The *Caenorhabditis elegans unc-31* Gene Affects Multiple Nervous System-Controlled Functions

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ABSTRACT

We have devised a method for selecting *Caenorhabditis elegans* mutants that execute feeding motions in the absence of food. One mutation isolated in this way is an allele of the gene *unc-31*, first discovered by S. Brenner in 1974, because of its effects on locomotion. We find that strong *unc-31* mutations cause defects in four functions controlled by the nervous system. Mutant worms are lethargic, feed constitutively, are defective in egg-laying and produce dauer larvae that fail to recover. We discuss two extreme models to explain this pleiotropy: either *unc-31* affects one or a few neurons that coordinately control several different functions, or it affects many neurons that independently control different functions.

GENETIC analysis can provide insights into the cellular and molecular components of a nervous system. One way to identify genes that act in the nervous system is by isolating mutants with defective behavior. However, the intrinsic complexity of the nervous system can make the analysis of behavioral mutants difficult. For example, since behaviors are generated by groups of neurons that act in concert, a single genetic defect can affect multiple neurons, a single neuron can affect multiple behaviors and multiple neurons can affect the same behavior. In practice, these complexities mean that understanding the effects of a behavioral mutation depends on understanding the neurons that generate and regulate the behavior.

We are approaching the problem of the genetic specification of nervous system development and function in the nematode *Caenorhabditis elegans*, which is genetically accessible and has a compact, well described nervous system. *C. elegans* has two almost independent nervous systems: the somatic nervous system, which consists (in the adult hermaphrodite) of 282 neurons of 104 types (WHITE *et al.* 1986), and the pharyngeal nervous system, which consists of 20 neurons of 14 types (ALBERTSON and THOMSON 1976). We have begun a genetic and neurobiological analysis of feeding, which is predominantly controlled by the pharyngeal nervous system. *C. elegans* eats bacteria (*Escherichia coli* in the laboratory). The pharynx filters bacteria from the liquid in which they are suspended, grinds them up, and passes them back to the intestine (DONCASTER 1962; SEYMOUR, WRIGHT and DONCASTER 1983). Pharyngeal muscles have two separately controlled motions: isthmus peristalsis, which carries bacteria from the anterior half of the pharynx to the posterior, and pumping, which takes bacteria into the anterior part of the pharynx from outside, and simultaneously breaks open bacteria in the posterior part of the pharynx and passes them to the intestine (AVERY and HORVITZ 1989). The rate of pumping depends on the presence of food (AVERY and HORVITZ 1990). A well fed worm suspended in liquid will pump only if bacteria are present. A worm starved for 6 hr or more will pump slowly and irregularly in the absence of bacteria and will accelerate its pumping in response to much lower amounts of bacteria than will a well fed worm. The stimulation of pumping by bacteria is almost entirely dependent on a single pharyngeal sensory neuron type, MC (AVERY and HORVITZ 1989).

To begin our genetic investigation of pharyngeal nervous system function, we decided to look for pumping-constitutive mutants: mutants that pump in the absence of bacteria even when not starved. We hoped in this way to find genes involved in the control of behavior, rather than its execution. Mutant worms that ate microscopic iron particles in the absence of bacteria were separated from normal worms by the attraction of the mutants to a small magnet. [This method is ultimately derived from one developed for the isolation of macrophages by ROUS and BEARD (1934).] One of the mutants we isolated in this way carried a mutation in the gene *unc-31*, first discovered by BRENNER (1974) because of the lethargic phenotype caused by *unc-31* mutations. [*unc* is short for uncoordinated, a general term for almost any defect

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in locomotion. *unc-31* mutants, although able to move almost normally under certain conditions, rarely move in response to the most commonly used stimuli (HODGKIN *et al.* 1988).] We found that *unc-31* loss-of-function mutations cause four defects in functions controlled by the nervous system: constitutive and rapid pumping, lethargy, defective egg-laying, and inability to recover from the dauer state. Like the rapid pumping induced by bacteria, the rapid pumping of *unc-31* worms depended on the MC neurons.

MATERIALS AND METHODS

General methods: General methods for handling and observing worms are described by SULSTON and HODGKIN (1988). Worms were routinely kept at 20°.

Strains and mutations used: Table 1 shows the strains used. General descriptions of the genes and mutations can be found either in this paper or in that of HODGKIN *et al.* (1988). The *unc-31* mutations *e714* and *e717* arose from the same mutagenesis (J. HODGKIN, personal communication) and are not known to be distinct. The mutations *e375* and *e379* were also not known to be distinct (J. HODGKIN, personal communication), but we show in this work that *e375* is suppressed by the amber suppressor *sup-7*, while *e379* is not.

Measurement of average pumping rates: Average pumping rates of worms suspended in liquid were measured by counting iron particles eaten by each of about 100 worms, as previously described (AVERY and HORVITZ 1990). The concentration of iron was 0.83 mg/ml, which usually gives results in the linear range of the assay (up to about 3 iron particles/worm). Pumping rate is expressed as the average number of iron particles/worm. Concurrent controls were included in all sets of assays, and the tubes were scored without knowledge of the identity of the assays within a set.

Magnetic selection of worms that pump iron: Starved and well fed worms were prepared as previously described (AVERY and HORVITZ 1990). For the experiment shown in Figure 1, from 50 to 88 worms were agitated for 5 min under the indicated conditions in a total volume of 600 ml. Bacteria, when present, were at 60% the density of a stationary LB culture. We have previously shown that a 60% density promotes vigorous pumping in both well fed and starved worms (AVERY and HORVITZ 1990). Pumping was stopped by the addition of sodium azide to 33 mM, which sedates but does not kill *C. elegans*. After removing excess iron with a horseshoe magnet held to the side of the tube, we separated the magnetic from nonmagnetic worms by inserting into the tube a 1 mm × 1 mm × 2 mm rare-earth cobalt magnet (Permag Northeast Corp, Billerica, MA) attached to the end of a Pasteur pipette with epoxy glue, lifting the magnet gently out of the tube along with any worms attracted to it, and shaking the worms off in a tube containing 1 ml SB (AVERY and HORVITZ 1990). (There was no difficulty in removing the worms from the magnet, since the attraction was weak.) We then counted the worms that had stuck to the magnet and the worms left behind.

Data for Table 2 were generated using a similar protocol. For this experiment the starved worms were *dpy-5* mutants, so that they could be easily recognized by their short body length (Dpy). Aliquots of the well fed wild-type worm suspension and the starved *dpy-5* suspension were counted, then varying volumes of the two were mixed for the selection. Well fed worms were agitated in the absence of bacteria for ten minutes immediately prior to selection. For one series, we killed the wild-type worms by exposure to a saturated solution of chloroform in SB, followed by washing. The numbers of worms of each type in the mixtures were estimated from the numbers counted in the aliquots. After magnetic selection the numbers of normal length and Dpy worms that had stuck to the magnet were counted. The enrichment was defined as [(no. magnetic Dpy)/(total magnetic)]/[(no. Dpy original)/(total original)].

