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Mutations in the *sup-38* Gene of *Caenorhabditis elegans* Suppress Muscle-Attachment Defects in *unc-52* Mutants

Erin J. Gilchrist and Donald G. Moerman

Department of Zoology, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z4

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ABSTRACT

Mutations in the *unc-52* locus of *Caenorhabditis elegans* have been classified into three different groups based on their complex pattern of complementation. These mutations result in progressive paralysis (class 1 mutations) or in lethality (class 2 and 3 mutations). The paralysis exhibited by animals carrying class 1 mutations is caused by disruption of the myofilaments at their points of attachment to the cell membrane in the body wall muscle cells. We have determined that mutations of this class also have an effect on the somatic gonad, and this may be due to a similar disruption in the myoepithelial sheath cells of the uterus, or in the uterine muscle cells. Mutations that suppress the body wall muscle defects of the class 1 *unc-52* mutations have been isolated, and they define a new locus, *sup-38*. Only the muscle disorganization of the Unc-52 mutants is suppressed; the gonad abnormalities are not, and the suppressors do not rescue the lethal phenotype of the class 2 and class 3 mutations. The suppressor mutations on their own exhibit a variable degree of gonad and muscle disorganization. Putative null *sup-38* mutations cause maternal-effect lethality which is rescued by a wild-type copy of the locus in the zygote. These loss-of-function mutations have no effect on the body wall muscle structure.

SUPPRESSION analysis has proven very useful in bacteria and viruses for examining gene interactions *in vivo* (Hartman and Roth 1973). Suppressor mutations have proved more difficult to isolate in multicellular organisms largely because of the lack of simple methods for examining sufficient numbers of animals. However, this powerful type of analysis has been possible with the nematode *Caenorhabditis elegans*. Large numbers of these worms can be grown in a very short period of time, and many specific mutations have already been described which permit easy detection of revertant phenotypes. Through this type of analysis several different mechanisms of suppression have been observed in *C. elegans* including intragenic rearrangement (Waterston, Hirsh and Lane 1984; Anderson and Brenner 1984), gene duplication (Riddle and Brenner 1978; Maruyama, Miller and Brenner 1989), epistasis (Moerman *et al.* 1982; Greenwald and Horvitz 1982) and informational suppression (Waterston and Brenner 1978; Waterston 1981; Hodgkin, Kondo and Waterston 1987; Hodgkin *et al.* 1989). We have used suppression analysis here to identify a new gene involved in maintaining the attachment of myofilaments to the cell membrane and the extracellular matrix of the body wall muscle cells.

In *C. elegans*, as in other nematodes, the muscles are obliquely striated as a result of the positioning of the dense bodies relative to one another along the length of the muscle cells (Rosenbluth, 1965; Francis and Waterston 1985; Waterston 1988). Dense bodies perform a function analogous to the Z-line in vertebrate muscle and to the dense plaques or focal adhesions in many types of cultured cells. Like the Z-line, they are responsible for anchoring the muscle cell thin filaments to the cell membrane and underlying extracellular matrix, and for maintaining the contractile units in register. The nematode dense body is also required to transmit force from the contractile apparatus to the hypodermis and outer cuticle, and in this function resembles the focal adhesions of cultured cells.

One class of mutation in the *unc-52* gene of *C. elegans* leads to disruption of the muscle cell dense bodies (see Waterston 1988). Animals homozygous for these class 1 mutations develop normally as young larvae, but adults are thin, paralyzed, and partially egg-laying defective (Brenner 1974). The paralysis is correlated with a gradual disruption of the muscle cell structure which begins at the third or fourth larval stage, and affects only the body wall muscle cells posterior to the pharynx (Mackenzie *et al.* 1978; Waterston, Thomson and Brenner 1980). Electron microscopy has revealed that the muscle cell dense bodies are fractured, so that the apical portion of the structure is no longer associated with the cell membrane (reviewed in Waterston 1988). The phenotype of these mutants indicates that the *unc-52* gene product is required for maintaining normal muscle structure.

Other classes of *unc-52* mutations result in embryonic lethality (B. Williams, personal communication;

K. KONDO and I. KATSURA, personal communication), suggesting that the gene also plays an essential role in early development. In class 2 and class 3 mutant embryos, elongation arrests at the two-fold stage and body wall muscle is severely disorganized. This phenotype is similar to that produced by mutations in the muscle-affecting genes *myo-3* (WATERSTON 1989), *unc-45* (VENOLIA and WATERSTON 1990), *deb-1* (BARSTEAD and WATERSTON 1991) and the *pat* genes (B. WILLIAMS and R. H. WATERSTON, personal communication).

The sequence of the *unc-52* gene has been determined (T. ROGALSKI and D. MOERMAN, unpublished results), and it resembles that of the mouse basement membrane heparan sulfate proteoglycan, perlecan (NOONAN *et al.* 1991). *In vitro*, proteoglycans have been shown to stabilize focal adhesions in cell cultures (reviewed in BURRIDGE *et al.* 1988). The sequence of *unc-52*, and its mutant phenotypes, suggest that this gene encodes a proteoglycan-like component which is important *in vivo* for stabilizing the interactions between muscle cells and the extracellular matrix.

In this paper we describe the isolation and characterization of intergenic suppressors of *unc-52*. All of these new mutations map to a single genetic locus, *sup-38*. Hermaphrodites homozygous for suppressor alleles of *sup-38* have defects in both muscle and gonad which are independent of the *unc-52* mutations. Putative null alleles of the *sup-38* locus have been isolated by selecting for loss of suppressor activity, and the terminal phenotype of animals homozygous for these mutations is maternal-effect lethal. Mutants homozygous for these *sup-38* loss-of-function alleles have wild-type muscle structure, suggesting that *sup-38* is not essential for normal muscle development.

