# Dominant *unc-37* Mutations Suppress the Movement Defect of a Homeodomain Mutation in *unc-4*, a Neural Specificity Gene in *Caenorhabditis elegans*

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ABSTRACT

The *unc-4* gene of *Caenorhabditis elegans* encodes a homeodomain protein that defines synaptic input to ventral cord motor neurons. *unc-4* mutants are unable to crawl backward because VA motor neurons are miswired with synaptic connections normally reserved for their sister cells, the VB motor neurons. These changes in connectivity are not accompanied by any visible effects upon neuronal morphology, which suggests that *unc-4* regulates synaptic specificity but not axonal guidance or outgrowth. In an effort to identify other genes in the *unc-4* pathway, we have devised a selection scheme for rare mutations that suppress the Unc-4 phenotype. We have isolated four, dominant, extragenic, allele-specific suppressors of *unc-4(e2322ts)*, a temperature sensitive allele with a point mutation in the *unc-4* homeodomain. Our data indicate that these suppressors are gain-of-function mutations in the previously identified *unc-37* gene. We show that the loss-of-function mutation *unc-37(e262)* phenocopies the Unc-4 movement defect but does not prevent *unc-4* expression or alter VA motor neuron morphology. These findings suggest that *unc-37* functions with *unc-4* to specify synaptic input to the VA motor neurons. We propose that *unc-37* may be regulated by *unc-4*. Alternatively, *unc-37* may encode a gene product that interacts with the *unc-4* homeodomain.

NEURAL networks are created when neurons send out axons to synapse with specific targets. Although recent work has identified molecules that guide process outgrowth (HYNES and LANDER 1992; GOODMAN and SHATZ 1993), little is known about what happens after the axon reaches the target area (JESSELL and KANDEL 1993). What are the signals exchanged between neuron pairs that result in the creation of a synapse between them?

This problem is well suited to a genetic approach, which does not require assumptions about the mechanism of synaptic specificity nor depend on *in vitro* assays of molecular function. Mutations that disrupt neural development in the nematode *Caenorhabditis elegans* produce mutant animals displaying abnormal or "uncoordinated" movement (BRENNER 1974). Many of the corresponding "Unc" genes have been cloned and shown to encode molecules that govern neural fate (FINNEY, RUVKUN and HORVITZ 1988), process outgrowth (LI, HERMAN and SHAW 1992) and axonal guidance (LEUNG-HAGESTEIJN *et al.* 1992; ISHII *et al.* 1992).

A mutation in one of these genes, *unc-4*, alters synaptic specificity in the motor neuron circuit. Reconstruction of the ventral nerve cord (WHITE *et al.* 1986) in the null allele *unc-4(e120)* has shown that the usual input to the VA motor neurons is replaced with synapses normally reserved for their sister cells, the VB motor neurons (Figure 1). *unc-4* mutants are unable to crawl backward and tend to coil dorsally when touched on the head (WHITE, SOUTHGATE and THOMSON 1992). The Unc-4 phenotype is primarily a result of the loss of proper input to the VA motor neurons, which normally mediate backward movement (CHALFIE *et al.* 1985). It is striking that this change in connectivity does not result from the proximity of VA motor neurons to new presynaptic partners; the absence of *unc-4* activity has no detectable effect on neuronal morphology or process placement in the ventral nerve cord. Thus, *unc-4* controls synaptic specificity but not axonal guidance or outgrowth (WHITE, SOUTHGATE and THOMSON 1992).

We have cloned the *unc-4* gene and shown that it encodes a homeodomain protein (MILLER *et al.* 1992). Recent experiments with an *unc-4-lacZ* reporter gene indicate that *unc-4* is expressed in the VA motor neurons but is not activated in VB motor neurons nor in the interneurons (AVA, AVD, AVE or AVB) that provide input to these cells (Figure 1). These findings suggest that *unc-4* controls some feature of the VA motor neurons that is specifically recognized by presynaptic partners and which distinguishes the VAs from their sister VB motor neurons (D. M. MILLER and C. J. NIEMEYER, unpublished data).

As a transcription factor, the *unc-4* protein is predicted to mediate neural specificity by regulating

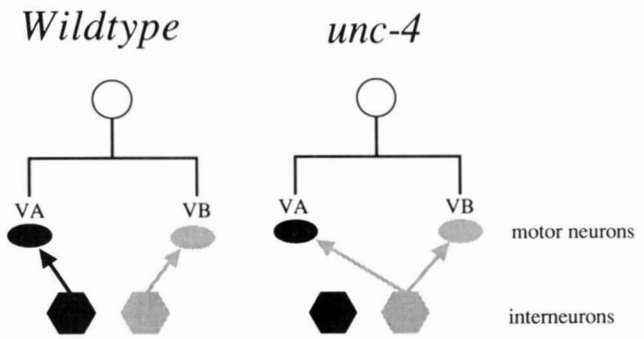


FIGURE 1.—Neuronal wiring defect in *unc-4* mutants. In the wild type, VA and VB motor neurons arise from a common precursor cell but accept synaptic input from different sets of interneurons (black hexagon, AVA, AVD, AVE; gray hexagon, AVB) and send out axons in opposite directions in the ventral nerve cord (VA, anterior; VB, posterior). In *unc-4(e120)* the usual input to most VA motor neurons is replaced with connections from an interneuron (AVB) that normally provides input to VB motor neurons. The *unc-4* mutation does not alter VA morphology or the direction of axonal outgrowth. Note that the arrows symbolize synapses between adjacent, parallel oriented neuronal processes and do not represent axonal branches (WHITE, SOUTHGATE and THOMSON 1992).

other genes. We have previously suggested that the limited effect of the *unc-4* mutation on VA cell fate may mean that *unc-4* regulates a small number of target genes and that these genes could encode molecules that are directly involved in synaptic choice (MILLER *et al.* 1992). In this paper, we describe a selection scheme for mutations in other genes in the *unc-4* pathway. The basis for the screen is our finding that the temperature sensitive allele *unc-4(e2322ts)* contains a point mutation in the *unc-4* homeodomain. It seemed reasonable to expect that a second-site mutation in an *unc-4* target gene or gene product that interacts with the *unc-4* homeodomain could compensate for this defect and suppress the Unc-4 phenotype. This simple model also predicts that loss-of-function mutations in such a gene could result in Unc-4-like movement.

We describe the isolation of four dominant, extragenic, allele-specific, suppressors of *unc-4(e2322ts)* and the evidence that all of these suppressors are gain-of-function mutations in the previously identified *unc-37* locus. The original loss-of-function mutation in this gene, *unc-37(e262)*, was initially described as a "coiler" (BRENNER 1974). We have observed that *unc-37(e262)* animals also phenocopy Unc-4 movement and may thus exhibit a similar wiring defect. On the basis of these findings, we propose that the *unc-37* gene functions with *unc-4* to control synaptic specificity in *C. elegans*.

## MATERIALS AND METHODS

**Nematode strains:** Nematodes were cultured on NGM plates inoculated with *Escherichia coli* strain OP50-1 using standard methods described in BRENNER (1974) and in SULSTON and HODGKIN (1988). All strains used in this work were isolated from N2, the canonical *C. elegans* wild-type strain. Nematode strains used in this work were obtained from the Caenorhabditis Genetics Center (University of Minnesota, St. Paul, Minn.), from A. ROSE (UBC, Vancouver, British Columbia) and H. R. HORVITZ (MIT, Cambridge, Mass.).

Mutations and strains that we have used include *unc-37(wd14dm), unc-37(wd16dm), unc-37(wd17dm), unc-37(wd18dm), unc-37(wd17wd19), unc-37(wd17wd20)* isolated in this work; *unc-37(s80), unc-37(h763)* (MCKIM, STARR and ROSE 1992); *unc-15(e1215) I* (WATERSTON, FISHPOOL and BRENNER 1977); *unc-4(e2322ts) II, unc-4(wd1)* (MILLER *et al.* 1992); *unc-4(e2322 wd11dm), unc-4(e2322 wd12dm), unc-4(e2322 wd13dm), unc-4(e2322 wd15dm)* (this work); *lon-2(e678)xol-1(y70)X* (MILLER *et al.* 1988; HENGARTNER, ELLIS and HORVITZ 1992). Balanced strains used in this work include *dpy-5 unc-37(h763) unc-13; sDp2(I;f), unc-37(s80) dpy-14 unc-13; sDp2* and *dpy-5 unc-13/hDf8* (MCKIM, STARR and ROSE 1992).

Other mutant strains that we used were described by BRENNER (1974) and by HODGKIN *et al.* (1988): Linkage group (LG) I: *dpy-5(e61), unc-37(e262), bli-4(e937), unc-87(e843), dpy-14(e188), unc-15(e73), unc-13(e450).* LG II: *rol-6(e187), unc-4(e120).* LG III: *lon-1(e185).* LG IV: *unc-24(e138).* LG V: *dpy-11(e224).* LG X: *lon-2(e678).*

The mutant *unc-4(e2322ts)* is temperature sensitive (MILLER *et al.* 1992); *unc-4(e2322ts)* animals are Unc-4 (cannot crawl backward) when grown at 25° but display wild-type movement if grown at 16°. *unc-4(e2322ts)* males were obtained by heat shock (SULSTON and HODGKIN 1988) and maintained at 16° by mating with *unc-4(e2322ts)* hermaphrodites.

