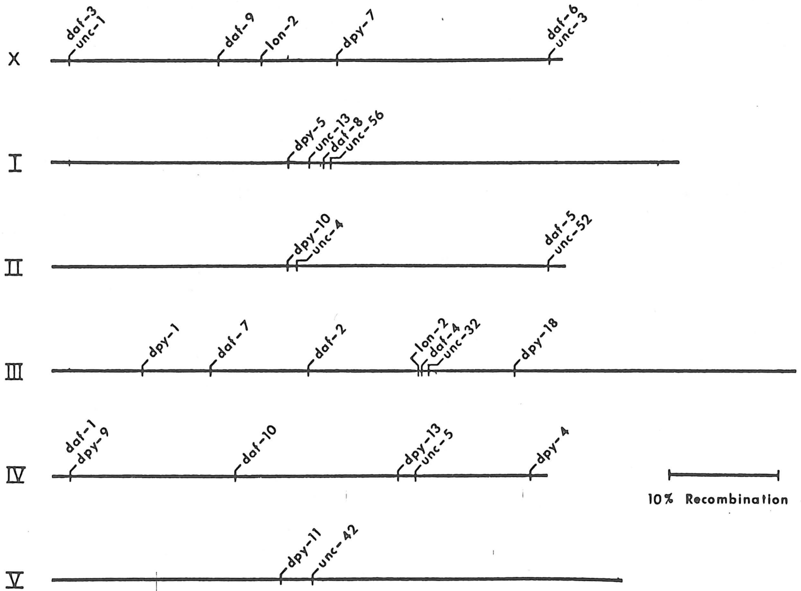

Figure 2. Simplified genetic map of *C. elegans* showing only *daf* loci and other markers used in mapping crosses. Gene designations other than *daf* are those used by Brenner (1974), including *unc* (uncoordinated), *dpy* (dumpy) and *lon* (long). The locations are shown only for mutations which have been unambiguously ordered. When two genes are joined at the same location, no recombination has been detected between them.

mutants are rare, and all of the dauer-constitutive genes may not have been detected as yet. Nevertheless, it seems likely that there are relatively few genes which can produce this phenotype.

All of the mutants are recessive. A search for dominant mutants among F_1 progeny of mutagenized worms established that such mutants do not occur at a frequency greater than 5×10^{-6} under standard mutagenesis conditions.

DAUER-DEFECTIVE MUTANTS

As part of the experiment described in the previous section in which 2000 F_1 clones were screened for constitutive mutants, some F_2 individuals were subcloned and the populations allowed to exhaust the bacteria. The starved F_2 subclones were visually screened for the presence of dauer larvae. From 205 F_1 clones tested in this way, eleven independent sub-clones were obtained which failed to form dauers when starved. Thus the frequency of dauer-defective mutants (about 5%) is very high relative to the frequency of dauer-constitutives. None of the dauer-defective mutants are temperature-sensitive. The eleven mutants fell into nine complementation groups, indicating that this class of mutant is not close to saturation.

I estimate there may be a total of thirty to sixty genes in this class. Four of the dauer-defective genes (*daf*3, 5, 6 and 10) have been located on the genetic map (Figure 1), and none of these is closely linked to a dauer-constitutive mutation. Since it is possible to construct uncoordinated derivatives of all the dauer-defective mutants, it seems likely that the mutants actually do not form dauers, rather than the alternative possibility, which is that they form dauers with a suicidal tendency to crawl off the petri plate. The dauer-defective phenotype is scored by cloning an animal, allowing its descendants to exhaust the food supply, then visually screening the population or treating the population with SDS to determine if dauers are present.

Many of the dauer-defective mutants have additional phenotypes. Of the eleven mutants, only two exhibit no secondary behavioral or morphological defects. Two mutants grow poorly and have an abnormal-appearing cuticle. These mutants may have molting defects, but have not been observed carefully as yet. Seven mutants grow well but exhibit sensory defects.

The sensory behavior of all the constitutive and defective mutants was tested by assaying their chemotaxis to sodium salts. Seven of the eleven dauer-defective mutants (representing six genes) are non-chemotactic, while "orientation assays" revealed no clear-cut chemotactic defects among dauer-constitutive mutants. The orientation assay developed by Ward (WARD 1973) is shown in Figure 3. The response of wild-type worms to a salt gradient (Figure 3A) is compared with the response of a dauer-defective mutant, E1378 (Figure 3B).