MATERIALS AND METHODS

Nematode strains and culture conditions: Nematodes were grown and maintained on NGM plates, streaked with *Escherichia coli* strain OP50, according to the standard techniques outlined by BRENNER (1974). Some strains used in this work were obtained from D. L. BAILLIE (Simon Fraser University, Burnaby, British Columbia), J. HODGKIN (MRC, Laboratory of Molecular Biology, Cambridge, United Kingdom), I. KATSURA (University of Tokyo, Tokyo, Japan), A. M. ROSE (University of British Columbia, Vancouver), and B. WILLIAMS and R. H. WATERSTON (Washington University, St. Louis, Missouri). Genetic nomenclature follows the recommendations of HORVITZ *et al.* (1979).

The class 1 *unc-52* alleles used in this study, *e444, e998, e669am, st196::*Tc1, *e1012, e1421* and *e699su250*ts are maintained as homozygous strains. The lethal, class 2 allele, *st549*, is maintained in a strain carrying a duplication for the *unc-52* region of linkage group (LG) II (*st549/st549/mnDp34*, B. WILLIAMS and R. H. WATERSTON, personal communication). The class 3 allele, *ut111*, is maintained in a heteroallelic strain which also carries the class 1 allele *e444* (K. KONDO and I. KATSURA, personal communication). The following chromosomal rearrangements were also used: *sDf21, sDf23, nDf27, nT1(IV, V)* (FERGUSON and HORVITZ 1985), and *nT1[(m435)IV, V]* (ROGALSKI and RIDDLE, 1988).

Microscopy: Living worms were examined using both polarized light microscopy and Nomarski differential interference contrast optics. Worms were removed from NGM plates, placed in a drop of M9 buffer on a slide, and then immobilized by gently placing a coverslip over them. Pictures were taken on KODAK TMAX400 film using a Zeiss Axiophot Photomicroscope (Carl Zeiss D-7082 Oberkochen).

Characterization of *unc-52* alleles: Heteroallelic class 1/class 1 *unc-52* animals (*e444/e1012, e444/e1421, e444/e669su250, e669/e1012, e669/e1421, e669/e669su250, e998/e1012, e998/e1421, e998/e669su250*) were constructed by crossing heterozygous males with hermaphrodites homozygous for another class 1 allele. Reciprocal crosses were done for all strains involving *e1421*. Heteroallelic class 1/class 2 *unc-52* animals (*st549/e998, st549/e444, st549/e669, st549/st196, st549/e1012* and *st549/e1421*) were constructed by: (1) mating heterozygous class 1 males with *st549/st549/mnDp34* hermaphrodites and (2) mating heterozygous *st549/+* males with homozygous class 1 hermaphrodites. Reciprocal crosses were done for all combinations. Class 1/class 3 complementation tests were originally done by K. KONDO and I. KATSURA at the University of Tokyo, Tokyo, Japan. In our laboratory, class 1 Unc-52 mutants were mated to males heterozygous for the *ut111* allele, and well moving F_1 progeny were selected and scored for the presence of class 3 lethals in the F_2 generation. The *ut111* allele was tested with *e998, e444, e669, st196, e1012, e1421* and *e669su250*. Class 2/class 3 complementation tests were done by mating *ut111/+* males to *st549/st549/mnDp34* hermaphrodites and screening for the presence of the *ut111* phenotype in the F_1 generation.

Brood sizes for homozygous and heteroallelic *unc-52* animals were determined by counting the total number of progeny from 30 hermaphrodites, and calculating the mean and the 95% confidence intervals for each strain.

Isolation of revertants: Mutagenesis was done using 0.05 or 0.025 M ethyl methanesulfonate (EMS) in M9 buffer as described by SULSTON and HODGKIN (1988). Dominant or semidominant revertants were obtained by screening the F_1 progeny of mutagenized Unc-52 hermaphrodites for animals that moved as adults. Briefly, worms were washed off 10 small (60 mm) plates into M9 buffer containing EMS, and gently agitated for 4–5 hr at room temperature. Then 20–30 worms per plate were transferred onto approximately 100 large (100 mm) NGM plates seeded with *E. coli*, and allowed to grow for 3–6 days before screening. In most cases, screening continued until the worms were near starvation. To reduce the probability of isolating tRNA amber suppressors when isolating revertants of *e669*, a strain was constructed which also carried an amber mutation in the *dpy-20(IV)* gene. The *unc-52(e669am);dpy-20(e2017am)* double mutants were mutagenized, and their F_1 progeny screened for animals that were able to move but were still Dpy in phenotype. Intergenic suppressors were maintained as homozygous *unc-52;sup-38, unc-52;dpy-20sup-38*, or *sup-38* strains after outcrossing.

Mapping of suppressors: Intergenic suppressor loci were mapped using the following *dpy* markers: *dpy-5(e61)I, dpy-10(e128)II, dpy-18(e364)III, dpy-13(e184)IV, dpy-4(e1166)IV, dpy-11(e224)V*, and *dpy-6(e14)X*. Males of genotype *unc-52/+;dpy/+* were mated to hermaphrodites from an *unc-52;sup-38* strain and *unc-52/unc-52;sup-38/+;dpy/+* F_1 progeny selected. The presence of 1/16 DpyUncs in the F_2 generation indicated that the suppressor was not on the same LG as the *dpy* marker. A frequency of greater than 1/16 DpyUncs indicated linkage of the suppressor locus to the *dpy* marker.