**Isolation of *unc-4* suppressors**: Dominant *unc-4* suppressors were obtained by screening the $F_1$ progeny of mutagenized NC25 *[unc-4(e2322ts) II; unc-24(e138) IV]* hermaphrodites. NC25 animals display a synthetic "stuck" phenotype due to the combined movement defects of the mutations in *unc-4* (cannot crawl backward) and *unc-24* (poor forward movement). An *unc-4*-suppressed animal should be able to crawl backward and can be detected as an nematode that can reach a patch of bacteria growing on the opposite side of a 100-mm agar plate as opposed to NC25 animals, most of which (>98%) are unable to traverse the plate (see below).

NC25 animals were synchronized by hypochlorite treatment (SULSTON and HODGKIN 1988) and mutagenized with 0.05 M ethyl methanesulfonate (EMS) (SULSTON and HODGKIN 1988) when most were L4 larvae. Large (100 mm) NGM plates seeded with *E. coli* strain OP50-1 were inoculated with 100–200 mutagenized nematodes and allowed to grow at 25° for 3–4 days. The $F_1$ progeny were collected from each plate by gentle rinsing with sterile water and transferred to a 15-ml plastic centrifuge tube. The nematodes were pelleted by a low speed spin in a clinical centrifuge and then washed three or four times with sterile water to remove residual bacteria. The total number of nematodes in each tube (5,000–10,000) was determined by counting a small aliquot on a glass slide under a stereo dissecting microscope. The selection scheme for *unc-4(e2322ts)* revertants relies on the chemotactic attraction for bacteria displayed by *C. elegans* (WARD 1973). The washed $F_1$ animals were taken up in a small volume and transferred to the bare surface of a 100 mm NGM plate with a small patch of bacteria growing on the opposite side. After standing on the benchtop for 15–30 min to dry, these chemotaxis selection plates were placed in a 25° incubator. Nematodes that reached the bacterial lawn after 24 and 48 hr of selection (<2% of $F_1$s) were tapped on the head with a platinum wire pick to test for the Unc-4 phenotype (cannot crawl back-

ward). Rare Unc-4 revertant hermaphrodites (can crawl backward) were transferred to individual small (60 mm) NGM plates seeded with OP50-1 and allowed to reproduce by self fertilization. Several individual non-Unc-4 hermaphrodite progeny were transferred to separate plates to establish homozygous lines that did not segregate Unc-4 progeny. Four extragenic suppressors and four intragenic revertants were isolated from approximately 500,000 $F_1$ progeny. Unc-24 revertants (can crawl forward) were also isolated but these were discarded.

**Mapping of suppressors and outcrossing:** Unless otherwise stated all crosses were performed at 25°. Extragenic suppressors and presumptive intragenic Unc-4 revertants were distinguished by linkage to *rol-6(e187)*, which is near *unc-4* on chromosome *II* (0.6 map units). All eight of the suppressors/revertants are dominant. *unc-4(e2322ts)* males (grown at 16° and therefore phenotypically wild type) were mated with Unc-4-suppressed hermaphrodites. Wild-type male progeny of genotype *sup/+; unc-4/unc-4; unc-24/+* (extragenic suppressor) or *unc-4(rev)/unc-4; unc-24/+* (intragenic revertant) were mated with *rol-6(e187) unc-4(e2322ts)* hermaphrodites (*rev* = revertant). Wild-type hermaphrodite offspring carrying an extragenic suppressor, genotype *sup/+; rol-6 unc-4/+ unc-4; unc-24/+* or *+/+*, segregate 3/16 Rol non-Unc-4 and 1/16 Rol Unc-4 whereas wild-type hermaphrodite progeny carrying an intragenic revertant, genotype *rol-6 unc-4/+ unc-4(rev); unc-24/+* or *+/+*, segregate 1/4 Rol Unc-4 and occasional Rol non-Unc-4 progeny due to recombination between *rol-6* and *unc-4*. Wild-type hermaphrodite siblings were picked for selfing and homozygous lines of genotype *sup/sup; unc-4/unc-4* (extragenic suppressors) or genotype *unc-4(rev)/unc-4(rev)* (intragenic revertants) established as populations that did not segregate Unc-4, Rol or Unc-24.

The absence of linkage to *rol-6* demonstrated that the extragenic suppressors did not map to chromosome *II*, and segregation of *unc-24* from *unc-4* suppressor activity eliminated chromosome *IV*. Linkage to the remaining four (*I, III, V, X*) chromosomes was established using the morphological mutations *dpy-5(e61) I, lon-1(e185) III, dpy-11(e224) V, lon-2(e678) X* in an *unc-4(ts)* background. For example, males of genotype *sup/+; unc-4/unc-4* were mated with *unc-4; dpy-11* hermaphrodites and wild-type hermaphrodite progeny, genotype *sup/+; unc-4/unc-4; dpy-11/+*, picked for selfing. Segregation of 3/16 Dpy non-Unc and 1/16 Dpy Unc indicated that the suppressor was not linked to chromosome V. The $F_2$ progeny of a similar mating with *dpy-5; unc-4* segregated 1/4 DpyUnc, indicating that the suppressor is linked to *dpy-5* on chromosome *I*. Rare Dpy non-Unc hermaphrodite siblings (from recombination between *dpy-5* and *sup*) were picked to establish *dpy-5 sup; unc-4*.

Two factor data were obtained from the initial chromosome mapping experiments described above. *dpy-5 +/+ sup; unc-4(ts)* hermaphrodites were allowed to self and the distance between *dpy-5* and the suppressor loci (~2 map units) calculated from the proportion of Dpy non-Unc to Dpy Unc progeny (R) according to the formula $P = R/(2 + R)$ (for small $p$) (BRENNER 1974).

Three factor mapping of the suppressors *wd14dm, wd16dm, wd17dm* and *wd18dm* was accomplished by mating *+ sup +/+ + +; unc-4(ts)* males with *bli-4 + unc-87; unc-4(ts)* hermaphrodites to obtain *+ sup +/bli-4 + unc-87; unc-4(ts)* hermaphrodites from which Bli non-Unc-87 were picked and scored as Unc-4 or non-Unc-4 [Unc-87 = muscle defective, flaccid, thin (WATERSTON, THOMSON and BRENNER 1980)]. In some cases the Bli phenotype (Blister) was so severe that the Bli non-Unc-87 recombinants were immobilized and the presence or absence of *unc-4*-suppressor activity was determined from larval progeny. The frequency at which crossovers occur between *unc-37* or *sup* and flanking markers *bli-4* and *unc-87* is directly related to the genetic map distance between them. For example, for three-factor crosses with *sup(wd16dm)*, 1/9 crossover events occurred between *sup* and *unc-87* (Table 1), which places *wd16dm* approximately 1/9 of 0.7 map units (*bli-4* to *unc-87* interval) or ~0.08 map units to the left of *unc-87*.

**TABLE 1**

**Three-factor crosses**

| Genotype of heterozygote | Phenotype of selected recombinants | Recombinant frequencies |
|---|---|---|
| *+ unc-37(e262) +/bli-4 +unc-87* | Bli | *bli-4*(19/21) *unc-37* (2/21) *unc-87* |
| *+ sup(wd14dm) +/bli-4 +unc-87;unc-4(ts)* | Bli | *bli-4*(12/13) *sup*(1/13) *unc-87* |
| *+ sup(wd16dm) +/bli-4 +unc-87;unc-4(ts)* | Bli | *bli-4*(8/9) *sup*(1/9) *unc-87* |
| *+ sup(wd17dm) +/bli-4 +unc-87;unc-4(ts)* | Bli | *bli-4*(22/22) *sup*(0/22) *unc-87* |
| *+ sup(wd18dm) +/bli-4 +unc-87;unc-4(ts)* | Bli | *bli-4*(10/10) *sup*(0/10) *unc-87* |

**Allele specificity:** Each of the extragenic suppressor alleles, *wd14dm, wd16dm, wd17dm* and *wd18dm*, was placed in an *unc-4*(+) background to test for a visible suppressor mutation phenotype. *dpy-5/+* males were mated with *sup; rol-6 unc-4(ts)* hermaphrodites, and individual non-Rol progeny, genotype *+/sup* or *dpy-5/sup; rol-6 unc-4(ts)/+ +* were allowed to self. Non-Rol hermaphrodite progeny were picked from plates segregating Dpy siblings and repeated for successive generations until a wild-type strain was obtained that segregated neither Rol nor Dpy and therefore should be *sup/sup; + +/+ +*.

None of the four extragenic suppressors showed a distinctive Unc phenotype as homozygotes. To test for suppressor activity in each of the outcrossed alleles, hermaphrodites of genotype *sup/sup; + +/+ +* were mated with *+ +/rol-6 unc-4(ts)* males. Wild-type $F_1$ hermaphrodite progeny segregating 3/16 Rol non-Unc and 1/16 Rol Unc were detected, indicating that each of the outcrossed lines still carries *unc-4(ts)* suppressor activity.