Previous electron microscopic studies on non-chemotactic mutants (LEWIS and HODGKIN 1977) showed that a high percentage of such mutants exhibit morphological defects in sensory neurons in the head. With this in mind, the anterior sensory neuroanatomy of three dauer-defective mutants was examined by serial section electron microscopy and compared with the wild-type neuroanatomy determined by Ward, Thomson, White and Brenner (1975). Adult hermaphrodites were fixed and stained with Osmium, and transverse sections were taken through the anterior ten microns of the head where most sensory processes are located. One mutant, E1376, which exhibits normal chemotaxis, has normal anatomy. Two non-chemotactic dauer-defectives, E1378 and E1387, have morphological alterations in a specific class of sensory neurons comprising the amphids. These neurons previously had been implicated in the chemosensory response to salts by Lewis and Hodgkin (1977).

The amphids are a pair of prominent sensilla, each consisting of twelve neurons, eight of which extend processes from cell bodies near the nerve ring to the tip of the nose, where the neuronal processes terminate in protective "pouches" on the lateral sides of the mouth. The ends of the neurons are exposed to the outside environment. The amphidial neurons are normally ciliated, that is, neuronal processes contain microtubules arranged in the typical "9+2" configuration. Ultrastructurally, the amphidial neurons of the two non-chemotactic, dauer-defective mutants examined are packed with electron-dense material in the space occupied by cilia in the wild type. In the mutants, normally cylindrical processes are enlarged, irregular, and fail to reach their normal length. In Figure 4, a transverse section through the normal amphids seen in E1376 (Figure 4a) is compared with the abnormal amphids seen in E1378 (Figure 4b). Other sense organs in the head of E1378 are normal in morphology.

The non-chemotactic dauer-defective mutants exhibit a third phenotype. Males homozygous for these mutations do not mate successfully even though they contain sperm and have a normal bursa. This suggests that the sensory defect in these animals involves sexual behavior as well as chemotaxis to sodium salts and dauer larva formation. Certain sensory neurons in the male tail may share common developmental programs with amphidial neurons in the head. Electron microscopic examination of tail neuroanatomy in the male (and in the hermaphrodite) might reveal anatomical defects similar to those seen in the amphids.

These results show great promise for further investigation, but they should be interpreted with caution. First, chemotaxis assays and electron microscopy have been performed only on adult hermaphrodites. The sensory defects, therefore, may result either from a failure in morphogenesis or from atrophy of cells which were formed normally in the embryo. Since these sensory mutants were isolated as dauer-defectives, the sensory defect presumably exists as early as the L2 stage. However, the time of onset of the sensory defects remains to be rigorously established. Secondly, only three individual animals (representing three distinct genes) have been examined by electron microscopy thus far. Additional animals must be examined if these results are to be verified and extended to other mutants. However, my working hypothesis is that there is a sensory signal for dauer larva formation mediated by the amphids. Any mutation which results in the failure of these neurons to develop properly might be detected as a dauer-defective mutant since