To obtain *sup-38 dpy-4* strains, DpySup segregants were selected from mapping experiments using *dpy-4* with the suppressor alleles *ra5, ra14, ra18, ra20* and *ra21*. These Dpy hermaphrodites were outcrossed to determine whether they still carried *unc-52*, and those which did were maintained as *unc-52;sup-38 dpy-4* strains. Strains homozygous for the suppressor locus on its own were constructed for *ra5, ra14, ra18, ra20* and *ra21*. This was done by crossing *dpy-4/+* males with *unc-52;sup-38* hermaphrodites, and selecting non-Dpy, well moving F_2 progeny from semi-Dpy F_1 hermaphrodites. The strains were then outcrossed to ensure that the *unc-52* locus was no longer present, and heterozygous males were crossed to *unc-52* hermaphrodites to ensure that the strain still carried the suppressor mutation. The presence of 1/16 instead of 1/4 Uncs in the F_2 generation of this last cross indicated that the suppressor was present.

Three factor mapping of the suppressor alleles *ra20* and *ra21* was done by mating heterozygous *unc-26(e345) dpy-4(e1166)/++* males with *unc-52(e1421);sup-38* worms to obtain *unc-52/+;unc-26 + dpy-4/+ sup-38 +* hermaphrodites, and then selecting Dpy and Unc recombinants from their progeny. These animals were heterozygous for the recombinant chromosome (*unc-26 + dpy-4/+ sup-38 dpy-4*, or *unc-26 + dpy-4/unc-26 sup-38 +*) and to test for the presence of the *sup-38* allele it was necessary to obtain homozygous strains (*sup-38 dpy-4*, or *unc-26 sup-38*). This was achieved by allowing the recombinant hermaphrodites to reproduce for one or two generations and then outcrossing these worms to wild-type males to determine whether they still carried a nonrecombinant chromosome or the *unc-52* mutation. If the worms were homozygous for one of the recombinant chromosomes but still carried the *unc-52* mutation then it was inferred that these strains were also carrying the suppressor mutation since none of the selected animals were paralyzed. Heterozygous males from strains which had lost *unc-52* but were homozygous for one of the recombinant chromosomes were mated to *unc-52(e1421)* hermaphrodites. The presence of DpyUnc-52, or Unc-26Unc-52 progeny in the F_2 generation indicated that the suppressor was absent, while the absence of these phenotypes indicated that it must be present. Strains carrying *unc-26* or *dpy-4* in cis with the suppressor have been maintained for both of these alleles.

Two-factor mapping of *sup-38(ra5)* and *sup-38(ra14)* relative to *dpy-4* was done as follows. Heterozygous *unc-52/+;dpy-4/+* males were crossed to homozygous *unc-52;sup-38* hermaphrodites, and *unc-52;dpy-4 +/+ sup-38* progeny were selected. These hermaphrodites were then allowed to reproduce, and the proportion of Unc and Dpy (non-Unc) recombinant worms in the F_2 progeny was used to determine the distance of the suppressor locus from *dpy-4* using the formula $p = 1 - \sqrt{1 - 2R}$ (BRENNER 1974).

Characterization of suppressors: Complementation testing between the five *sup-38* alleles, *ra5, ra14, ra18, ra20* and *ra21*, was accomplished by mating heterozygous *dpy-4sup-38/+* males with homozygous *sup-38* hermaphrodites carrying a different *sup-38* allele. The total number of progeny from each *dpy-4 sup-38/+ sup-38* worm was determined and compared to the number of progeny produced by *sup-38/+* animals. If the mean brood size of the heteroallelic mutants was less than that produced by heterozygous *sup-38/+* animals the two alleles were said to fail to complement.

To test the allele specificity of the suppressors, class 1 *unc-52/+;sup-38 dpy-4/++* hermaphrodites were obtained by mating *sup-38 dpy-4/++* males to *e444, e669, e998, st196, e1012, e1421* or *e669su250* hermaphrodites. If DpyUncs were seen in the progeny of these worms, it was assumed that the allele being tested was not suppressed. The *sup-38* alleles were also tested against a lethal *unc-52* allele, *st549*, by the following procedure. Hermaphrodites of genotype *unc-52* (class 1)/*unc-52* (class 2);*sup-38 dpy-4/++* were obtained by mating *unc-52* (class 1)/+;*sup-38 dpy-4/++* males to *unc-52(st549)/unc-52(st549)/mnDp34* hermaphrodites. Approximately 18–24 Dpy segregants were selected in the F_2 generation, and these Dpy worms were then outcrossed to determine their genotype. Nine or 10 outcrossed hermaphrodites from each mating were selected and their progeny screened for homozygous class 1 and class 2 progeny. If the Dpy animals being tested had been homozygous for *st549*, then class 1 animals should not be present. The absence of animals homozygous for the class 2 allele, *st549*, indicated that this allele was never suppressed. The *ra5* and *ra14* alleles were also tested for their ability to suppress the class 3 lethal allele, *ut111*. In this case, heterozygous *unc-52(ut111)/+* males were mated with homozygous *sup-38 dpy-4* hermaphrodites to produce *unc-52(ut111)/+;sup-38 dpy-4/++* hermaphrodites. The Dpy progeny of these animals were then outcrossed and their F_2 progeny screened to determine whether any of these Dpy animals was homozygous for the *ut111* mutation. If the original Dpy animal had been homozygous for *ut111*, then all of the outcrossed hermaphrodites would be expected to segregate homozygous *ut111* larvae. The absence of *ut111/ut111;sup-38 dpy-4/sup-38 dpy-4* animals indicated that the *sup-38* mutation did not suppress *ut111*.