The extragenic suppressors *wd14dm, wd16dm, wd17dm* and *wd18dm* were tested for their ability to suppress the null alleles *unc-4(wd1)* and *unc-4(e120)*. Approximately 20 *++/rol-6 unc-4(0)* males were mated with two *unc-4(ts)* hermaphrodites to ensure that most of the $F_1$s were cross progeny. For all eight combinations of the four suppressors and two *unc-4* null alleles, the $F_1$ cross progeny hermaphrodites, genotype *sup/+; + unc-4(ts)/rol-6 unc-4(0)*, segregated 1/4 Rol Unc and less than 1/16 Rol non-Unc progeny, which indicate that the suppressor alleles *wd14dm, wd16dm, wd17dm* and *wd18dm* do not suppress *unc-4(e120)* and *unc-4(wd1)*.

**Reverting suppressor activity:** Revertants of the extragenic suppressor mutation *wd17dm* were isolated in an effort to define a loss-of-function phenotype for the suppressor locus (*lf* = loss-of-function). The strain *dpy-5 sup(wd17dm); unc-4(ts)* was mutagenized with EMS (0.05 M) (SULSTON and HODGKIN 1988), and five to eight L4 hermaphrodites were mated with 15 *unc-4(ts)* males (grown at 16°) on individual 60-mm plates. Typically, 15 mating plates were set up for each mutagenesis. After 3 and 4 days at 25°, individual $F_1$ non-Dpy hermaphrodite progeny were tested for Unc-4 (cannot crawl backward) by tapping on the head with a

platinum wire pick. Approximately 10,000 $F_1$ progeny were screened from three EMS mutagenesis experiments. In one case, *unc-4(ts)* males were mated with EMS-mutagenized *dpy-5 sup(wd17dm); unc-4(ts);lon-2(e678) xol-1(y70) X*. The *xol-1* mutation is lethal in males (MILLER *et al.* 1988) and was utilized to effectively prevent male/hermaphrodite matings among the $F_1$ progeny, which complicates genetic analysis (HENGARTNER, ELLIS and HORVITZ 1992).

Two revertants, *wd17dmwd19* and *wd17dmwd20*, were isolated as Unc heterozygotes, *dpy-5 sup(lf)/+ + ; unc-4(ts)*. Both segregated small, DpyUnc progeny, which developed to the adult stage but were sterile. Because *dpy-5* is semidominant, the heterozygous strain could be maintained initially by picking slightly Dpy (short, fat) hermaphrodites from plates with small, Dpy Unc siblings. These heterozygotes were mated with *dpy-14* males to obtain *dpy-5 sup(lf) +/+ + dpy-14*, which, because of the temperature sensitive lethal phenotype of *dpy-14*, is balanced at 25°. The balanced strain *sup(wd17wd20) +/+ dpy-14* was obtained from the *dpy-5 sup(wd17wd20) +/+ dpy-14* parent by picking wild-type progeny from a plate segregating Unc non-Dpy siblings. Outcrossing also removed *unc-4(ts)* and *xol-1* mutations from these strains.

**Genetic tests:** *Revertants fail to complement unc-37 mutations: unc-37(e262)/+* males mated with heterozygous *wd17wd19* and *wd17wd20* hermaphrodites produced cross progeny that display the viable Unc phenotype of *unc-37(e262)*. *wd17wd20 +/+ dpy-14* males were mated with *sDp2;dpy-5 unc-37(s80) dpy-14 unc-13* hermaphrodites; small, Unc-37 non-Dpy, sterile cross progeny were detected, indicating that *wd17wd20* fails to complement *s80*.

*The unc-37 null phenotype: wd17wd19* and *wd17wd20* were placed *in trans* to the deficiency *hDf8* by mating *+ dpy-14/ unc-37(wd17wd19)* or *+ dpy-14/unc-37(wd17wd20)* males with *hDf8/dpy-5 unc-13* hermaphrodites. *hDf8/wd17wd19* and *hDf8/wd17wd20* cross progeny displayed the slow growing, Unc, adult sterile phenotype of homozygous *wd17wd19* and *wd17wd20* animals.

*cis/trans suppression:* A *cis/trans* suppression test was carried out to determine if the revertants of the *unc-4(ts)* suppressor mutations are intragenic. This is an important experiment because of the formal possibility that *unc-4(ts)* suppressor activity could be inhibited by a second-site mutation in another nearby gene. If the revertant blocks suppressor activity in *cis* but not in *trans*, then it is unlikely to be extragenic to the suppressor mutation and more likely to have occurred in the gene in which the suppressor mutation resides (GREENWALD, STERNBERG and HORVITZ 1983). *dpy-5 unc-37(wd17d)/+ +; unc-4(ts)* males were mated with *dpy-5 unc-37(wd17wd19)/ + +; unc-4(ts)* hermaphrodites; all Dpy male progeny were non-Unc-4, which demonstrates that *unc-37(wd17wd19)* is not a *trans* dominant suppressor of *unc-37(wd17dm)* and must therefore correspond to an intragenic revertant of *unc-37(wd17dm)*. The same result was obtained for *wd17wd20* from a similar experiment.

*Constructing unc-37(e262); unc-4 double mutants: unc-4(ts)* males were mated with *unc-37(e262);rol-6* hermaphrodites and non-Rol non-Unc progeny, genotype *unc-37/+; unc-4(ts)/rol-6*, were allowed to self at 16°. One-third of the Unc $F_2$s were the desired *unc-37(e262); unc-4(ts)* genotype and were detected as Unc hermaphrodites that did not segregate Rol animals.

*+/unc-37;+/rol-6* males were mated with *unc-87; unc-4(wd1)* hermaphrodites. One-fourth of the $F_1$ cross progeny were *unc-37 +/+ unc-87; + unc-4(wd1)/rol-6 +* and were detected as wild-type hermaphrodites segregating Unc-87 and Rol Unc progeny. One-fifth of their Unc siblings were the desired genotype, *unc-37(e262); unc-4(wd1)*. Because both of these *unc-37; unc-4* double mutants exhibit phenotypes that are indistinguishable from either single mutant, the genotypes of these strains were verified by complementation tests with *unc-37/+* and *unc4/+* males.

**Molecular biology:** A 552-bp cDNA fragment spanning the *unc-4* homeodomain and flanking regions was generated by reverse transcription-PCR (KAWASAKI 1990) from total RNA isolated from *unc-4(e2322ts)* mutant animals. The PCR primers were as described in MILLER *et al.* (1992). The PCR product was subcloned into Bluescript. Templates from three independent clones were sequenced using Sequenase (United States Biochemicals).

**Transgenic lines:** *unc-4-lacZ* refers to a reporter gene in which β-galactosidase expression in transgenic nematodes is governed by a 3.2-kb *PstI-SmaI* genomic fragment spanning the *unc-4* promoter region (D. M. MILLER and C. J. NIEMEYER, unpublished data). The *unc-4-unc-76-lacZ* construct includes a cDNA fragment from the axon-specific *unc-76* protein (L. BLOOM and H. R. HORVITZ, unpublished data). A detailed description of these reporter genes and their pattern of expression in transgenic lines will be presented elsewhere (D. M. MILLER and C. J. NIEMEYER, manuscript in preparation). Conventional genetic crosses were used to transfer the reporter genes from transgenic lines into an *unc-37(e262)* background. *C. elegans* strains that are transgenic for *lacZ* reporter constructs typically display a mosaic pattern of β-galactosidase expression (WAY *et al.* 1991). In this case, all *unc-4*-positive neurons do not stain in every animal but can be nonetheless reliably identified by comparing the patterns of *lacZ* expression observed in several separate animals. For example, in Figure 8a, 2 of 10 VA motor neurons (VA5 and VA6) and 1 of 6 DA motor neurons (DA3) in the ventral nerve cord are not stained in this particular animal, but *unc-4-lacZ* expression in these motor neurons has been observed in many other animals, *unc-4-lacZ* expression in transgenic lines was detected by either histochemical staining with X-gal (Figure 8a) or by immunofluorescence with anti-β-galactosidase (Figure 8b) (D. M. MILLER and C. J. NIEMEYER, unpublished data).

**Microscopy:** Live N2, *unc-37* and *unc-4* animals were photographed on agar plates mounted in an inverted microscope (Nikon Diaphot). *unc-4-lacZ* transgenic animals were photographed in a Nikon Microphot microscope.