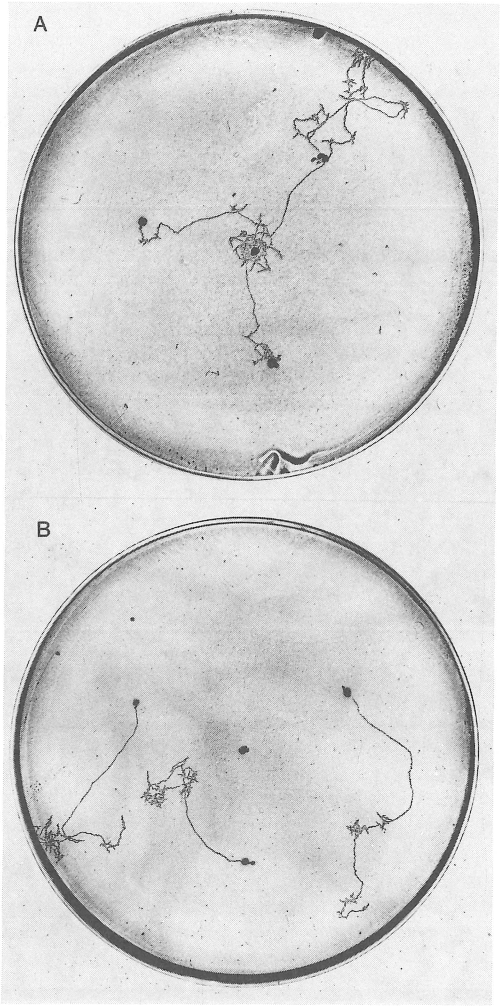

Figure 3. (A) Tracks of wild-type adults responding to a gradient of sodium acetate as described by Ward (1973). 5 μl of 0.5M $NaC_2H_3O_2$ were applied to the center of an 8.5 cm petri plate coated with 4 ml of 1.5% agarose. 12 hr. later another 5 μl were applied. 3 hr. later 3 worms were placed on the plate at the points marked by dots (at the outer periphery of the tracks). They were allowed to make tracks for 15 min. (B) Plates were prepared as in (A), but dauer-defective mutants of strain E1378 were placed on the plate. In a typical experiment, a total of 30 to 50 individuals are tested.

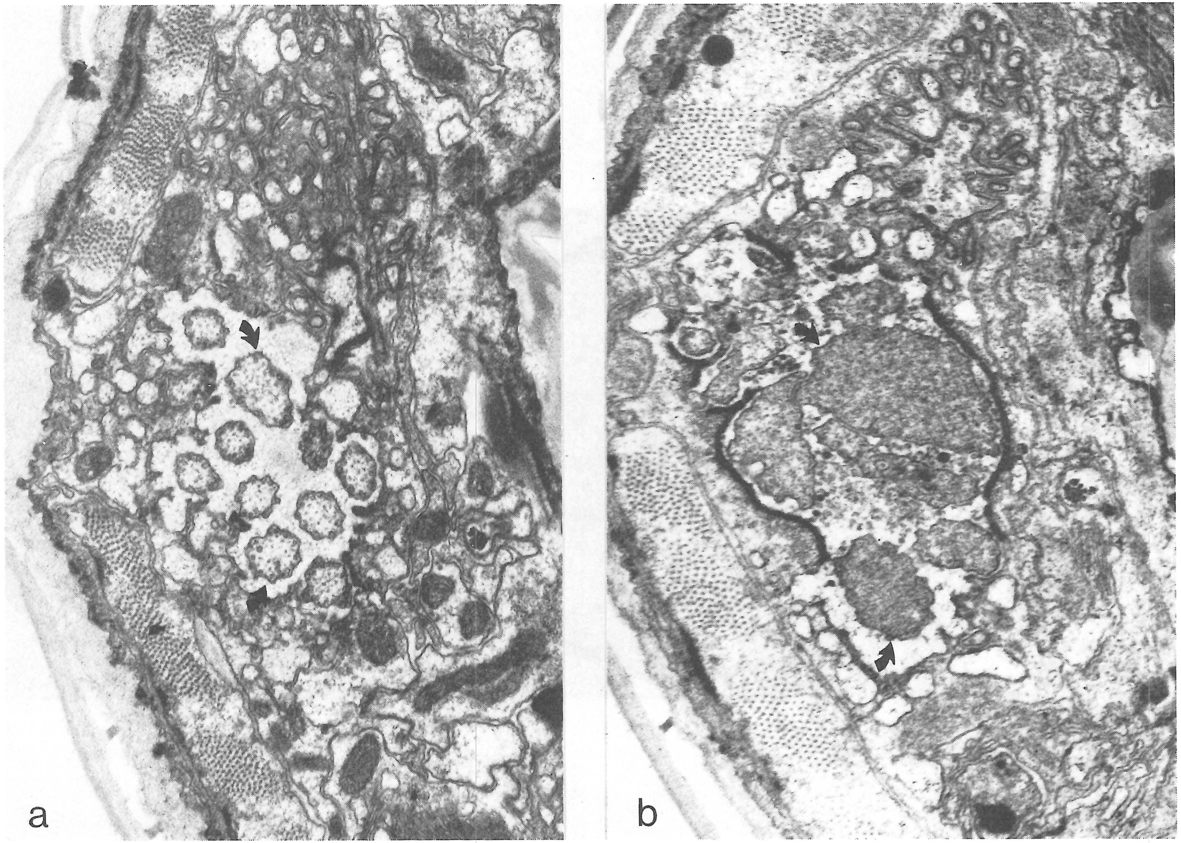

Figure 4. Transverse sections through the head of E1376 (part a) and E1378 (part b). Only the right lateral quadrant is shown (at 5000X). Arrows indicate normal amphidial cilia in E1376 and abnormal structures in E1378.

the animal's central nervous system cannot receive the starvation signal from the environment.