After the isolation of pumping-constitutive (Puc) mutants, we were able to repeat these experiments, selecting Puc mutants instead of starved wild-type worms. The enrichment of Puc mutants varied in the same range as did enrichment of starved worms.

Isolation of mutants: The Puc mutants described in this paper were isolated in four independent mutagenesis experiments. In each of the first three experiments 20 wild-type L4 hermaphrodites mutagenized with 50 mM ethyl methanesulfonate (EMS) as described by BRENNER (1974) were placed on a 10-cm plate seeded with *E coli*. Five days later we harvested 2000 gravid F_1 hermaphrodite progeny and treated them with basic hypochlorite (SULSTON and HODGKIN 1988) to prepare about 45,000 eggs, which were washed and agitated overnight in SB without bacteria to give 14,000 live F_2 L1s. (The death of two-thirds of the F_2 eggs is caused by two factors: killing by hypochlorite and the segregation of EMS-induced zygotic lethal mutations. Since the level of hypochlorite killing is variable in our hands, we cannot estimate the number of lethal mutations induced.) About 2800 L1s were placed on each of three seeded 10-cm plates and allowed 3 days to become gravid adults. The worms from each of the three plates were then harvested in 5 ml SB, pelleted by spinning briefly in a tabletop clinical centrifuge, transferred to 10 ml SB, pelleted again, and transferred to 6 ml SB for magnetic enrichment at a concentration of 7.8 mg/ml iron. Between 31 and 44 worms total were selected from the three plates in each of the three experiments. These animals were then examined in the dissecting microscope for iron in the pharyngeal lumen, which, if present in sufficient quantity, is visible as a black line down the center of the pharynx. (Our goal in this secondary visual screen was to improve on the rather poor enrichment achieved by the magnet alone. Unfortunately, subsequent reconstruction experiments showed the visual screen to be completely ineffective.) Strains were derived by self-fertilization of between six and eight F_2 adults from each experiment. *n1445* came from one of these experiments, *n1304* from a second, and *ad80*, *ad81*, and *ad83* from the third.

The fourth experiment was on a larger scale. We designed this experiment so as to make it statistically unlikely that more than one F_2 animal would be homozygous for any given EMS-induced mutation. Eggs were prepared from wild-type hermaphrodites by basic hypochlorite treatment, synchronized by starvation, allowed to grow 2 days, at which time they had become 20,000 L4 hermaphrodites, and mutagenized with 50 mM EMS. The mutagenized hermaphrodites were grown 1 day at 25° then 1 day at 10°, in which time they became gravid adults. 66,000 live F_1 L1 progeny synchronized by hypochlorite treatment and starvation became gravid hermaphrodites after 3 days at 25° and were in turn used to prepare 12,000 synchronized F_2 L1s, which were also grown to adulthood at 25°. After magnetic selection (without a secondary visual screen) and production of self-progeny broods, we were left with 25 strains. We measured pumping rates in 21 of these strains. Data from the 15 strains isolated in the first three mutageneses were pooled with data from the 21 strains isolated in the fourth mutagenesis to produce Figure 2.

TABLE 1

***C. elegans* strains used**

Strain	Genotype	Source[a]
N2	+	MIT
CB61	*dpy-5(e61) I*	MIT
CB69	*unc-31(e69am) IV*	MRC
CB86	*unc-31(e86) IV*	MRC
CB128	*dpy-10(e128) II*	MIT
CB164	*dpy-17(e164) III*	MIT
CB169	*unc-31(e169) IV*	MIT
CB184	*dpy-13(e184) IV*	MIT
CB224	*dpy-11(e224) IV*	MIT
CB375	*unc-31(e375am) IV*	MRC
CB379	*unc-31(e379) IV*	MRC
CB636	*unc-31(e636) IV*	MRC
CB714	*unc-31(e714) IV*	MRC
CB717	*let(n1327ts) unc-31(e717) IV*	MRC
CB928	*unc-31(e928) IV*	MRC
CB1141	*cat-4(e1141) V*	MIT
CB1512	*unc-31(e1512) IV*	MRC
CB2488	*dpy-11(e224) ali-1(e1934) him-5(e1490) unc-76(e911) V*	MRC
DA80	*puc(ad80) II*	This work
DA81	*puc(ad81) II*	This work
DA83	*puc(ad83) II*	This work
DA316	*dpy-20(e2017am) IV; sup-7(st5sd) dpy-7(e1324ts) X*	LA
DA436	*unc-31(e928) IV; cat-4(e1141) V*	This work
ING7	*unc-31(e928) IV; Ex[unc-31(+)]*	DL/RH
ING8	*unc-31(e928) IV; Ex[unc-31(+)]*	DL/RH
ING10	*unc-31(e928) IV; Ex[unc-31(+)]*	DL/RH
INH1	*unc-31(u280am) IV; Ex[unc-31(+)]*	DL/RH
INH3	*unc-31(u280am) IV; Ex[unc-31(+)]*	DL/RH
MT464	*unc-5(e53) IV; dpy-11(e224) V; lon-2(e678) X*	MIT
MT465	*dpy-5(e61) I; bli-2(e768) II; unc-32(e189) III*	MIT
MT1067	*egl-31(n472) I*	MIT
MT1073	*egl-4(n478) IV*	MIT
MT1207	*unc-31(n577) IV*	MIT
MT1232	*egl-12(n602sd) V*	MIT
MT1356	*sup-7(st5sd) dpy-7(e1324ts) X*	MIT
MT1445	*egl-35(n694ts) III*	MIT
MT2069	*egl-42(n996sd) II*	MIT
MT2071	*egl-44(n998) II*	MIT
MT2072	*egl-45(n999) III*	MIT
MT2315	*egl-46(n1126) V*	MIT
MT2940	*unc-31(n1304) IV*	This work
MT3300	*unc-31(e717) IV*	This work
MT3387	*puc(n1445) X*	This work
MT3408	*unc-30(e191) unc-26(e205am) IV; sup-7(st5sd) dpy-7(e1324ts) X*	LA
MT3399	*unc-31(n1304) IV; sup-7(st5sd) dpy-7(e1324ts) X*	This work
MT3400	*unc-31(e86) IV; sup-7(st5sd) dpy-7(e1324ts) X*	This work
MT3401	*unc-31(e379) IV; sup-7(st5sd) dpy-7(e1324ts) X*	This work
MT3402	*unc-31(e636) IV; sup-7(st5sd) dpy-7(e1324ts) X*	This work
MT3403	*let(n1327ts) unc-31(e717) IV; sup-7(st5sd) dpy-7(e1324ts) X*	This work
MT3404	*unc-31(e1512) IV; sup-7(st5sd) dpy-7(e1324ts) X*	This work
MT3405	*unc-31(e714) IV; sup-7(st5sd) dpy-7(e1324ts) X*	This work
MT3406	*unc-31(e169) IV; sup-7(st5sd) dpy-7(e1324ts) X*	This work
MT3407	*unc-31(e928) IV; sup-7(st5sd) dpy-7(e1324ts) X*	This work
MT3409	*unc-31(e375am) IV; sup-7(st5sd) dpy-7(e1324ts) X*	This work
MT3410	*unc-31(e69am) IV; sup-7(st5sd) dpy-7(e1324ts) X*	This work
MT3412	*unc-31(u280am) IV; sup-7(st5sd) dpy-7(e1324ts) X*	This work
TJ413	*unc-31(z1) IV*	TJ
TU280	*unc-31(u280am) IV*	MC