## RESULTS

***unc-4* temperature sensitive mutant has a point mutation in the *unc-4* homeodomain:** The mutant *unc-4(e2322ts)* is temperature sensitive; when grown at 25°, *unc-4(e2322ts)* animals display an Unc-4 phenotype (cannot crawl backward, coil dorsally when touched on the head), whereas growth of *unc-4(e2322ts)* animals at 16° produces almost wild-type movement (MILLER *et al.* 1992). Our data show that the temperature-sensitive defect in *unc-4(e2322ts)* is correlated with a C to T transition (CTT to TTT) with the result that phenylalanine (F) is substituted for leucine (L) at amino acid residue 34 in the *unc-4* homeodomain (Figure 2). The term homeodomain refers to a 60 amino acid sequence that binds DNA and thereby mediates the regulation of gene expression by this class of protein transcription factors (HAYASHI and SCOTT 1990). The *unc-4* homeodomain is

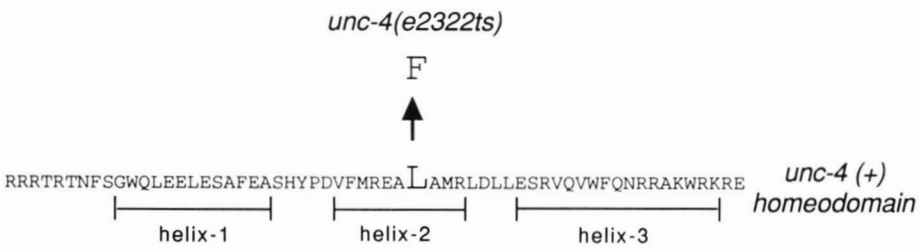


FIGURE 2.—A *unc-4* temperature-sensitive mutation is an amino acid substitution in the homeodomain. The predicted locations of alpha helical regions are indicated (MILLER *et al.* 1992). In *unc-4(e2322ts)*, a C to T transition (CTT to TTT) replaces leucine$_{34}$ (L) with phenylalanine$_{34}$ (F).

most closely related to the *paired* family but is also sufficiently different that it may represent a novel class (MILLER *et al.* 1992).

The homeodomain is a compact structure in which most of the amino residues are folded into three separate alpha-helices. Helix-3 interacts with the major groove of double-stranded DNA. Helix-1 and helix-2 are arranged at right angles to helix-3 to create a hydrophobic core that includes amino acid side chains contributed by all three helices (KISSINGER *et al.* 1990). In the NMR structure of the antennapedia homeodomain, ile-34 is on the inside surface of helix-2 and projects into the interior (QIAN *et al.* 1989). Thus, the substitution of the bulkier phenylalanine side chain for leucine in this position in the *unc-4(e2322ts)* sequence may distort the homeodomain structure such that interactions with the DNA-binding site or other components of the transcription complex are perturbed. The effect is relatively minor, however, because the mutant homeodomain appears to function almost as well as wild-type at lower temperature (MILLER *et al.* 1992).

**A strategy for isolating *unc-4* suppressors:** Because the *unc-4(e2322ts)* mutation alters the DNA-binding domain of the *unc-4* protein, we reasoned that second site mutations in the promoter/regulatory region of an *unc-4* target gene could conceivably compensate for the defect (SCHIER and GEHRING 1992). Alternatively, suppressor mutations might occur in a cofactor that facilitates DNA binding of the *unc-4* homeodomain protein or its interaction with the transcription complex. It also seemed possible that intragenic suppressor mutations would occur, such as the restoration of Leu$_{34}$ or substitutions of other amino acids in the *unc-4* homeodomain to offset the *unc-4(e2322ts)* defect. All of these suppressor mutations, however, should be rare (BOTSTEIN and MAURER 1982). Loss-of-function mutations arise in *C. elegans* at a rate of about 1 per 2000 gametes (BRENNER 1974; GREENWALD and HORVITZ 1980) whereas allele-specific suppressors are typically isolated at a much lower frequency or $10^{-5}$–$10^{-6}$ per gamete (GILCHRIST and MOERMAN 1992). Therefore, we could expect to screen about 100,000 $F_1$ animals before finding an *unc-4(e2322ts)* suppressor in this case.

A suppressor mutation should restore the ability to crawl backward to an *unc-4(ts)* animal. In principle, an animal displaying this trait could be found within a large population of Unc-4 animals by systematically touching each candidate on the head to induce an avoidance response; *unc-4* animals coil dorsally as they attempt to move backward, whereas a wild-type or suppressed animal will immediately back away from the stimulus.

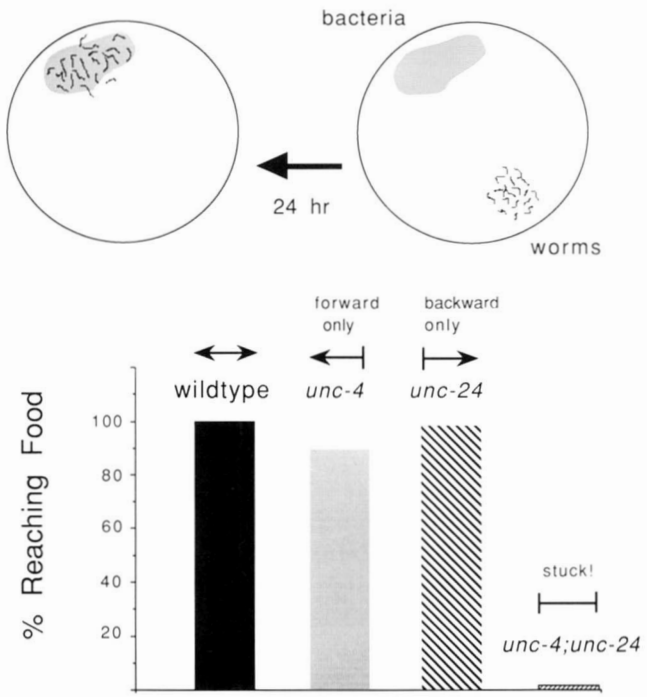


FIGURE 3.—A selection strategy for *unc-4* suppressors. One side of a 100-mm nutrient agar plate was inoculated with bacteria. Groups of 100 L4 animals from each of the indicated gentotypes were placed on the bare side of separate plates. After 24 hr, nematodes in the bacterial patch were counted and plotted as a fraction of the animals applied to the plate.

As an alternative to the touch assay, we devised a method of selection that greatly reduces the number of animals that must be individually examined to detect suppressor activity. *C. elegans* displays a chemotactic response (WARD 1973) and will crawl across a bare 100-mm agar plate to reach a patch of bacteria. Initially, we imagined that *unc-4* animals would not be able to reach the bacteria as readily as wild type such that this simple strategy could be used to select for suppressors. However, as shown in Figure 3, most *unc-4* animals (89%) are able to traverse the plate in

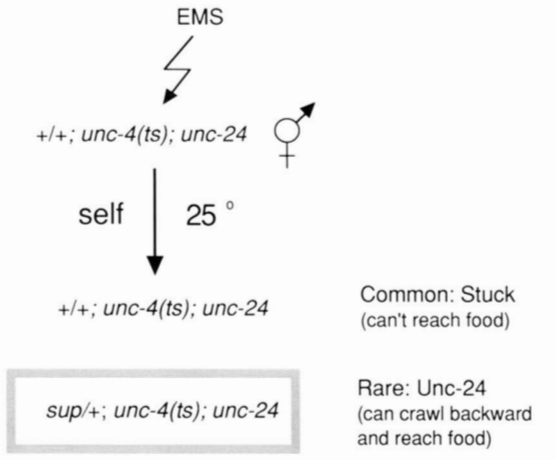


FIGURE 4.—Mutagenesis to produce *unc-4* suppressors. *unc-4(ts); unc-24* hermaphrodites were mutagenized with EMS and their $F_1$ progeny applied to chemotaxis plates (see Figure 3). Rare, Unc-4-suppressed (*i.e.*, Unc-24 non-Unc-4) animals were able to crawl across the selection plate to a patch of bacteria whereas the parental Stuck (Unc-4 Unc-24) animals were unable to reach the food.

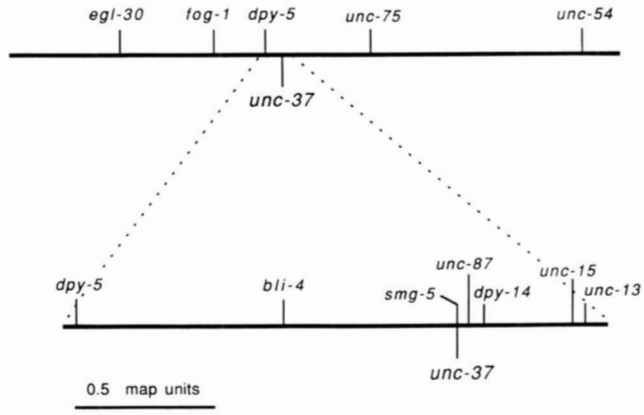


FIGURE 5.—*unc-37* region of chromosome *I*. *unc-37* is 0.05 map units to the left of *unc-87*. *smg-5* also maps to this interval but its position relative to *unc-37* has not been established by recombination.

an overnight test. The problem with this strategy is that *unc-4* animals can move forward almost as well as wild type. What we needed then was another mutation that could specifically inactivate forward movement in *unc-4* mutants. We examined several different Unc mutants and discovered that alleles of *unc-24* are primarily defective in forward movement; *unc-24(e138)* animals tend to stop and then reverse direction when stimulated to move forward but exhibit nearly normal backward movement. In spite of this movement defect, most *unc-24* animals (98%) are able to reach food on the chemotaxis selection plates (Figure 3). The double mutant, *unc-4(e2322ts)II; unc-24(e138)IV*, however, is essentially immobilized by the combination of the Unc-4 and Unc-24 phenotypes and no more than 2% of these "Stuck" animals can crawl across a 100-mm plate in a 24 hr period (Figure 3). Thus, if an Unc-4-suppressed animal arose from a population of Stuck animals, then it should be able to reach the bacterial lawn because Unc-24 animals can do so. Although this scheme would also select for Unc-24-suppressed animals (Unc-4 phenotype only), these can be easily discriminated from Unc-4-suppressed animals by the touch assay.