These results and the results of Lewis and Hodgkin (1977) on other classes of non-chemotactic mutants suggest that it is possible to identify by mutation genes which specify the development of a discrete part of the animal's sensory nervous system. The anatomical defects seen in E1378 and E1387 specifically involve the amphids, and it is possible that all the dauer-defective mutants exhibiting sensory defects are amphid mutants of one sort or another. The fact that a high proportion of sensory mutants are found to have morphogenetic defects (LEWIS and HODGKIN 1977) suggests that much of the functioning of the sensory nervous system is reflected in its morphology. Electron microscopy is therefore a valuable tool in the analysis of these mutants. In addition, complete characterization of sensory defects in these mutants will help establish the amphid functions.

EPISTATIC RELATIONSHIPS

There is a relationship between the constitutive and defective mutants in the pathway of dauer larva formation. The epistatic relationships can be determined by constructing double mutants, homozygous for both a "constitutive" and a "defective" mutation. However, the first such double mutants were not constructed in genetic crosses, but were obtained as revertants of dauer-constitutive mutants. The temperature-sensitive lethal dauer-constitutives are quite suitable for reversion experiments. The mutant strain is mutagenized and allowed to grow for one generation at 15°. Homozygous revertants, arising in the F_2 generation, grow and reproduce at 25°, while parental-type larvae form dauers and stop growing. Revertants of *daf*2, *daf*4, and *daf*7 alleles were selected, and nearly all revertants were found to be dauer-defective, that is, the revertants not only escape dauer formation at 25°, but they fail to form dauers even when starved. Backcrossing the revertants revealed that they are, in fact, double mutants which not only carry the parental constitutive mutation, but are also homozygous for an epistatic dauer-defective mutation. Thus some dauer-defective mutations are suppressors of dauer-constitutives. Suppression is generally recessive.

Many revertants carry alleles of dauer-defective genes which already had been isolated and mapped. Reversion of the dauer constitutive mutants provides a direct positive selection for these mutants. The spectrum of revertants quickly approached saturation, and many alleles of a few dauer-defective genes were obtained. Twenty-six independently isolated revertants were distributed among six complementation groups. Thus it is clear that some, but not all, dauer-defective mutations can suppress the constitutive mutations.

In order to determine which dauer-constitutive mutants could be suppressed by various defectives, a series of multiple mutants was constructed by genetic crosses. Seventy-five cross-suppression tests were performed on dauer-constitutive mutants representing four genes. Suppression patterns were determined for mutants representing seven dauer-defective genes (including mutants from the original collection as well as revertants). These data are summarized in Table 1. The dauer-defective mutants fell into four suppressor classes based on suppression of the four dauer-constitutives listed in the table.

Table 1. Suppression of Dauer-Constitutive Mutants by Dauer-Defective Alleles

Suppressor Class	Representative Suppressor Mutants		Dauer-Constitutive Mutants E1393 (*daf*8)	E1372 (*daf*7)	E1364 (*daf*4)	E1370 (*daf*2)
1	E1377	(*daf*6)	+	0	0	0
	E1375	(autosomal)				
2	M27	(autosomal)	+	+	0	+
	M25	(x-linked)				
3	E1376	(*daf*3)	+	+	+	0
	E1386	(*daf*5)				
4	M26	(autosomal)	0	0	0	+

Strain designations of representative *ts* dauer-constitutive mutants and dauer-defective (suppressor) mutants are given. A "+" signifies suppression (growth of the double mutant at 25°). A "0" signifies lack of suppression (double mutants form dauers at 25°).