[a] Codes for sources are as follows: DL/RH, D. LIVINGSTONE and R. HOSKINS (Medical Research Council Laboratory of Molecular Biology, Cambridge, England); MIT, HORVITZ laboratory strain collection, Massachusetts Institute of Technology (Cambridge, Massachusetts); MRC, Medical Research Council Laboratory of Molecular Biology strain collection (Cambridge, England), courtesy of JONATHAN HODGKIN; MC, MARTIN CHALFIE, Columbia University (New York, New York); TJ, TOM JOHNSON, University of Colorado (Boulder, Colorado); LA, LEON AVERY (unpublished).

Genetic mapping and complementation testing: The mutation *n1304* was mapped to chromosome *IV* using its Unc phenotype. The mutation *unc-31(e169) IV*, which produces a similar Unc phenotype, failed to complement the Unc phenotype produced by *n1304*. *e169* proved on testing to be Puc. To test whether *e169* would fail to complement the Puc as well as the Unc phenotype of *n1304*, we mated *e169* hermaphrodites with *n1304/+* males (*unc-31* homozygous males are incapable of mating) and assayed pumping as usual, counting iron particles in male progeny, expected to be a 1:1 mixture of *n1304/e169* and *e169/+* heterozygotes. This average was 0.82 particles/worm. The pumping rate of the *n1304/e169* heterozygote was estimated from the equation $(x + 0.06)/2 = 0.82$, where 0.06 is the measured pumping rate of *n1304/+* males and x the estimated pumping rate of *n1304/e169* males. This estimate is conservative, because if *unc-31* mutants were slow growing or subviable, compared to wild type, their proportion in the mixture would have been less than 0.5. Casual observations indicate that *unc-31* homozygotes are fully viable and reach adulthood in the same time as wild-type worms.

To test if *n1304* caused the Egl (egg-laying defective) as well as the Unc phenotype of MT2940, the self-progeny of 24 Unc and 20 Egl self-progeny of *n1304/+* hermaphrodites were examined. All progeny of 20/20 Egl worms were Unc, and all progeny of 24/24 Unc worms were Egl. These results imply that the recombination distance between the mutations causing the Unc and Egl phenotypes is <1/88, *i.e.*, <1.1 cM.

To test the *puc* mutations *n1445*, *ad80*, *ad81* and *ad83* for dominance and *X*-linkage, the progeny of wild-type males and *puc* homozygous hermaphrodites (*n1445*, *ad81* and *ad83*) or of *puc* males and *dpy-11* hermaphrodites (*n1445* and *ad80*) were assayed for pumping in the absence of bacteria. These crosses were done under conditions that favored efficient outcrossing (SULSTON and HODGKIN 1988).

To map these *puc* mutations, double heterozygotes *puc/+;m/+* were constructed for different markers *m*, their Puc progeny enriched by magnetic selection, and self-progeny of worms that were not homozygous for *m* were examined for *m/m* homozygotes. In this way, we confirmed the *X*-linkage of *n1445* by demonstrating linkage to *lon-2 X*. *ad80*, *ad81* and *ad83* were shown to be linked to *bli-2 II*, and *ad80* was additionally shown to be linked to *dpy-10 II*. To test for complementation, *ad80* males were mated with *ad81* and *ad83* hermaphrodites and the progeny assayed.

Measurement of dauer recovery: Wild-type or *unc-31* dauer larvae generated by crowding and starvation were placed on bacterial lawns and observed on the following days. A dauer larva was considered to have recovered when frequent pharyngeal pumping was observed.

Temperature dependence of *unc-31* phenotypes: *e69*, *e86*, *e169*, *e375*, *e379*, *e636*, *e714*, *e717*, *e1512*, *n1304* and *z1* mutants were viable, fertile, and Unc at 25°, 20°, and 15°, although the Unc phenotype of *e636* animals was slightly weaker at 15°. (CB717, the original *unc-31(e717)* strain, was inviable at 25°. This inviability proved to be caused by a maternal-effect temperature-sensitive lethal mutation, *n1327*, linked to but separable from *unc-31*. We constructed a strain MT3300 that is homozygous for *unc-31(e717)* and viable and fertile at 25°.) *n1304* was Puc at 15°, 20° and 25°. None of the other alleles was tested for pumping-constitutivity at 15° or 25°. Most or all *unc-31* mutants were Egl at 25°. At 15° the Egl phenotype was absent or weak.

Amber suppression: To test for amber suppression, we constructed *unc-31; sup-7 dpy-7* triple mutant strains. *sup-7* is a strong semidominant amber suppressor (WATERSTON 1981). *sup-7* homozygotes are sterile at 15°. *dpy-7* homozygotes are dumpy at 25°, rollers (Rol) at 22.5°, and normal at 15° and 20°. Constructions were done at 22.5°, so that *dpy-7* homozygotes could be recognized as Rol worms.

Strains were constructed by one of two alternative methods. In the first, we looked at the self-progeny of eight Unc hermaphrodite self-progeny of *unc-31/dpy-20(e2017am); sup-7 dpy-7/+ +* heterozygotes. If the *unc-31* mutation was not suppressed by *sup-7*, some of these Unc worms should have produced *unc-31; sup-7 dpy-7* self-progeny, recognizable as Unc Rol worms whose progeny were sterile at 15°. The construction succeeded for *e86*, *e169*, *e379*, *e636*, *e714*, *e717*, *e928* and *e1512* but failed for *e69*, *e375* and *u280*.