**Isolation of dominant *unc-4* suppressors:** The $F_1$ progeny of EMS-mutagenized *unc-4(ts); unc-24* hermaphrodites were applied to chemotaxis plates to select for dominant, Unc-4 suppressors (see MATERIALS AND METHODS) (Figure 4). We screened approximately 500,000 $F_1$s and isolated eight independent *unc-4* suppressor lines in which normal backward movement was restored. We also detected several suppressors of *unc-24(e138)* in this screen but these were discarded.

Each of the *unc-4* suppressors was tested for linkage to *rol-6*, which is near *unc-4* on LGII to distinguish between intragenic revertants and extragenic suppressors (MATERIALS AND METHODS). Four of the suppressors (*wd11dm*, *wd12dm*, *wd13dm* and *wd15dm*) are closely linked to *rol-6* and are therefore presumptive intragenic revertants of *unc-4(e2322ts)* but have not been studied further. The remaining four suppressor mutations, *wd14dm*, *wd16dm*, *wd17dm* and *wd18dm* segregated independently of *rol-6* in linkage tests and were therefore presumed to be extragenic and not located on chromosome *II*.

***unc-4* suppressors are closely linked and allele specific:** Linkage tests *vs.* genetic markers on each of the remaining five chromosomes (*I*, *III*, *IV*, *V*, *X*) established that all of the extragenic suppressor mutations are positioned within 2 map units of *dpy-5* on LGI. Two-factor crosses with *bli-4* (blister) and *unc-15* (muscle defect) placed *wd17dm* about 0.7 map units to the right of *bli-4* or near *unc-87* (data not shown) (Figure 5). Three-factor crosses (Table 1) with *bli-4* and *unc-87* indicate that all four of the extragenic suppressors map to within 0.08 map units to the left of *unc-87* (Figure 5). The close proximity of the extragenic suppressors and their similar phenotypes suggest that *wd14dm*, *wd16dm*, *wd17dm* and *wd18dm* are allelic (see below).

The extragenic suppressors are strong suppressors of *unc-4(e2322ts)*. Animals that are homozygous for a suppressor mutation and *unc-4(e2322ts)* can crawl backward essentially as well as wild type. The one exception is the *sup(wd18dm); unc-4(ts)* homozygote, which can back smoothly but tends to be "loopy" in its movements with the head almost touching the tail at the end of each body wave. All of the extragenic suppressors are dominant. A heterozygous animal *wd17dm/+; unc-4(ts)*, for example, may be slightly less mobile than wild type but cannot be reliably distinguished from *wd17dm/wd17dm; unc-4(ts)* animals (Table 2) by simple inspection.

**TABLE 2**

**Suppressors are dominant and allele specific**

| Genotype[a] | Movement |
|---|---|
| *unc-4(e2322ts)* | Unc-4 |
| *unc-37(wd17dm);unc-4(e2322ts)* | Wild type |
| *unc-37(wd17dm)/+;unc-4(e2322ts)* | Wild type to slightly slow |
| *unc-4(el20)* | Unc-4 |
| *unc-37(wd17dm);unc-4(el20)* | Unc-4 |
| *unc-4(wd1)* | Unc-4 |
| *unc-37(wd17dm);unc-4(wd1)* | Unc-4 |
| *unc-37(wd17dm)/+;unc-4(e2322ts)/unc-4(wd1)* | Slightly slow |
| *unc-37(wd17dm);unc-4(+)* | Wild type |
| *unc-37(wd17dm)/unc-37(0);unc-4(ts)*[b] | Wild type to slightly slow |

[a] Similar results were obtained for all of the *unc-4* suppressor alleles, *wd14dm*, *wd16dm*, *wd17dm* and *wd18dm*.

[b] This experiment was not performed for *wd14dm*, *wd16dm* or *wd18dm*. *unc-37(0)* corresponds to the revertants *wd17wd19* and *wd17wd20*.

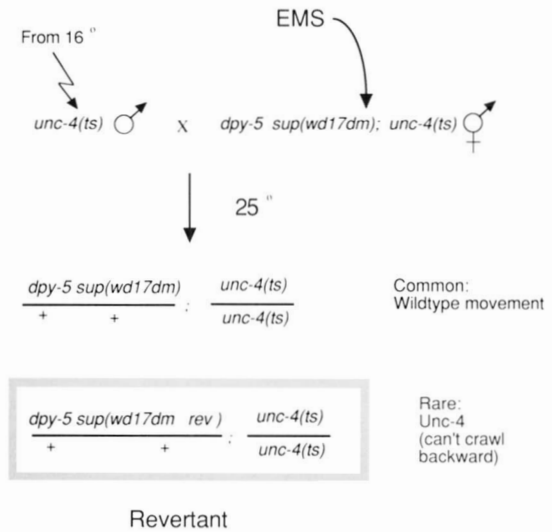


FIGURE 6.—Strategy for isolating revertants of *unc-4* suppressor. (See RESULTS and MATERIALS AND METHODS for a detailed description.)

The extragenic suppressors were tested for allele specificity against representative null alleles *unc-4(wd1)* and *unc-4(e120)*. The *unc-4(wd1)* mutation is a deletion of most of the *unc-4* coding region and does not express any detectable *unc-4* mRNA (MILLER *et al.* 1992). The mutation in *unc-4(e120)* has not been determined but it displays the Unc-4 null phenotype (WHITE, SOUTHGATE and THOMSON 1992; MILLER *et al.* 1992). Because similar results were obtained for all four of the extragenic suppressors, only the data for *wd17dm* are listed in Table 2. These experiments show that *wd14dm*, *wd16dm*, *wd17dm* and *wd18dm* do not suppress the Unc-4 phenotypes of *unc-4(e120)* or *unc-4(wd1)*. This finding is important because it rules out a model in which the suppressor mutations upregulate the expression of an *unc-4* homolog, which then substitutes for the missing *unc-4* protein. If that were true, then *unc-4* null mutations would be suppressed (ROGGE, KARLOVICH and BANERJEE 1991). Our results indicate that suppression depends on the presence of the *unc-4(ts)* protein. (Models of suppression are described in the DISCUSSION.) It is interesting that the heterozygous strain, *wd17dm/+; unc-4(e2322ts)/unc-4(wd1)*, displays almost wild-type movement, indicating that one copy each of a suppressor allele and *unc-4(e2322ts)* is sufficient to restore movement in an otherwise *unc-4* null background (Table 2).

It is also significant that the suppressor mutations do not show an obvious Unc phenotype in a wild-type [*i.e.*, *unc-4(+)*] background (Table 2). We interpret these data to mean that the suppressor mutations do not interfere with the normal function of the wild-type *unc-4* protein.

**Reversion of an *unc-4(ts)* suppressor mutation:** Because the *unc-4(ts)* extragenic suppressors arose at a low frequency ($4 \times 10^{-6}$) and are dominant, we reasoned that these alleles are not likely to represent null mutations. To obtain loss-of-function (lf) alleles, we devised a genetic screen to isolate revertants of the extragenic suppressor allele, *wd17dm*, under the assumption that mutations that knock out suppressor activity should also disable the gene in which the suppressor mutation resides (GREENWALD, STERNBERG and HORVITZ 1983; HENGARTNER, ELLIS and HORVITZ 1992). As shown in Figure 6, our strategy relies on the logic of a typical noncomplementation screen in the sense that a revertant of an *unc-4(ts)* suppressor will fail to complement a wild-type version of the suppressor gene and produce an animal with an Unc-4 phenotype. Because this screen detects revertants as heterozygotes, it is capable of isolating recessive lethal or sterile alleles.

The suppressed strain, *dpy-5 sup(wd17dm); unc-4(e2322ts)* was mutagenized with EMS and mated at 25° with *unc-4(e2322ts)* males (grown at 16° and therefore wild type and able to mate). Most of the progeny of this mating are wild type because the suppressor mutation, *wd17dm*, is dominant. Mutations that inactivate the suppressor mutation, however, will produce a rare Unc-4 animal which can be identified by the touch assay described above. Two independent revertants, *wd17dmwd19* and *wd17dmwd20*, were isolated. That both the *wd17wd19* and *wd17wd20* mutations are recessive and the frequency at which they arose (1/5000 gametes) are consistent with loss-of-function mutations (BRENNER 1974; GREENWALD and HORVITZ 1980).