The five suppressor alleles, *ra5, ra14, ra18, ra20* and *ra21*, were also tested against three other muscle-affecting genes to determine whether they were capable of suppressing different aspects of muscle disorganization. Male *sup-38 dpy-4/++* animals were mated with hermaphrodites homozygous for *unc-23(e25)V, unc-54(st1008)I*, or *unc-112(r367)V*. The F_2 progeny of these crosses were screened for the presence of DpyUnc hermaphrodites and a ratio of 1/16 DpyUncs was taken to indicate lack of suppression.

Elimination of suppressor activity: Reversion of suppression was achieved by mutagenizing *unc-52;sup-38 dpy-4/nT1[let(m435)IV,V]* worms with 0.025 M EMS, and selecting F_1 offspring which were paralyzed. A total of approximately 240,000 worms of this genotype were screened. Six mutagenesis runs allowed us to screen 600 plates carrying approximately 400 worms each (of the correct genotype), 4–6 days after mutagenesis. All but one of the nine strains isolated in these screens carried a lethal mutation balanced by the nT1 translocation. Hermaphrodites from all of the strains carrying lethal mutations were mated to wild-type males, and the F_2 progeny were counted and scored to determine whether or not the lethal mutation was closely linked to the *dpy-4* locus. The proportion of viable Dpy recombinants was used to calculate the approximate map distance between the lethal mutation and *dpy-4* using the formula $p = 1 - \sqrt{1 - 2R}$. The frequency of Unc-52 progeny was used to determine if suppressor activity was still present in the strain. A ratio of 3 wild-type:1 Unc indicated that suppression had been eliminated, while a ratio of 11 wild-type:1 Unc was seen when suppressor activity was present. The lethal mutations which did not map to the *sup-38* locus were eliminated by rebalancing the new *sup-38* mutations in *cis* with *dpy-4*, over the nT1 chromosome. These were then maintained as heterozygous strains.

Three factor mapping of the maternal-effect lethal (Mel) mutation *ra60* was done by mating *ra5ra60 dpy-4/++* males to homozygous *unc-22(s8)* hermaphrodites. Recombinant DpyUnc progeny (*unc-22 ? dpy-4/unc-22 ? dpy-4*) were selected in the F_2 generation and scored for the presence of the Mel phenotype. If *ra60* was to the right of *dpy-4* then

all of the recombinants would be homozygous for *ra60* and exhibit the Mel phenotype, whereas if it was between *dpy-4* and *unc-22* only a certain proportion of the recombinants should be Mel.

Complementation testing of the null suppressor alleles *ra61* and *ra65* was done with two essential genes which map to the same region, *let-323* and *let-324* (CHAREST *et al.* 1990). Males heterozygous for *let-323(s1719)* or *let-324(s1727)* linked to *unc-22(s7)* were mated to hermaphrodites of genotype *unc-52;sup-38(ra5ra61) dpy-4/nT1* or *unc-52;sup-38(ra14ra65) dpy-4/nT1*. Well moving F_1 progeny were selected and plated individually to allow self-fertilization. The F_2 progeny of this cross were then screened for the presence of the *dpy-4* marker and the *unc-22*-linked lethal mutation. The presence of both of these markers on the same plate indicated the ability of the *sup-38* allele to complement the lethal mutations.

To determine whether the maternal-effect lethal phenotype could be zygotically rescued, heterozygous *dpy-4 sup-38*++ hermaphrodites were allowed to reproduce, and their Dpy progeny were mated to wild-type males. The semi-Dpy progeny of this cross were then scored for their ability to produce offspring. The presence of fertile F_2 hermaphrodites in this experiment indicated that a wild-type copy of the *sup-38* locus in the zygote was sufficient to rescue the maternal-effect lethal phenotype produced by the null mutations.

RESULTS

Characterization of *unc-52*: Seven of the class 1 *unc-52* alleles have been ranked here, based on the severity of the paralyzed phenotype (Figure 1; Table 1). The *e998* and *e444* alleles were the most severe, causing paralysis to begin early in the fourth larval (L4) stage. As adults, animals homozygous for these mutations were completely paralyzed, except for their heads, and were very thin and smaller than wild-type worms. The phenotype produced by the *e669* allele was milder than that of the two above alleles, and mutants carrying this allele did not become paralyzed until later in the L4 stage. They also grew to a larger overall size, and were not as completely paralyzed as *e998* or *e444* worms. The *e669* allele has been shown to be well suppressed by the amber suppressors, *sup-5 III* and *sup-7 X* (WATERSTON 1981). The *st196*, *e1012* and *e1421* alleles did not cause paralysis until early in adulthood although movement was slower than normal by the L4 stage. Mutants carrying these alleles were also larger, as mature adults, than the more severe *unc-52* alleles. The *st196* allele carries the transposon, Tc1, inserted in the *unc-52* locus (D. MOERMAN, unpublished results). The other class 1 *unc-52* allele which we ranked here was the temperature-sensitive mutation, *e669su250* (S. EMMONS and L. JACOBSON, personal communication). Animals carrying this mutation exhibited the mildest phenotype of all. Even at the restrictive temperature (25°) paralysis did not begin until adulthood, and the worms rarely became totally paralyzed.