For these three alleles, we used a different method involving mutations in the linked genes *unc-30* and *unc-26*, which produce Unc phenotypes distinct from that of *unc-31*. The *unc-26* mutation we used, *e205*, turned out to be fully suppressed by a single copy of *sup-7* (data not shown and H. ELLIS, personal communication). As a result, only *unc-30* was useful in the construction. Rol hermaphrodites from the self-progeny of *unc-31/unc-30 unc-26; sup-7 dpy-7/+ +* were allowed to produce self-progeny. Ignoring occasional easily recognized recombinants, the Rol non-Unc hermaphrodites were of two types. One (*unc-31/unc-30 unc-26; sup-7 dpy-7*) produced 1/4 Unc-30 progeny, and the other (*unc-31; sup-7 dpy-7*) did not. All progeny of worms in this second class were somewhat lethargic, although much less so than *unc-31* single mutants. The genotype of these putative *unc-31; sup-7 dpy-7* strains was verified by showing that they were sterile at 15°, and that *unc-31* could be recovered among the F2 progeny of a mating with wild-type males. That they were homozygous for the *unc-31* mutation was shown by the fact that their self-progeny were all moderately lethargic. Quantization of the movement of these *unc-31(amber); sup-7 dpy-7* worms showed that *u280* is suppressed more strongly than *e69*, suggesting that the three ambers include at least two distinct mutations.

Effects on behavior of laser killing of neurons: NSM and MC pharyngeal neurons and ADF, ASG, ASI and ASJ chemosensory neurons were killed as previously described (AVERY and HORVITZ 1987; BARGMANN and HORVITZ 1991a).

Construction of an *unc-31; cat-4* double mutant: Twelve Unc-31 non-Dpy non-Unc-76 worms were picked from the self-progeny of *unc-31(e928)/+; cat-4/dpy-11 ali-1 him-5 unc-76* hermaphrodites. (*ali-1*, a recessive mutation that affects the morphology of the L1 alae (HODGKIN *et al.* 1988), was not scored in any of the crosses that follow. *cat-4* maps between *dpy-11* and *unc-76* (SULSTON, DEW and BRENNER 1975), which are about 7 cM apart (EDGLEY and RIDDLE 1990).) *cat-4(e1141)* mutants do not look obviously different from wild-type worms when examined with a dissecting microscope. We expected 30% of Unc-31 non-Dpy non-Unc-76 worms to be *unc-31; dpy-11(+) cat-4 unc-76(+)* homozygotes. (The number is 30% rather than 33% because of recombination between *dpy-11* and *unc-76*.) However, there was none among the 12 we picked. If *unc-31; cat-4* and *unc-31; cat-4(+)* were indistinguishable, the probability of such a failure would be <0.02.

On the hypothesis that *unc-31; cat-4* mutants were eating less than *unc-31; cat-4(+)* and growing slowly, we looked at the self-progeny of one scrawny, irregularly pumping worm from each of eleven *unc-31; cat-4/dpy-11 ali-1 him-5 unc-76* heterozygotes. Two were recombinants, but the remaining nine had no Dpy or Unc progeny, the result expected for an *unc-31; cat-4* homozygote. In contrast to our previous failure to isolate the desired homozygote, this result is far better than expected by chance, confirming our hypothesis.

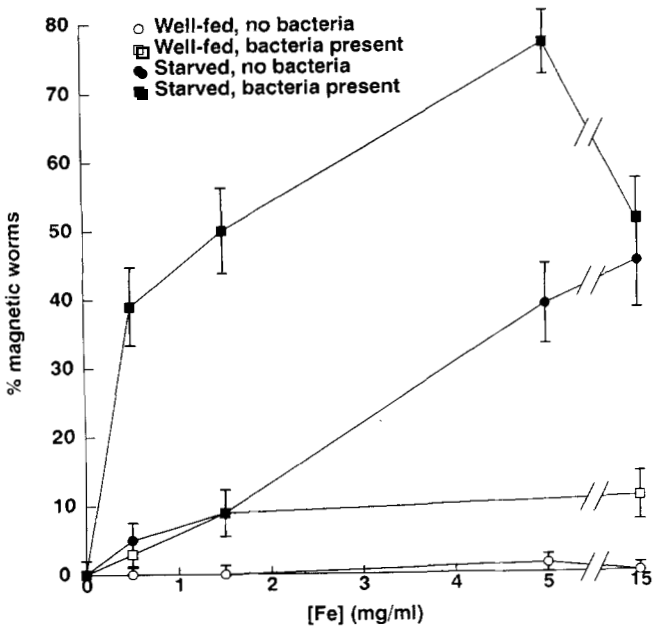

FIGURE 1.—Attraction to a magnet of worms that have pumped iron. Starved (filled symbols) or well fed (open symbols) wild-type worms were suspended in liquid in the presence (squares) or absence (circles) of bacteria for 5 min with iron particles at the indicated concentration. After removal of excess iron, worms attracted to the magnet were separated from those that were not, and both groups were counted. The plot shows the ratio of the number of worms attracted to the magnet to the total number of worms. Each point represents a single assay, containing between 50 and 88 worms. Error bars represent a ±1 SEM range.

The nine presumed *unc-31; cat-4* homozygotes showed slightly slower pumping than *unc-31* worms, slightly more activity, and were much more strongly Egl, especially at 15°. We mapped the source of this enhanced Egl phenotype between *dpy-11* and *him-5*, consistent with the known location of *cat-4* between *dpy-11* and *unc-76*.

RESULTS

A magnetic selection for pumping-constitutive mutants: We previously described an assay for measuring pumping rate in populations of worms suspended in liquid (AVERY and HORVITZ 1990). In this assay, a small number of 5-mm iron particles are suspended in liquid with worms, the worms are allowed to pump for 5 min, and iron particles inside worms are counted. To enrich for mutants that pump in the absence of bacteria, we used a variation of this assay. As before, worms were suspended in liquid with iron particles and allowed to pump iron. After addition of anesthetic and removal of excess iron, worms that had swallowed large amounts of iron were selected by their attraction to a small magnet.

Figure 1 shows that pumping worms can take in enough iron to be attracted to a magnet, that conditions that cause rapid pumping (previous starvation and the presence of bacteria in the suspension) result in more worms being attracted to the magnet, and that attraction is absolutely dependent on iron. Table 2 shows that pumping worms can be enriched 5–10-fold from a mixture of worms that do not pump (well fed wild-type worms) and a small proportion (<10%) of worms that do (starved *dpy-5* worms, where the *dpy-5* mutation allowed the starved worms to be recognized by their shorter body length).