Homozygous *wd17wd19* and *wd17wd20* animals display an uncoordinated phenotype in an *unc-4(+)* background that resembles Unc-4 (Table 3 and see below). They are also small, slow growing, show vulval and

**TABLE 3**

***unc-37* alleles and phenotypes**

| *unc-37* allele | Phenotype | Reference |
|---|---|---|
| *e262* | Unc, fertile | BRENNER (1974) |
| *h763* | (Unc), adult sterile[a] | MCKIM, STARR and ROSE (1992) |
| *s80* | Early larval lethal[b] | ROSE and BAILLIE (1980) |
| *wd17wd19* | Unc, adult sterile | This work |
| *wd17wd20* | Unc, adult sterile | This work |
| *unc-37(e262)/ unc-37(0)*[c] | Unc, fertile | This work and MCKIM, STARR and ROSE, 1992 |
| *wd17wd20/s80* | Unc, adult sterile | This work |
| *wd17wd19/hDf8* | Unc, adult sterile | This work |
| *wd17wd20/hDf8* | Unc, adult sterile | This work |

[a] *unc-37(h763)* is maintained with the closely linked *unc-13* mutation, which obscures the contribution of *h763* to the severely Unc phenotype of this strain.

[b] *unc-37(s80)* carries the closely linked mutation *dpy-14(e188)*, which is a synthetic lethal in combination with certain other mutations and may account for early larval lethality of *s80* (ROSE and BAILLIE 1980; MCKIM, STARR and ROSE 1992).

[c] *unc-37(0)* refers to null alleles, *s80*, *h763*, *wd17wd19* and *wd17wd20*.

gonadal defects and are sterile but can be maintained as heterozygotes using a balancer mutation (see MATERIALS AND METHODS).

***unc-4(ts)* suppressors are dominant *unc-37* mutations:** The *unc-4* (ts) suppressors are closely linked to the *unc-37* locus (Table 1), which is also positioned just to the left of *unc-87* on the genetic map (Figure 5). The original mutant, *unc-37(e262)*, was initially described as a "coiler" (BRENNER 1974). We have observed that the movement defect displayed by *unc-37(e262)* animals is also very similar to that of *unc-4(0)* mutants (see below and Figure 7).

Because the revertants *wd17wd19* and *wd17wd20* show an Unc-4-like phenotype, they were placed *in trans* to *unc-37(e262)* to test for allelism. In both cases, the revertants fail to complement *unc-37(e262)*, which indicates that *wd17wd19* and *wd17wd20* are *unc-37(lf)* mutations (Table 3). Furthermore, because the revertants are *cis* dominant suppressors of the *unc-4(ts)* suppressor *wd17dm* and not *trans* dominant suppressors of *wd17dm* (see MATERIALS AND METHODS), it follows that *wd17dm* is likely to be an *unc-37(gf)* mutation and not a mutation in some other nearby gene. Although we have not directly reverted the other three *unc-4(ts)* suppressor alleles, *wd14dm*, *wd16dm* and *wd18dm*, the similarity of their suppressor phenotypes to that of *wd17dm* and their close linkage on the genetic map, suggests that *wd14dm*, *wd16dm* and *wd18dm* are also *unc-37(gf)* mutations (*gf* = gain of function).

Recently, two additional recessive alleles, *unc-37(s80)* and *unc-37(h763)* have been described (MCKIM, STARR and ROSE 1992). *unc-37(h763)* displays an adult, sterile phenotype similar to that of *wd17wd19* and *wd17wd20* (Table 3). *unc-37(s80)* is an early larval lethal but the severity of the *s80* phenotype may be enhanced by a closely linked *dpy-14* allele (ROSE and BAILLIE 1980). The similar lethal phenotypes of the *unc-37 (lf)* mutations *wd17wd19*, *wd17wd20* and *h763* and the fact that this phenotype is not more severe in heterozygotes with the deficiency *hDf8* suggests that these alleles are *unc-37* null mutations [symbolized as *unc-37(0)* ] (Table 3). The *unc-37(e262)* mutation, however, is a viable Unc mutant and therefore may represent a rare mutation in which the *unc-37* gene is only partially defective. Heteroalleles of *unc-37(e262)* in combination with each of the *unc-37(0)* mutations, exhibit the fertile, Unc phenotype of the *unc-37(e262)* mutation (Table 3), which suggests that either *unc-37* is not dose sensitive or that the *e262* mutation affects an *unc-37* function that is required for movement but not for reproduction (MCKIM, STARR and ROSE 1992).

**A *unc-37* mutant phenocopies the Unc-4 movement defect:** *unc-37(e262)* animals display a movement defect that is strikingly similar to the Unc-4 phenotype. Neither *unc-37(e262)* nor *unc-4* mutants can crawl backward and both tend to coil dorsally (vulva on outside of loop) when touched on the head (Figure 7, d and g); the midbody region can flex dorsally but not ventrally whereas the head and tip of the tail can bend in either direction such that the coiled animals frequently assume a characteristic "omega" shape (Figure 7, e and h). Forward movement is inhibited in both *unc-4(0)* and *unc-37(e262)* but to a much lesser extent than backward movement. The Unc phenotypes of these mutants are so similar, in fact, that it is not possible to distinguish *unc-37(e262)* nematodes from *unc-4(0)* animals by simple inspection. In addition, the Unc phenotypes of double mutants, *unc-37(e262); unc-4(ts)* and *unc-37(e262); unc-4(wd1)* are no more severe than either Unc-37 or Unc-4 animals, which suggests that *unc-4* and *unc-37* act in the same genetic pathway.

In separate experiments, we have shown that synaptic input to the VA motor neurons is directly dependent on *unc-4* expression in these cells (D. M. MILLER and C. J. NIEMEYER, unpublished data). Thus, an Unc-4 phenotype would be expected if *unc-37(e262)* were to block *unc-4* expression in the VA motor neurons. As shown in Figure 8a, however, the *unc-37(e262)* mutation does not prevent the expression of an *unc-4lacZ* transgene in the VA motor neurons. In addition, the steady state level of mRNA transcribed from the endogenous *unc-4* gene in *unc-37(e262)* is comparable to wild type during the developmental period (MILLER *et al.* 1992) in which the VA motor neurons accept synaptic input from interneurons (data not shown). These results indicate that

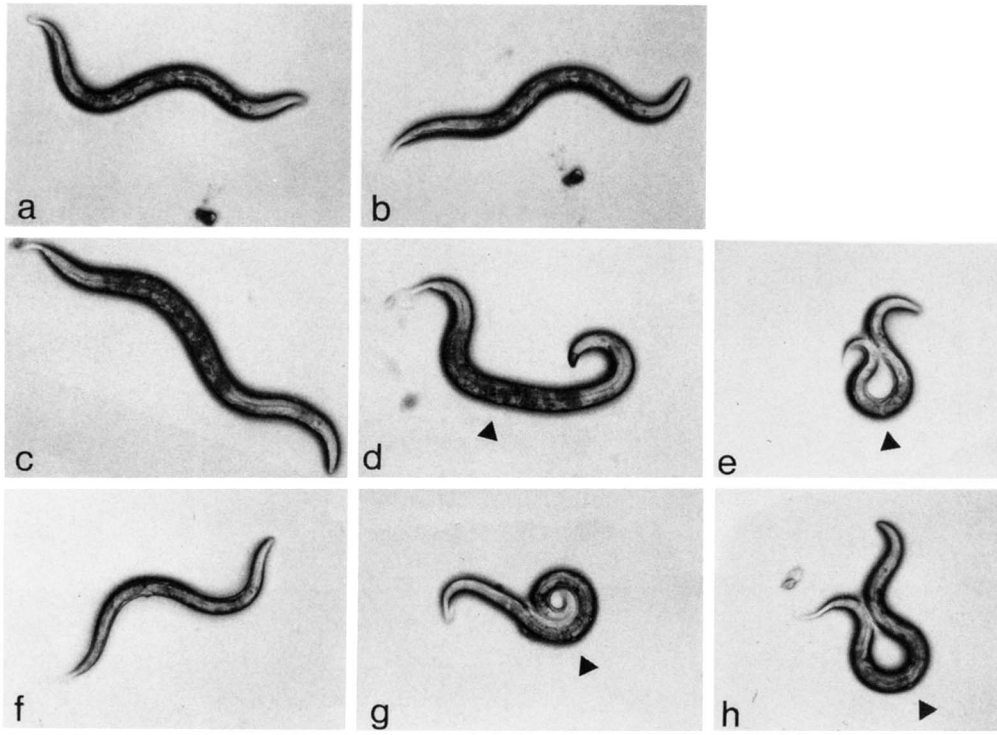


FIGURE 7.—*unc-37(e262)* phenocopies the Unc-4 movement defect. Wild-type (N2) animals display smooth sinusoidal waves along the length of the body when crawling either forward (a) or backward (b). *unc-4(wd1)* animals are able to crawl forward (c) but coil dorsally (d) and frequently show an "omega" shape when attempting to crawl backward (e). *unc-37(e262)* animals can move forward (f) but cannot crawl backward and either coil dorsally (g) or show the "omega" shape when touched on the head (h). Arrowheads point to the vulva on the ventral side.

*unc-4* transcription is not dependent on *unc-37* gene activity and thus that *unc-37* does not act upstream of *unc-4* in a regulatory pathway specifying neural input to VA motor neurons.