We have observed defects in the somatic gonads of the various Unc-52 mutants which had not been previously described (Figure 2). These defects were primarily limited to the proximal arm of the gonad which was sometimes enlarged (Figure 2b) or, in older adults, completely disorganized. This aspect of the phenotype was not fully penetrant and in some worms the gonad was not noticeably disorganized. In some animals, the anterior arm of the gonad was significantly shorter than the posterior arm, although this did not appear to be correlated with the overall degree of disorganization. The brood size of Unc-52 animals was very variable for any given allele (Table 1) and was correlated with the degree of disorganization of the gonad. In worms heteroallelic for a class 1 and a class 2 allele this defect was more severe, and in some worms the basement membrane, or the sheath cells surrounding the gonad appeared to have disappeared in places, allowing the oocytes to leak out into the body cavity of the worm (data not shown). Presumably, this contributed to the near-sterility of these animals. In worms heteroallelic for a class 1 and the class 3 allele the gonad appeared normal indicating that the *ut111* allele complemented the class 1 alleles in this function.

All of the *unc-52* mutations used here were completely recessive. Complementation testing between the different class 1 alleles, and between class 1 alleles and the class 2 allele, *st549*, indicated a failure to

TABLE 1

Phenotypes of Unc-52 and Sup-38 mutants

unc-52 allele	*sup-38* allele	Muscle defects[a]	Gonad defects[b]	Mean brood size[c]
e998	+	Severe	Severe	20
e998	*ra5*	Moderate	Severe	22
e444	+	Severe	Severe	18
e444	*ra14*	Moderate	Severe	21
e669	+	Severe	Severe	19
st196	+	Severe	Severe	27
e1012	+	Severe	Severe	57
e1421	+	Severe	Severe	31
e1421	*ra18*	Very mild	Mild	158
e1421	*ra21*	Very mild	Mild	143
e669su250	+	Moderate	Mild	133
e669su250	*ra5*	Mild	Severe	8
+	*ra5*	Mild	Severe	29
+	*ra14*	Mild	Severe	6
+	*ra18*	None	Mild	79
+	*ra21*	None	Mild	207
+	+	None	None	258

[a] Severe: all adult animals exhibit severe disorganization in all body wall muscle cells posterior to the pharynx; moderate: most animals exhibit some muscle disorganization, but not in all body wall muscle cells; mild: less than 50% of animals exhibit some muscle disorganization; very mild: less than 90% of animals exhibit any muscle disorganization, and only in 1–2 cells per animal; none: wild-type muscle structure in all animals examined.

[b] Severe: gonad structure always appears abnormal in some way, and animals are frequently sterile; mild: only mild abnormalities are present in the gonad, and animals are rarely sterile; none: wild-type gonad structure in all animals examined.

[c] $n = 30$.