TABLE 2

Magnetic enrichment of starved worms

[Fe] (mg/ml)	Original mix (estimate) Well-fed non-Dpy	Original mix (estimate) Starved Dpy	Attracted to magnet non-Dpy	Attracted to magnet Dpy	Enrichment[a]
8.3	0	6	0	0	
8.3	29	6	0	0	
8.3	74	6	2	0	0
8.3	294	6	0	3	50
8.3	735	6	20	2	11
8.3	0	18	0	2	1
8.3	25	18	0	5	2
8.3	62	18	0	5	4
8.3	246	18	3	6	10
8.3	615	18	32	7	6
0.0	330	22	2	0	0
0.3	330	22	1	2	11
0.8	330	22	11	4	4
1.7	330	22	10	5	5
3.3	330	22	4	3	7
8.3	0	9	0	5	1
8.3	31[b]	9	1	4	4
8.3	78[b]	9	2	5	7
8.3	310[b]	9	0	8	35
8.3	775[b]	9	2	6	65

Well-fed wild-type and starved Dpy worms mixed in the proportions shown were allowed to eat iron particles in the absence of bacteria. Worms that stuck to a magnet were selected, and the non-Dpy and Dpy worms among them counted. Estimates of the numbers of worms of each type in the original mix are based on counts of worms in aliquots of the suspensions mixed for the assay.

[a] Enrichment is defined as [(no. magnetic Dpy worms)/(total no. magnetic worms)]/[(no. Dpy worms in original mix)/(total worms in original mix)]. Note that this value cannot exceed the reciprocal of the proportion of Dpys in the original mixture, so that the highest enrichments can be obtained only when most of the worms are non-Dpy.

[b] These worms were killed with chloroform before testing.

To isolate pumping-constitutive mutants, we did magnetic selections on the F_2 self-progeny of EMS-mutagenized wild-type worms and created strains from the worms that stuck to the magnet (see MATERIALS AND METHODS). Figure 2 is a histogram of the pumping rates of 36 such strains, measured by the pumping iron assay (AVERY and HORVITZ 1990). Pumping rates ranged from 0.03 to 2.74. Concurrent wild-type controls ranged from 0.02 to 0.15, so at least 24/36 of the strains, those whose pumping rates were greater than 0.2, are likely to carry mutations that confer a pumping-constitutive (Puc) phenotype. Ten of these strains had pumping rates greater than 1, which we consider a strong Puc phenotype. Five of the strongest Puc mutant strains from the first three

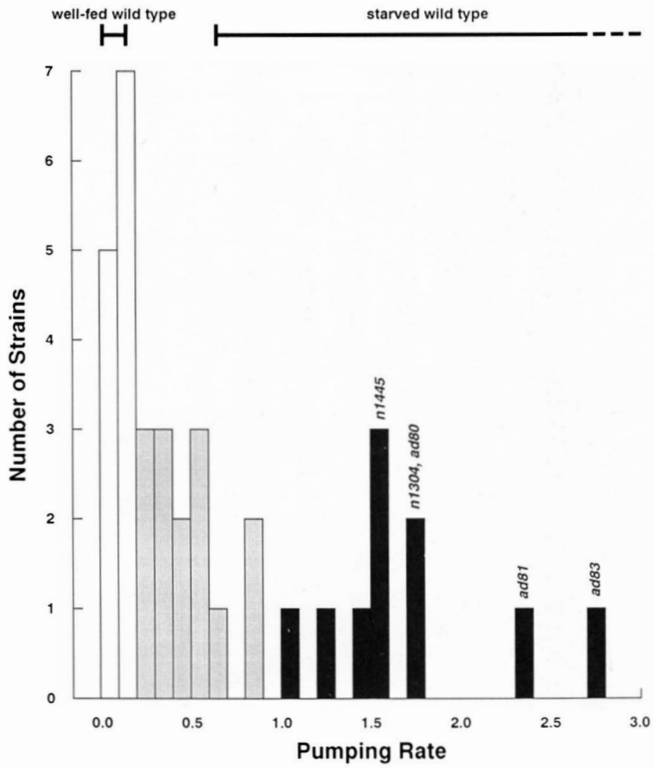

FIGURE 2.—Pumping rates of strains created from magnetically selected mutants. The F_2 progeny of EMS-mutagenized wild-type hermaphrodites were subjected to magnetic selection. Thirty-six strains derived from worms that were attracted to the magnet were assayed by the pumping iron assay (AVERY and HORVITZ 1990) for average pumping rate in the absence of bacteria after growth in the presence of abundant bacteria. An average of 90 worms was scored in each assay; the minimum was 25. This figure is a histogram of the measured average pumping rates. For comparison, bars over the histogram indicate the range of pumping rates of well fed wild-type worms in parallel controls (0.02–0.15) and an interval expected to include 95% of assays of starved wild-type worms under similar conditions (0.64–3.6; AVERY and HORVITZ 1990; our unpublished data). Pumping rates <0.2 (white bars) are not distinguishable from those of wild-type worms. Pumping rates between 0.2 and 1.0 (gray bars) are probably different from wild-type rates. We consider a pumping rate greater than 1.0 (black bars) to indicate a strong Puc mutant. Five of the strong mutants were analyzed further. Their positions in the histogram are indicated.

experiments, MT2940, MT3387, DA80, DA81 and DA83, were selected for further analysis.

DA80, DA81 and DA83 were derived from the same experiment. Each appeared to carry a single recessive Puc mutation linked to *bli-2 II*, and the three mutations failed to complement (data not shown). We therefore believe they are multiple isolates of the same mutation, *ad80*. The Puc phenotype of MT3387 was caused by a single *X*-linked recessive mutation, *n1445* (data not shown). Neither *n1445* nor *ad80* worms were visibly different from wild-type worms in the presence of bacteria. Despite persistent attempts, especially with *ad80*, we had little success in mapping these mutations more accurately, because of the difficulty of scoring the Puc phenotype reliably. (Well fed wild-type worms on an agar surface pump in the absence of bacteria (AVERY and HORVITZ 1990), so the mutant phenotype could be scored only by assaying populations in liquid.) MT2940 worms, in contrast, had two visible defects in addition to their Puc phenotype: they moved very little (an uncoordinated or Unc phenotype), and retained late-stage eggs (an egg-laying-defective or Egl phenotype). We therefore concentrated our further efforts on MT2940.