The wiring defects in an *unc-4* null mutant, *unc-4(e120)*, have been identified by reconstructing the ventral nerve cord from electron micrographs of serial sections (Figure 1) (WHITE, SOUTHGATE and THOMSON 1992). The inability of *unc-4* mutants to crawl backward is correlated with the loss of appropriate input to nine VA motor neurons (VA2–VA10), which are required for activating ventral muscles during backward movement (CHALFIE *et al.* 1985). Input to DA motor neurons, which innervate dorsal muscles, is normal in *unc-4(e120)*, however, and accounts for the dorsal coiling response. The dorsal-ventral flexures of the head and tip of the tail are attributed to VA motor neurons at each end of the ventral nerve cord (VA1 and VA11, VA12, respectively) that are not miswired in *unc-4(e120)* and thus function normally (WHITE, SOUTHGATE and THOMSON 1992).

Although the remarkable resemblance of *unc-37(e262)* to *unc-4(0)* mutants is indicative of the same pattern of wiring defects, it does not exclude other possible explanations. Unc-4-like movement would result from any mutation that blocks proper differentiation of VA motor neurons. If, for example, VA motor neurons failed to send out axonal processes in *unc-37(e262)*, then ventral body wall muscles would not be innervated and the affected animals would be expected to resemble *unc-4(0)* mutants. To examine this possibility, we created a chimeric *lacZ* reporter gene for visualizing VA motor neuron morphology. A cDNA fragment encoding a portion of the *unc-76* axonal protein (L. BLOOM and H. R. HORVITZ, unpublished data) was incorporated into the *unc-4-lacZ* reporter gene. In the presence of the *unc-76* protein fragment, most of the *lacZ* staining occurs in neuronal cell bodies and axons instead of in the nucleus (D. M. MILLER and C. J. NIEMEYER, unpublished data). In the wild type, VA motor neurons extend anteriorly directed axons in the *C. elegans* ventral nerve cord (WHITE *et al.* 1986). As shown in Figure 8b, VA motor neuron morphology and process outgrowth are apparently normal in *unc-37(e262)* animals expressing the *unc-4-unc-76-lacZ* transgene. These results argue against a model in which the *unc-37(e262)* mutation either prevents or drastically misguides VA axonal outgrowth.

In summary, the *unc-37(e262)* mutation confers an Unc-4 like movement defect but exhibits no detectable effect on either *unc-4* expression or VA motor neuron morphogenesis. These findings are consistent with the hypothesis that VA motor neurons are miswired in *unc-37(e262)* just as they are in *unc-4(0)* mutants but do not rule other kinds of defects in VA motor neuron function that would not be detected by our methods. Ultimately, it will be necessary to reconstruct the ventral nerve cord from an *unc-37(e262)* animal to distinguish among these possibilities.

## DISCUSSION

We have isolated rare, dominant, extragenic mutations that fully suppress the movement defect con-

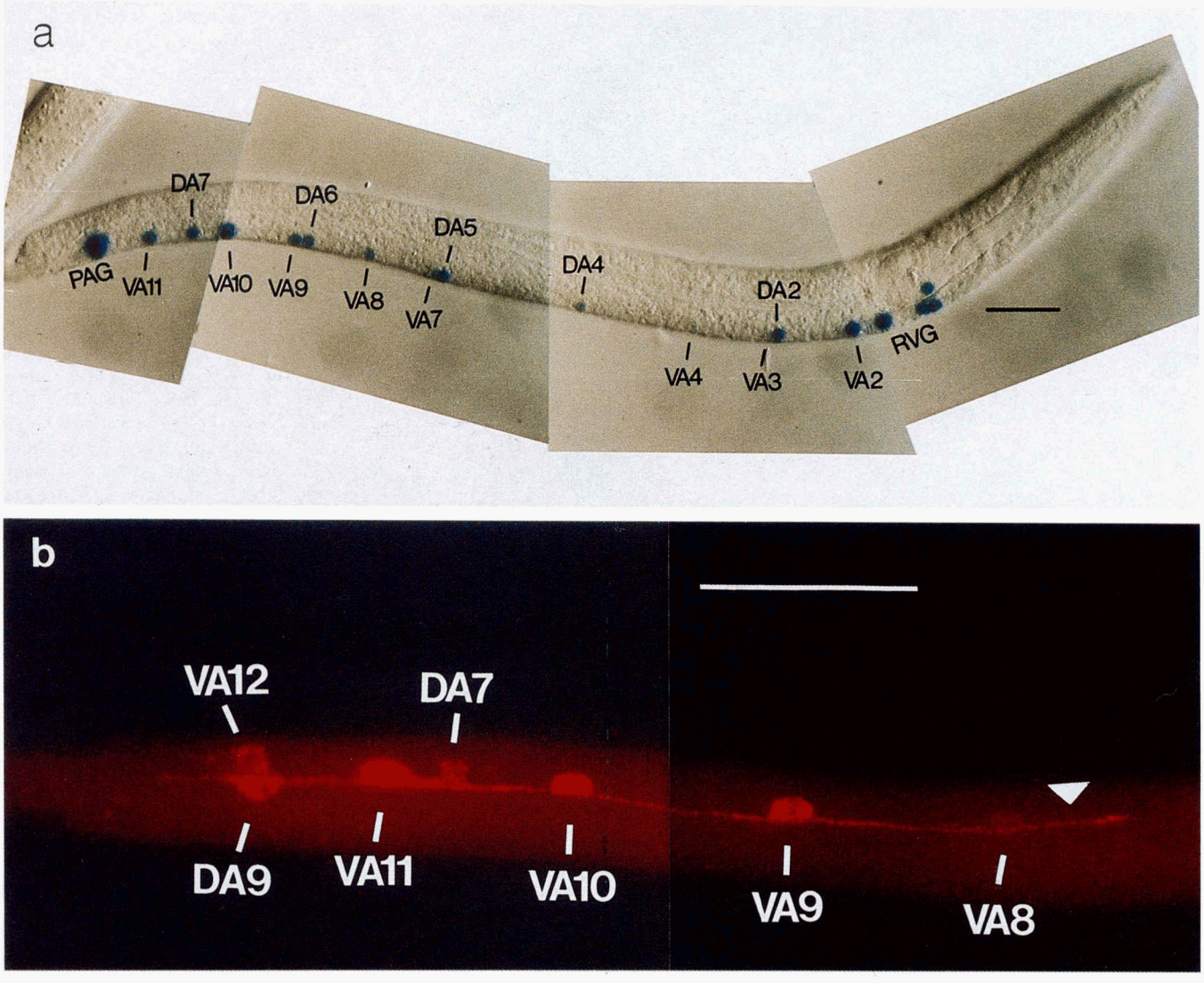


FIGURE 8.—*unc-37(e262)* does not prevent *unc-4* expression nor alter VA motor neuron morphology. (a) Expression of *unc-4-lac Z* transgene in VA motor neurons in a representative *unc-37(e262)* larva (early L2) detected by staining with X-gal. *unc-4-lacZ* is also expressed in DA motor neurons in the ventral nerve cord, in three motor neurons (VA12, DA8, DA9) in the preanal ganglion (PAG) and in certain other neurons in the retrovesicular ganglion (RVG). A single neuron in the posterior bulb of the pharynx is frequently stained (MILLER and NIEMEYER 1993). (b) Expression of *unc-4-unc-76-lacZ* reporter gene in the posterior region of an *unc-37(e262)* larva (late L1 stage). Anti-β-galactosidase stains motor neuron cell bodies and processes due to the axon-specific *unc-76* protein sequence (L. BLOOM and H. R. HORVITZ, unpublished data) in the reporter gene. Arrowhead points to anteriorly directed axon from VA9. Anterior is to the right. Bars are 20 um.

ferred by the *unc-4(ts)* mutation at restrictive temperature. All of these mutations specifically suppress the *unc-4(ts)* allele; none suppress *unc-4* null mutations. This finding argues against a mechanism in which the suppressors enhance the expression or function of an *unc-4* homolog, which then substitutes for the missing *unc-4* protein (BOTSTEIN and MAURER 1982). An example of this kind of suppression is the amplification of the myosin heavy chain gene, *myo-3*, to compensate for null mutations in the *C. elegans unc-54* myosin gene (MARUYAMA, MILLER and BRENNER 1989). Thus, the *unc-4(ts)* suppressors do not bypass the *unc-4* pathway. It seems more likely that the *unc-4(ts)* suppressors are mutations that compensate for the amino acid substitution in the *unc-4(e2322ts)* homeodomain by either functional or structural interaction with the defective *unc-4* protein (see below).

In principle, it is possible to obtain such a dominant suppressor by ectopically expressing a gene product that is normally not active in the VA motor neurons in which *unc-4* functions (HERMAN 1988). If this were the case, then loss-of-function mutations in the suppressor locus would not be expected to produce a phenotype resembling that of *unc-4* but would exclusively result in defects in other tissues. To address this possibility, we generated revertants of an *unc-4(ts)* extragenic suppressor. All of these revertants map to the previously identified *unc-37* locus (BRENNER 1974). We have observed that the original mutation in this gene, *unc-37(e262)*, results in a movement de-

fect that is strikingly similar to that of *unc-4* (Figure 7). In fact, in our hands, it is not possible to distinguish *unc-37(e262)* animals from *unc-4* null mutants except by complementation. Thus, this observation supports the idea that *unc-37* interacts with *unc-4* in some way in the wild type and that both of these genes are required for specifying proper synaptic input to VA motor neurons. *unc-37* function is also required in other tissues because the revertants that we isolated, *wd17wd19* and *wd17wd20*, are *unc-37* null mutants and display a severely defective reproductive system in addition to uncoordinated movement.