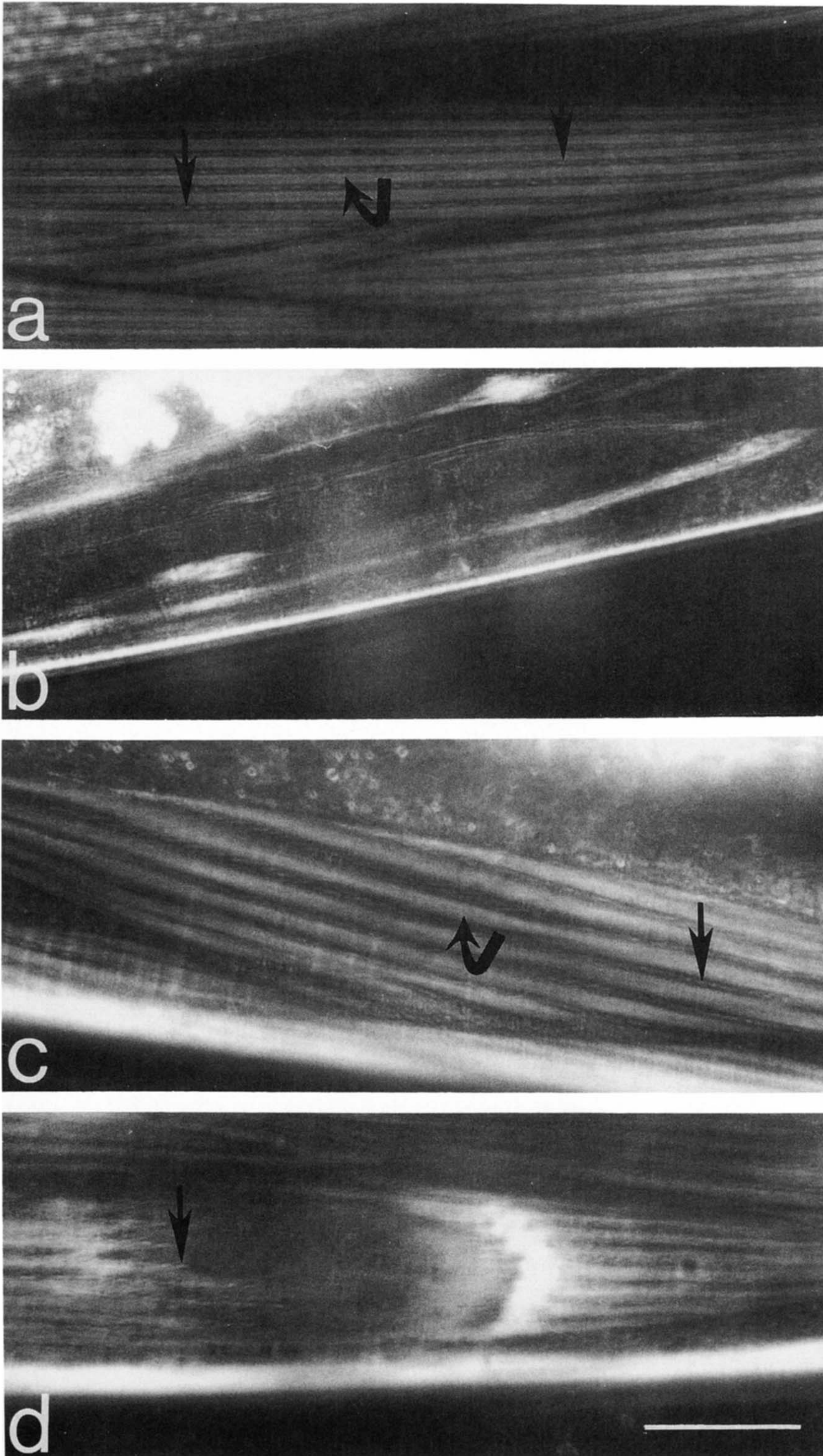

FIGURE 1.—Polarized light micrograph of body wall muscle cells of adult hermaphrodites grown at 20°: (a) N2, (b) *unc-52(e998)*, (c) *unc-52(e998);sup-38(ra5)*, (d) *sup-38(ra5)*. Straight arrows point to dense bodies, curved arrows show thick filaments. Bar is 5 μm.

complement. Animals which were heteroallelic for this class 2 allele and a class 1 allele became paralyzed by about the second larval stage and had very small brood sizes (0–15 progeny). The *e1421* allele of *unc-52*, although similar in phenotype to the other class 1 alleles, behaved somewhat differently in these crosses. When *st549/e1421* animals were obtained by mating homozygous *e1421* hermaphrodites to males heterozygous for the *st549* allele, they became paralyzed by about the L2 stage, but were still viable and produced some offspring. These worms were similar in phenotype to those of genotype *st549/e998* or *st549/e669*. However, when the reciprocal cross was done, the heteroallelic *unc-52* progeny were early larval lethals which resembled class 3, *ut111*, animals in phenotype. Similar crosses with any of the other class 1 alleles

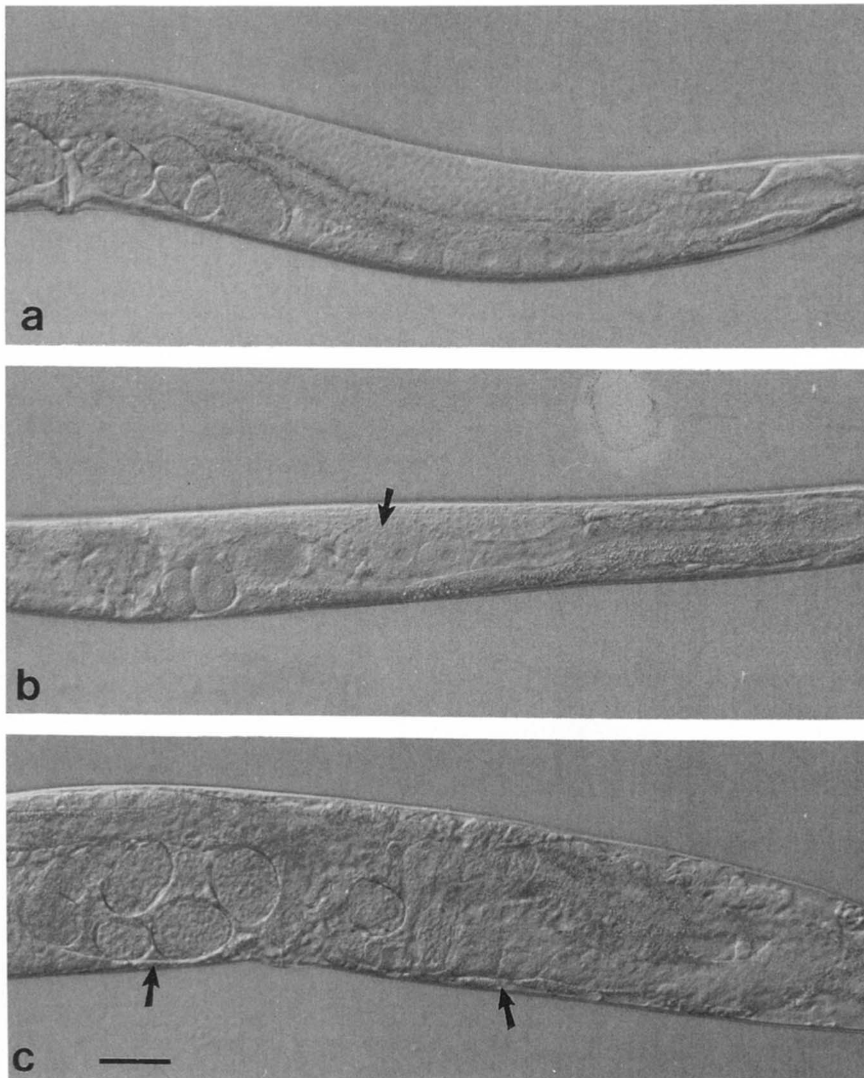

FIGURE 2.—Nomarski micrograph showing one arm of the gonad of young adult hermaphrodites grown at 20°: (a) N2, (b) *unc-52(e444)*, (c) *sup-38(ra5)*. Arrows point to abnormalities in the structure. Bar is 5 μm.

produced only the viable, paralyzed, heteroallelic animals described above.