TABLE 3

Pumping-constitutive phenotype of *unc-31* mutants

Experiment	*unc-31* genotype	Mutant pumping rate	Wild-type control pumping rate
1	*e69am*	2.9, 5.9	0.19, 0.21
	e86	4.0, 3.7	
	e375am	4.6, 1.6	
	e379	5.7, 3.0	
	e636	6.0, 3.4	
	e714	5.3, 6.2	
	e717	6.7, 5.6	
	e928	6.7, 5.1	
	e1512	5.5, 6.2	
2	*e169*	2.75	0.07
3	*n1304/e169*	1.58[a]	0.03
	n1304	1.45	
4	*n1304/+*	0.06	

Pumping rates were assayed for worms of the indicated genotypes suspended in liquid in the absence of bacteria by the pumping iron assay (AVERY and HORVITZ 1990). Pumping rates are indicated as the average number of iron particles taken up by a worm in 5 min. In all but experiment 4 wild-type worms were assayed concurrently, all the assays were scored without knowledge of the genotype of the worms, and the results correlated with the genotypes only after all assays had been scored. At least 80 worms were scored in each assay except in three of the assays of experiment 1 where the numbers were 20, 27, and 52. Assays carried out in parallel and scored at the same time are grouped together by experiment numbers. Numbers from the same experiment can be directly compared. In experiment 1, two assays were carried out for each strain. We show both numbers to indicate the variability of the measurements.

[a] Calculated from the average pumping rate of a 1:1 mixture of *n1304/e169* and *e169/+* males. See MATERIALS AND METHODS.

***unc-31* mutations cause a pumping-constitutive, egg-laying-defective, uncoordinated, dauer-recovery-defective phenotype:** The Unc phenotype of MT2940 was caused by a single recessive mutation, *n1304*, in the previously known gene *unc-31* (BRENNER 1974) (see MATERIALS AND METHODS). Table 3 shows that each of the 10 *unc-31* mutations isolated by BRENNER (1974) on the basis of their Unc phenotypes also produced a strong Puc phenotype. Furthermore, *n1304* failed to complement the Puc phenotype of one of BRENNER's *unc-31* mutations, *e169*. We conclude that *unc-31* mutations cause constitutive pumping. Even on a Petri dish with abundant bacteria, *unc-31* pumping is visibly different from wild type. Wild-type worms pump rapidly but irregularly, with frequent brief pauses. *unc-31* worms, in contrast, pump rapidly, regularly and continuously.

unc-31 mutations also cause a temperature-sensitive egg-laying-defective (Egl) phenotype. Hermaphrodites homozygous for the *unc-31* mutations *e69*, *e86*, *e169*, *e375*, *e379*, *e636*, *e717*, *e928*, *e1512*, *n1304*, *u280* and *z1* were viable, Unc, and Egl at 25°. Furthermore, the mutation responsible for the Egl phenotype of MT2940 was closely linked to *n1304*, defined as the mutation responsible for the Unc phenotype (<1.1 cM; see MATERIALS AND METHODS). The Egl phenotype was more or less temperature-sensitive in all *unc-31* homozygotes, since at 15° all were either weakly Egl or normal. These conclusions have been supported by independent observations by G. GARRIGA (personal communication), who discovered that mutations in the gene *egl-22*, all of which confer a temperature-sensitive Egl phenotype (TRENT, TSUNG and HORVITZ 1983), are alleles of *unc-31*.

All *unc-31* mutations, even those isolated on the basis of Egl or Puc phenotypes, conferred a lethargic phenotype (Unc) on homozygotes. In the presence of abundant food, *unc-31* mutants were almost completely immobile and appeared thoroughly relaxed. However, this immobility seems to be caused more by lack of motivation than inability to move. In the absence of food *unc-31* worms were active and moved with a normal sinusoidal waveform, although more slowly than wild-type worms (HODGKIN *et al.* 1988, and our observations). Also, *unc-31* adult males were very active, and in fact difficult to distinguish from wild-type males based on their behavior, although they mate inefficiently or not at all (HODGKIN *et al.* 1988; and our data not shown). *unc-31* hermaphrodites also moved briefly when disturbed. Thus *unc-31* worms are lethargic, not paralyzed.

unc-31 mutants are also defective in recovery from the dauer larva state, a nonaging stress-resistant stage formed when worms are crowded and there is little food (RIDDLE 1988). As shown in Figure 3, dauer larvae homozygous for several different *unc-31* alleles recovered less efficiently than wild-type dauer larvae. Five of the seven mutants tested recovered more efficiently at 15° than at 25°, probably as a consequence of the intrinsic bias toward the dauer state at high temperature (GOLDEN and RIDDLE 1984). To test whether the dauer-recovery (Dar) defect was caused by the *unc-31* mutations or by other unlinked mutations in these strains, we tested *unc-31(e928)* and *unc-31(u280)* mutants that had been rescued by germline transformation with the cosmid C14G10, which contains the wild-type *unc-31* gene (D. LIVINGSTONE and R. HOSKINS, personal communication). The C14G10 cosmid can rescue the Dar phenotype of these mutants (Figure 3). (The failure to achieve 100% recovery in these strains is not surprising, since the *unc-31(+)* gene was carried on an unstable extrachromosomal element, and was therefore probably not present in all cells of all worms tested.)

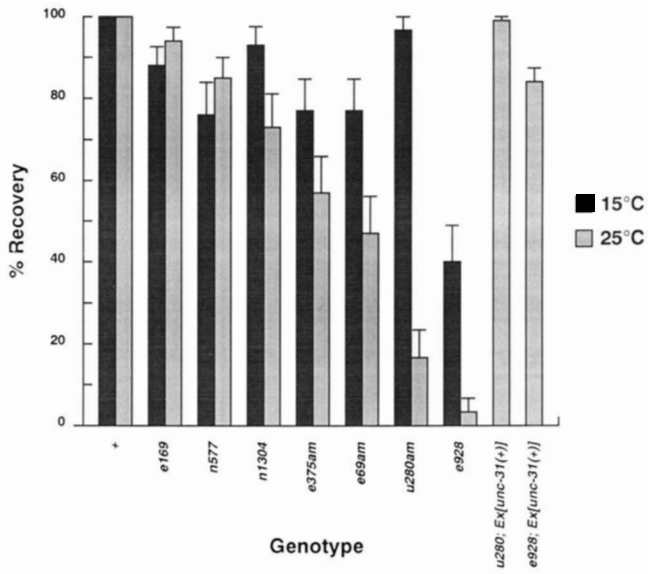

FIGURE 3.—Dauer recovery in *unc-31* mutants. Dauer larvae generated by crowding and starvation were placed on bacterial lawns and observed on the following days. A dauer larva was considered to have recovered when frequent pharyngeal pumping was observed. This figure plots the proportion that recovered in 5 days at 15 or 25°. Between 29 and 84 larvae were tested in each experiment; the average number was 40. Error bars are placed one SEM above the mean. Genotypes are shown along the *x* axis. The *u280; Ex[unc-31(+)]* and *e928; Ex[unc-31(+)]* strains are transgenic strains mutant for the chromosomal copies of *unc-31* and carrying a wild-type *unc-31* gene extrachromosomally.