Our finding that *unc-37* null mutations are generated by direct reversion of the *unc-4(ts)* suppressor *wd17dm* indicates that this suppressor is a gain-of-function (*gf*) mutation in the *unc-37* gene. Although, the remaining three *unc-4(ts)* suppressors were not directly reverted, their close linkage and phenotypic similarity to *wd17dm* argues that *wd14dm*, *wd16dm* and *wd18dm* are also *unc-37(gf)* alleles. The suppressors display wild type movement and apparently normal reproductive function in an *unc-4*(+) background, which indicates that these *unc-37(gf)* mutations do not disrupt *unc-37* activity and have no significant effect on the interaction of *unc-37* with the wild-type *unc-4* protein (Table 2).

We have considered the possibility that the restoration of wild-type movement to *unc-4(ts)* animals by the suppressors could result from a novel set of neuronal connections or altered motor neuron function, either of which could conceivably offset the existing Unc-4 wiring defect. If this were true, however, the suppressor mutations might be expected to display a distinctive Unc phenotype in a wild type [*i.e.*, *unc-4*(+)] background, which is not the case. In addition, suppression of *unc-4* null mutants is also predicted by this model since it is unlikely that these hypothetical changes in neural connectivity or function would depend on *unc-4*. We favor the explanation that the normal set of synaptic inputs has been restored to the VA motor neurons by suppressor mutant function. This assumption can be readily tested by reconstruction of the ventral nerve cord in an *unc-4(ts)* suppressed animal.

**Models of *unc-37* action:** We have shown that *unc-37(262)* produces a movement defect strongly resembling that of *unc-4(0)* mutants and that the severity of the Unc-4 or Unc-37 phenotype is not enhanced in double mutants of *unc-37(e262); unc-4(ts)* or *unc-37(e262); unc-4(0)*. A reasonable interpretation of these findings is that *unc-4* function depends on an active *unc-37* gene and that *unc-37* action also depends on *unc-4* in the ventral nerve cord. These are the properties of genes that are sequential components of a pathway or that make equally important contributions to a single step in that pathway (BOTSTEIN and MAURER 1982). Because of the similar phenotypes of *unc-37(e262)* and *unc-4(0)* mutants, it is not possible to order these genes by epistasis (HERMAN 1988). Other evidence, however, argues against *unc-37* acting upstream of *unc-4*; the loss-of-function mutation, *unc-37(e262)*, does not block *unc-4* expression, which would be expected if *unc-4* transcription were dependent on *unc-37* (Figure 8). This leaves open the alternatives that *unc-37* is downstream of *unc-4* or that *unc-37* and *unc-4* mediate a common step in this pathway. Both of these models are considered below.

*unc-4 regulates unc-37:* The *unc-4* homeodomain is likely to function as a transcription factor and therefore would be expected to control the expression of other genes in order to mediate synaptic input to the VA motor neurons. We have previously suggested that the limited effect of *unc-4* mutations on VA cell fate may mean that *unc-4* regulates a small number of target genes (MILLER *et al.* 1992). Our finding that all of the *unc-4(ts)* suppressor mutations map to a single genetic locus is consistent with this idea. We believe that *unc-37* is a strong candidate for a downstream gene that is regulated by *unc-4* and could be directly involved in the choice of synaptic partners for the VA motor neurons.

It is reasonable to imagine that *unc-37* encodes a protein that mediates specific cell-cell interactions in both the ventral nerve cord and in the developing reproductive system. In this scenario, the viable allele, *unc-37(e262)* corresponds to a mutation in an *unc-37* protein domain that governs interaction between VA motor neurons and specific interneurons but is not required for cell fate decisions in the vulva or gonad. A separate *unc-37* protein domain that is needed for gonadal development would function normally in this case. With the elimination of an active *unc-37* protein in null mutants, neither of these *unc-37* mediated events occurs. This model parallels the recent finding that separate "src homology" or SH2/SH3 domains in the *C. elegans sem-5* protein are required for sex muscle cell migration and vulval induction (CLARK, STERN and HORVITZ 1992). Similarly, the *Drosophila* Notch protein utilizes distinct and independent domains to interact with different effector proteins (REBAY *et al.* 1991; LIEBER *et al.* 1992). In the pathway that we have proposed, *unc-37* expression is regulated by *unc-4* in VA motor neurons but is controlled by a different transcription factor in the gonad or vulval precursor cells, which do not appear to express *unc-4* (D. M. MILLER and C. J. NIEMEYER, unpublished data).

If *unc-37* is regulated by *unc-4*, what is the basis for suppression? The *unc-4(e23322ts)* mutation is predicted to perturb the structure of the *unc-4* homeodomain (Figure 2). Perhaps the *unc-4(ts)* suppressor mutations correspond to specific nucleotide changes in the *unc-37* promoter that compensate for altered

DNA binding by the *unc-4(ts)* homeodomain (SCHIER and GEHRING 1992). This model would account for the failure of the *unc-37(gf)* mutations to suppress the *unc-4(0)* mutations that do not produce an *unc-4* protein. If this model is correct, however, the proposed changes in the *unc-37* promoter sequence must also accommodate the wild-type *unc-4* protein since the suppressor mutations do not show a homozygous phenotype in an *unc-4*(+) background. Alternatively, the *unc-4(e2322ts)* protein may retain residual function, which is insufficient to specify proper input to the VA motor neurons unless it is enhanced by overproduction or elevated activity of the downstream *unc-37* protein. Suppression by these *unc-37(gf)* mutations would depend on at least a low level of *unc-4* activity because *unc-4* null mutations are not suppressed (Table 2). This model also predicts that suppression should be dose sensitive (ROGGE, KARLOVICH and BANERJEE 1991). That is, the level of suppression should increase according to the series: *unc-37(gf)/unc-37(0)* < *unc-37(gf)/unc-37*(+) < *unc-37(gf)/unc-37(gf)*, which we do not observe. *unc-4(ts)* animals carrying *unc-37(gf)/unc-37*(+) may be slightly slower than *unc-4(ts)* animals that are homozygous for the suppressor but cannot be distinguished from *unc-4(ts)* mutants that are *unc-37(gf)/unc-37(0)* (Table 2). Thus, we do not favor the idea that suppression of the *unc-4(ts)* defect is achieved by constitutive expression of *unc-37* and prefer the idea that the suppressor mutations could define a region of the *unc-37* promoter that is recognized by the *unc-4* homeodomain.

*unc-37 and unc-4 act in concert:* We have presented a model in which *unc-37* transcription is regulated by *unc-4* as a reasonable explanation for the genetic interactions that we have observed. It is possible, however, to devise an equally plausible model in which *unc-4* and *unc-37* act in concert to specify input to the VA motor neurons. For example, *unc-37* could encode a cofactor protein that binds to the *unc-4* homeodomain in a transcription complex in VA motor neurons. The helix-2 region of the homeodomain in which the *unc-4(e2322ts)* mutation occurs has been shown to mediate specific binding between the Oct-1 homeodomain and the VP16 cofactor protein (STERN and HERR 1991). Thus, the suppressor mutations may alter amino acids in an *unc-37* cofactor protein that interact with the *unc-4* homeodomain. The *unc-37(e262)* mutation could specifically disable this interaction but have no effect on a separate *unc-37* cofactor domain that binds to a different transcription factor in the developing reproductive system. As in the model described above that predicts direct binding of the *unc-4* homeodomain to the *unc-37* promoter, the presumptive mutations in the *unc-37* cofactor that compensate for the *unc-4(ts)* defect must also accomodate binding to the wild-type *unc-4* homeodomain (Table 2).

***unc-37* is a neural specificity gene?** Mutations in the *unc-4* gene alter synaptic input to VA motor neurons but do not affect VA axonal outgrowth or process placement in the ventral nerve cord (WHITE, SOUTHGATE and THOMSON 1992). The *unc-37(e262)* mutation may have a similar effect on the VA motor neurons; *unc-37(e262)* animals display a movement phenotype that is virtually identical to that of *unc-4* mutants, and VA motor neurons are morphologically normal in *unc-37(e262)* animals (Figure 8b). In addition, we have isolated gain-of-function mutations in the *unc-37* locus that fully suppress the *unc-4(ts)* movement defect and therefore are likely to have restored the proper set of synaptic connections to VA motor neurons in *unc-4(ts)* mutant animals. We interpret our data to mean that *unc-37* functions with *unc-4* to specify synaptic input to VA motor neurons in the ventral nerve cord but is not required for either *unc-4* expression or VA motor neuron morphogenesis. We have proposed that *unc-37* could represent an *unc-4* target gene or encode a gene product that interacts with the *unc-4* homeodomain. Although our data do not distinguish between these possibilities, they do indicate that *unc-37* may be just as necessary as *unc-4* for the specification of synaptic input to the VA motor neurons. The *unc-37* mutants that we have generated coupled with the availability of the *C. elegans* physical map (COULSON *et al.* 1988) should facilitate our effort to clone the *unc-37* gene for sequence analysis and for studies of *unc-37* gene expression.

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