When complementation tests were done with the class 1 alleles and the class 3 allele, *ut111*, the heteroallelic progeny appeared much healthier than either of the parental alleles. This indicated that alleles of these two classes complement one another at least partially. Complementation testing of *ut111* with *e444* was originally done by K. KONDO and I. KATSURA (personal communication). We tested *ut111* with *e998*, *e669*, *st196*, *e1012*, *e1421* and *e669su250* by mating *ut111*/+ males with class 1 hermaphrodites as described in MATERIALS AND METHODS. Heteroallelic class 1/class 3 animals appeared nearly wild-type in size and ability to move, but muscle disorganization was consistently apparent when the body wall muscle cells were examined using polarized light microscopy (data not shown). The same type of disorganization was seen when strains heteroallelic for *ut111* and any of the class 1 alleles were constructed, but was never seen in *ut111*/+ worms. Reciprocal crosses were done for *e1421*, and the *ut111/e1421* heteroallelic progeny were found to be larval lethals. The lethal stage of arrest was later in *ut111/e1421* animals (L3–L4) than in *st549/e1421* animals (L1–L2). Complementation testing between the class 2 allele, *st549*, and *ut111* was also carried out by crossing *ut111*/+ males to *st549*/*st549*/+ hermaphrodites. These two alleles failed to complement one another and heteroallelic *st549*/*ut111* animals arrested at the same stage and had the same general appearance as homozygous *ut111* animals.

Isolation of revertants: We have isolated revertants of class 1 Unc-52 mutants in order to identify other genes which normally interact with this locus. Forty-four *unc-52* revertants were isolated after screening approximately 1.3×10^8 animals homozygous for a class 1 allele (Table 2). Since screens were done in the F_1 generation, all of the animals selected were heterozygous for the new mutation. Revertant worms were picked and allowed to reproduce for several generations until they were homozygous (*i.e.*, no longer

TABLE 2

Screen for suppressors of Unc-52

unc-52 allele	No. of revertants isolated	Total No. screened	Intragenics	Intergenics	Untested	Suppressors mapped to LGIV(R)
e998	12	3.5×10^7	9	2	1	*ra5*
						ra40
e444	2	1×10^7	0	2	0	*ra13*
						ra14
e669	6	2.5×10^7	1	5	0	*ra10*
						ra17
e1012	2	1×10^7	2	0	0	
e1421	22	2.5×10^7	10	11	1	*ra18*
						ra20
						ra21
Total	44	13.5×10^7	22	20	2	

segregated Unc progeny), and then were outcrossed to wild-type males to determine whether the revertant phenotype was due to a second mutation within the *unc-52* locus or to a mutation at a separate locus which suppressed the original *unc-52* mutation. The results are presented in Table 2. Twenty-two of the revertants produced no Unc-52 worms after being outcrossed, indicating that they now carry a second mutation within, or very close to, the *unc-52* locus. Twenty of the revertants did segregate Unc-52 progeny when outcrossed and were presumed to carry unlinked suppressors of *unc-52*. The remaining two revertants were not outcrossed because they showed only partial suppression of the *unc-52* mutation and, as a result, were difficult to work with. Both intragenic revertants and intergenic suppressors were obtained for three *unc-52* alleles, but the screen using the *e444* allele did not produce any intragenic revertants, and we did not obtain any intergenic suppressors of the *e1012* allele.

Mapping of intergenic suppressors: Seven intergenic suppressor mutations (*ra5, ra13, ra14, ra18, ra20, ra21* and *ra40*) were positioned near *dpy-4* on the right arm of chromosome *IV* (Figure 3). Genetic mapping was performed using Dpy markers from each of the *C. elegans* linkage groups as described in MATERIALS AND METHODS. In each case, when *dpy-4* on LG IV was used, approximately one quarter of the F_2 progeny were DpyUnc, indicating linkage of the suppressor mutations. In contrast, only 1/16 DpyUnc progeny were seen with the *dpy* genes on the other chromosomes.

The *ra10* and *ra17* suppressors were isolated in strains carrying *dpy-20(e2017)IV* (see MATERIALS AND METHODS). When these strains were outcrossed to wild-type males we observed that the suppressor locus segregated with *dpy-20* in the F_2 generation, indicating that these suppressors were also on LG IVR.

Two of the suppressor mutations, *ra20* and *ra21*, were positioned to the left of *dpy-4* by three-factor

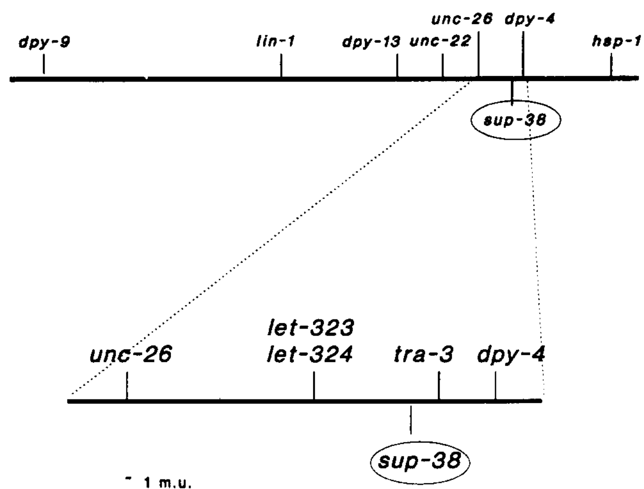

FIGURE 3.—Approximate map location of *sup-38* on LG IV(R).

mapping. Approximately one third (5/16) of the Dpy-4 and two thirds (8/14) of the Unc-26 recombinants obtained from *unc-52;+ sup+/unc-26 + dpy-4* hermaphrodites carried the suppressor, suggesting that both mutations lie within the three map unit interval between *unc-26* and *dpy-4*, slightly more than one map unit from *dpy-4*. Two-factor mapping of *ra5* and *ra14* positioned these alleles approximately 1.1 map units away from the *dpy-4* locus.