The acetylcholine agonist carbamyl choline (MOLITOR 1936) can partially substitute for food in initiating dauer recovery in wild-type animals (C. I. BARGMANN, unpublished results). Over 50% of *unc-31(e928)* and *unc-31(u280)* dauer larvae recovered from the dauer stage in the presence of carbamyl choline. Since the *unc-31* dauer larvae can recover under these conditions, their failure to recover in food must reflect a specific defect in dauer recovery, and not death or irreversible metabolic arrest.

The Puc Egl Unc Dar phenotype is caused by loss of *unc-31* gene function: The high frequency at which *unc-31* mutations arise (BRENNER 1974; TRENT, TSUNG and HORVITZ 1983) suggests they might be loss-of-function mutations. In confirmation of this suggestion, the Unc phenotype of *e69*, *e375* and *u280* worms was suppressed by the strong amber suppressor *sup-7* (see MATERIALS AND METHODS).

Amber mutations in *C. elegans* usually cause complete or nearly complete loss of gene function (WATERSTON 1981). We would therefore expect amber mutations to be among those that cause the most complete loss of *unc-31* function. Consistent with this expectation, *u280*, *e69* and *e375* produce strong Unc-31 phenotypes. The strength of the amber mutant phenotypes is most clearly shown by examining dauer recovery (Figure 3), which seems to be highly

sensitive to small amounts of *unc-31(+)* activity. The three amber mutants recovered less efficiently than any others tested, with the exception of *e928*. The strongest allele, *e928*, is a 5.2-kb deletion that removes much of the *unc-31* gene (D. LIVINGSTONE and R. HOSKINS, personal communication).

Serotonin and the Unc-31 phenotype: *unc-31* mutations stimulate feeding and inhibit locomotion and egg-laying. The neurotransmitter serotonin also affects these three behaviors, stimulating feeding and egg-laying and inhibiting locomotion (HORVITZ *et al.* 1982). *unc-31* mutations have been reported to increase the level of serotonin immunoreactivity in neurons (DESAI *et al.* 1988). These observations suggested the *unc-31* phenotype might be caused by abnormalities in serotonergic transmission. It was unlikely that the sole effect of *unc-31* mutations is a decrease in serotonergic transmission, because mutations in three different genes that reduce serotonin levels, *bas-1(ad446)* (G. GARRIGA, personal communication), *cat-1(e1111)* and *cat-4(e1141)* (SULSTON, DEW and BRENNER 1975; DESAI *et al.* 1988), do not cause Unc or Egl phenotypes (our unpublished observations). *cat-1* mutants have also been tested for the Puc phenotype: their basal pumping rate (0.006 ± 0.004, $n = 4$ assays) was actually lower than concurrent wild-type controls (0.21 ± 0.035, $n = 4$ assays). *cat-4* mutants also have a reduced pumping rate in a different assay (AVERY and HORVITZ 1990). In summary, the phenotype caused by a reduction in serotonergic transmission does not resemble that caused by *unc-31* mutations.

The possibility remained that the Unc and Puc phenotypes of *unc-31* mutants might be caused by increased serotonergic transmission. (In this case, the Egl phenotype, which is as expected for a decrease in serotonergic transmission, might be explained by desensitization of serotonin receptors constantly exposed to ligand.) We did several experiments to test this hypothesis. While inconclusive, our results do not support the idea that the Unc-31 phenotype can be explained solely by abnormally high serotonin levels.

The NSMs are a pair of serotonergic pharyngeal neurons (SULSTON, DEW and BRENNER 1975; HORVITZ *et al.* 1982; AVERY and HORVITZ 1989) thought from their anatomy to be secretory (ALBERTSON and THOMSON 1976). They have free endings appropriately positioned to detect bacteria in the pharyngeal lumen. Bacteria have the same effects on locomotion, egg-laying, and feeding as does serotonin (HORVITZ *et al.* 1982). We therefore thought the Unc-31 Unc and Puc phenotypes might be caused by constitutive release of serotonin from the NSMs in the absence of food. To test this hypothesis, we killed the NSMs in *unc-31(e928)* mutants with a laser microbeam. NSM-killed *unc-31* worms were lethargic, like intact *unc-31* worms. NSM-killed wild-type worms, in contrast to NSM-killed *unc-31* worms, were active. *unc-31* must therefore be acting in cells other than the NSMs, and these other cells must be capable of affecting locomotion in an NSM-independent way.

As a more general test of the importance of serotonin in the *unc-31* mutants, we constructed an *unc-31(e928); cat-4* double mutant. The *cat-4* mutation partially reduces dopamine levels (SULSTON, DEW and BRENNER 1975) and drastically reduces serotonin levels, but probably does not altogether eliminate serotonin (DESAI *et al.* 1988; AVERY and HORVITZ 1990). *unc-31; cat-4* worms are lethargic, unlike *unc-31(+); cat-4* worms. Since *cat-4* had little effect on the Unc phenotype, it is unlikely that this phenotype is caused solely by elevated serotonin. To our surprise *cat-4* strongly enhanced the Egl phenotype caused by *unc-31* mutations. This effect was especially obvious at 15°, at which temperature *unc-31* single mutants were nearly normal in egg-laying, but all *unc-31; cat-4* double mutant hermaphrodites were clearly Egl. The enhancement of the *unc-31* Egl phenotype by *cat-4* is unexpected under the hypothesis that elevated serotonin causes the Egl defect.

The MC neurons and Unc-31 constitutive pumping: We have previously shown that the pharyngeal MC neurons are necessary for the normal stimulation of pumping rate by bacteria (AVERY and HORVITZ 1989). Two possible explanations for the constitutive pumping of *unc-31* mutants are: (1) *unc-31* mutations bypass the MCs, allowing rapid pumping even when they are inactive, and (2) *unc-31* mutations cause the MCs to be active even in the absence of bacteria. To test these models, we killed the MCs in *unc-31(n1304)* worms. The effect on feeding was indistinguishable from that produced by killing the MCs in wild-type worms: pumping was slow and irregular, growth of the worms was retarded by about a day, and they had a starved appearance. MC-killed *unc-31* worms were Unc; the Egl and Dar phenotypes were not examined. Thus the Puc phenotype of *unc-31* mutants requires the MCs. This result is inconsistent with the first model, in which *unc-31* mutations bypass the MCs. It is consistent with but does not prove the second model.