These data have been considered in the following way. The dauer-defective mutants are blocked in the "natural" pathway of dauer formation. The dauer-constitutives, on the other hand, generate a false internal signal which causes the mutant to form a dauer even in the absence of the natural signal (which accompanies starvation). If the pathway is blocked after the false signal, the double mutant will be dauer-defective. However, if the false signal is generated after the block, the double mutant will be dauer-constitutive. Thus the data in Table 1 can be organized in the form of a genetic pathway for dauer larva formation (Figure 5). The boxes in the pathway represent points at which a false signal can be generated. The dashed lines represent points in the pathway where blocks occur. The pathway in Figure 5 is almost certainly incomplete and may become more complex as more mutants are characterized. As shown in the figure,an unambiguous order can be assigned to the dauer-constitutive genes *daf*8, *daf*7, and *daf*4. However, the *daf*2 gene seems to be in a distinct pathway, pathway II. Pathway II shares at least two steps in common with pathway I since the class 2 suppressors (which block both pathways) include two distinct suppressor genes (see Table 1). However, pathway II must not be functional in wild-type animals since any block in pathway I produces the dauer-defective phenotype. In other words, pathway II must not respond to the natural signal for dauer larva formation, but only functions in *daf*2 mutants. It is interesting to note that *daf*2 is the frequently mutated gene, while *daf*8, *daf*7, and *daf*4 mutants are rare.

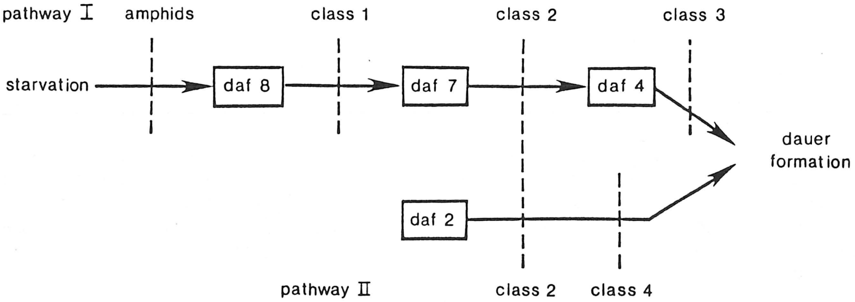

Figure 5. A partial genetic pathway for dauer larva formation. Dauer-constitutive genes are represented in boxes. Dauer-defective genes block the pathway at the positions shown by dashed lines. Class 2 genes and Class 4 genes have not been ordered with respect to each other.

A block in pathway I is both necessary and sufficient to produce a dauer-defective phenotype. In contrast to blocks in pathway I, the M26 mutation (which blocks only pathway II) is not completely dauer-defective. The M26 mutation was selected as a suppressor of *daf*2, and that is its most obvious phenotype.

As described in the previous section, over half of the original collection of dauer-defectives are sensory mutants, at least some of which have defects in amphid structure. Such sensory mutants are not found among revertants of dauer-constitutives. Furthermore, one sensory mutant with abnormal amphids, E1387 (*daf*10), has been combined with dauer-constitutive *daf*2 and *daf*8 alleles and fails to suppress dauer formation at 25°C in either case. Thus it is concluded that *daf*10 functions early in the pathway, prior to *daf*8. This is consistent with the hypothesis that the amphids mediate the primary sensory signal for dauer larva formation, while the dauer-constitutive mutations generate a false, internal signal.

What physiological processes are represented by the genetic pathway in Figure 5? The pathway almost certainly represents a signal pathway preceding the actual process of dauer formation (which involves cessation of development and synthesis of the special cuticle).

Although the methods used to deduce the genetic pathway are basically those of the biochemical geneticist, it seems unlikely that the pathway represents a series of steps converting intermediate compounds into some metabolic product. It seems more plausible that the normal pathway is a sequence of cellular events involving reception of an environmental signal and the conversion of that signal into a neuro-endocrine response. Ultrastructural studies have revealed neurosecretory cells associated with the pharynx

(ALBERTSON and THOMSON 1976) and with the nerve ring. Underlying the neural pathway would be the developmental processes which produce a functional set of nerve cells. For example, a mutation apparently affecting morphogenesis of amphidial neurons is assigned to a position in the pathway corresponding to amphid function.

The genetic pathway is potentially useful in suggesting ways to investigate particular mutants assigned to various positions in the pathway. If the genetic pathway corresponds to a pathway of neurotransmission, electron microscopic analysis of the circumpharyngeal nerve ring might reveal anatomical defects in *daf* mutants which have been assigned to internal positions in the genetic pathway. On the other hand, if a dauer-defective mutant is found which is positioned at the beginning of the pathway yet exhibits normal chemotaxis and normal sensory ultrastructure, that mutant would be suspected to have a specific defect in the receptor for the starvation signal.

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Front row from left to right: Drs. Feldman, Riddle, Thompson and Janet Stein