Chemosensory neurons and the Unc-31 dauer-recovery phenotype: When uncrowded and well fed, wild-type worms do not become dauers. They will become dauers, however, if the chemosensory neurons ADF, ASG, and ASI are killed (BARGMANN and HORVITZ 1991b). If left alive, any one of the three neuron types can prevent dauer formation. The chemosensory neuron ASJ is necessary for recovery from the dauer state (BARGMANN and HORVITZ 1991b). When mutations in *unc-31* are combined with mutations in *aex-3* (the only obvious phenotype of *aex-3*

TABLE 4

Dauer arrest after sensory cell kills in *unc-31(e928)* and wild-type animals

Cells left intact				Genotype	
ADF	ASG	ASI	ASJ	*unc-31*	+
+	+	+	+	0/31	0/>100[a]
−	−	−	−	4/4	30/37[a]
+	+	−	+	12/15	0/10
−	−	+	−	0/6	0/8[a]

This table shows the number of worms arrested at the dauer stage as a proportion of the total number of worms operated. Worms were considered arrested if they formed dauers and failed to recover within 1 day.

[a] These data are from BARGMANN and HORVITZ (1991b).

single mutants is a defect in defecation; THOMAS 1990), the double mutants become dauers and do not recover (AVERY 1993). Neither single mutant is dauer-constitutive. A possible explanation for the synthetic effect of mutations in these two genes on dauer formation is that each eliminates the function of a subset of the four sensory neurons. To test this hypothesis we killed individual sensory cell types in *unc-31(e928)* animals.

When ADF, ASG, ASI and ASJ were killed in *unc-31(e928)* animals, they became dauer larvae, as did wild-type animals (Table 4). Unlike wild-type animals, however, *unc-31(e928)* animals became dauer larvae when only the ASI neurons were killed (Table 4). They did not become dauers when ADF, ASG and ASJ were killed. Thus only ASI is able to activate normal non-dauer development in *unc-31* animals. Apparently *unc-31* mutations directly or indirectly exclude ADF and ASG from the regulation of dauer formation and ASJ from the regulation of dauer recovery, perhaps by inactivating these cells or making their targets unresponsive.

DISCUSSION

Loss of *unc-31* function causes defects in feeding, locomotion, egg-laying, and dauer-recovery: By selecting for worms that pump constitutively, we isolated a new mutation in the previously known gene *unc-31*, first described by BRENNER (1974). *unc-31* homozygotes had four defects: they were lethargic (Unc), pumping-constitutive (Puc), egg-laying defective (Egl), and defective in recovery from the dauer state (Dar). That all four phenotypes result from mutations in the same gene is shown by several lines of evidence: (1) *unc-31* mutants have been isolated independently by screening for Unc (BRENNER 1974), Egl (TRENT, TSUNG and HORVITZ 1983; G. GARRIGA, personal communication), and Puc (this work) phenotypes. (2) Four independent *unc-31* mutants (*e69*, *e375*, *e928* and *u280*) have been shown to have all four defects. (3) Two alleles fail to complement for both the Unc and Puc phenotypes (Table 3). (4) Several alleles isolated by screening for the Egl phenotype fail to complement for Egl and Unc phenotypes (TRENT, TSUNG and HORVITZ 1983; G. GARRIGA, personal communication). (5) The cosmid clone C14G10 rescues the Unc and Dar defects of two alleles in transgenic worms (Figure 3).

These defects result from reduction or loss of *unc-31* function. Three *unc-31* mutations, two demonstrated to produce all four *unc-31* phenotypes, are suppressed by the amber suppressor *sup-7*. In *C. elegans* amber mutations usually cause complete or nearly complete loss of function (WATERSTON 1981). Another *unc-31* mutation, *e928*, is a 5.2-kb deletion that removes much of the *unc-31* gene (D. LIVINGSTONE and R. HOSKINS, personal communication). *e928* is the strongest known *unc-31* allele based on its Dar phenotype (Figure 3) and might be a null mutation. Thus, the Unc Egl Puc Dar phenotype is caused by loss of *unc-31* function and might be the null phenotype.

The Unc-31 phenotype is probably caused by nervous system defects: Pharmacological studies suggest that at least the Egl defect of *unc-31* worms is caused by nervous system dysfunction. *unc-31* mutant worms lay eggs in response to the neurotransmitter serotonin or the serotonin uptake inhibitor imipramine (G. GARRIGA, personal communication; TRENT, TSUNG and HORVITZ 1983). Worms with defective vulvae or egg-laying muscles do not lay eggs in response to these drugs, whereas egg-laying-defective worms known to have nervous system defects do (TRENT, TSUNG and HORVITZ 1983; DESAI *et al.* 1988). The ability of *unc-31* mutants to move almost normally when disturbed or deprived of food also suggests a neural rather than muscular cause for the Unc defect. Our laser killing studies of sensory neurons involved in the dauer-formation and dauer-recovery decisions suggest that the sensory neurons ADF, ASG and ASJ are unable to exert their normal effects in *unc-31* mutants. More direct evidence for a nervous system locus of *unc-31* action comes from molecular studies by D. LIVINGSTONE (personal communication), who has shown that *unc-31* is expressed in all neurons but not in any other cells.

We do not know whether *unc-31* is necessary for the development of the nervous system, or only for its subsequent function. The tests done so far indicate that *unc-31* mutants have anatomically normal nervous systems: the pharynxes of *unc-31* mutants have the normal number of nuclei of normal morphology in their normal locations (our unpublished observations), and serotonergic neurons have normal morphology (DESAI *et al.* 1988). Nevertheless, we cannot exclude the possibility that *unc-31* mutants have anatomical defects in one or more neurons that have not

been examined, or that they have developmental defects that do not result in an anatomical defect.

How do mutations in *unc-31* affect four different neural functions? Two extreme possibilities are: (1) *unc-31* is necessary for the normal function of four or more distinct neurons that separately control feeding, locomotion, egg-laying, and dauer-recovery. (2) *unc-31* is necessary for the normal function of a single neuron, which coordinately controls all four behaviors. (Although no such neuron is known, fewer than half of the neuron types have been tested for effects on pumping, egg-laying, dauer recovery, and locomotion.) Between the two extremes are models in which *unc-31* affects multiple neurons, some of which control more than one behavior. D. LIVINGSTONE's finding that *unc-31* is expressed in all neurons (personal communication) suggests that the *unc-31* gene product might be widely required, thus supporting multiple neuron models, but this finding is also consistent with a single-neuron model.

Despite the complex defects of *unc-31* mutants and the broad expression of the *unc-31* product, *unc-31* mutants can still perform many of the behaviors regulated by the *C. elegans* nervous system. Therefore, *unc-31* cannot be essential for all activity of the neurons in which it is expressed. How can the relatively mild *unc-31* phenotype be reconciled with its broad expression? One possibility is that *unc-31* functions in only some of the cells that express it. A second possibility is that the *unc-31* product is one of a family of proteins with similar functions, so that *unc-31* is required only in that subset of neurons in which it is the only family member expressed. A third possible explanation is that loss of *unc-31* activity causes some change in neuronal activity different from loss of neuronal function, *e.g.*, low level constitutive release of neurotransmitter.

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