Characterization of intergenic suppressors: The five suppressor alleles we have analyzed confer an essentially wild-type phenotype in terms of appearance and movement even when the *unc-52* mutation is still present in the strain. Suppressor strains free of the *unc-52* mutations were obtained for the *ra5, ra14, ra18, ra20* and *ra21* alleles as described in MATERIALS AND METHODS. When the muscle cells of animals carrying a suppressor mutation were observed using polarized light microscopy, most of the cells exhibited an almost wild-type pattern of birefringent filaments and dense bodies (Figure 1c). However, very rarely an occasional cell in an otherwise wild-type worm was disrupted (Figure 1d). This disruption appears to be due to fragility in the muscle structure of worms carrying the suppressor mutation since it appeared more often after the worms had been rolled under the coverslip to expose the different muscle quadrants. This fragility is not apparent in wild-type animals.

Worms carrying any of the suppressor mutations also exhibited some degree of gonad disorganization when observed using Nomarski microscopy (Figure 2c). The *ra5* and *ra14* alleles consistently exhibited more severe defects which were reflected in the greatly reduced brood sizes of homozygous animals. The *ra18, ra20* and *ra21* mutants had only slightly reduced brood sizes compared to wild-type worms (Table 1), and showed a variable amount of gonadal disorganization under Nomarski optics.

TABLE 3

Complementation testing between *sup-38* alleles ($n > 10$) (mean brood size)

	+	*ra5*	*ra14*	*ra18*	*ra20*	*ra21*
+	259	187	144	156	200	228
ra5		29	20	108	119	166
ra14			6	84	ND	ND
ra18				79	ND	ND
ra20					79	ND
ra21						207

ND = not determined.

Strains carrying *ra5*, *ra14* and *ra18* also exhibited a slight Dpy phenotype, and those carrying the *ra5* and *ra14* alleles were Him, producing approximately 5% male progeny. To determine whether the Him phenotype was due to a mutation in the *tra-3* locus (HODGKIN and BRENNER 1977) which maps near the suppressor alleles, these males were mated to wild-type hermaphrodites to determine whether they were fertile. Although their fertility was well below that of wild-type males, they did produce offspring, indicating that they were not carrying a *tra-3* mutation. Like their hermaphrodite siblings, the gonads of these males appeared variably abnormal when observed under Nomarski microscopy (data not shown).

The fact that all of the suppressor mutations mapped to the same location on LG IV suggested that they might be alleles of the same gene. The low fecundity of the *ra5* and *ra14* alleles was found to be semidominant. Homozygous *ra5* and *ra14* hermaphrodites had, on average, less than 20 progeny each and exhibited a high incidence of sterility (>10%). Heterozygous animals, on the other hand, produced more than 140 progeny each (Table 3) and were rarely sterile. Using this phenotype, we determined that *ra5* and *ra14* failed to complement one another since hermaphrodites heteroallelic for these two mutations produced less than 20 progeny each and were frequently sterile. The *ra21* mutation was also found to be allelic with *ra5* and *ra14* based on the fact that heteroallelic *ra21*/*ra5* mutants produced fewer progeny than homozygous or heterozygous *ra21* animals (Table 3). The *ra18* and *ra20* alleles can also be said to fail to complement *ra5* since *ra18*/*ra5* and *ra20*/*ra5* animals had smaller brood sizes than *ra18*/+ or *ra20*/+ animals. However, unlike the *ra21* allele, heteroallelic *ra18*/*ra5* and *ra20*/*ra5* animals had greater brood sizes than homozygous *ra18* or *ra20* mutants. The locus defined by these mutations has been designated *sup-38*.

Allele specificity of suppressors: The allele specificity of the five suppressor mutations described above was determined as described in MATERIALS AND METHODS. The *ra5* and *ra14* mutations suppress the *e998*, *e444*, *e669am*, *st196::*Tc1, *e1012*, *e1421* and *e669su250*ts alleles of *unc-52*. These results are based on the absence of DpyUnc progeny produced in the F_2 generation of *unc-52*/+;*sup-38 dpy-4*/++ hermaphrodites. In all cases, suppression by these two alleles was semidominant. Homozygous *unc-52* hermaphrodites carrying two copies of *ra5* or *ra14* were able to move better than those with a single copy of the suppressor mutation, but movement was significantly improved even with one copy of the suppressor. The three suppressors derived from *e1421* screens (*ra18*, *ra20* and *ra21*) appear to be specific to that allele, since DpyUnc animals were always seen in the F_2 progeny at the expected ratio when these suppressors were tested with the *e998*, *e444*, *e669am*, *st196::*Tc1, *e1012* and *e669su250* alleles. These three suppressor alleles were completely dominant in the *e1421* background, and there was no visible difference in phenotype when one or two copies of the suppressor alleles were present.

The *ra5*, *ra14*, *ra18*, *ra20* and *ra21* alleles were also tested for their ability to suppress a class 2 allele, *unc-52(st549)*, by crossing class 1 *unc-52*/+;*sup-38 dpy-4*/++ males with hermaphrodites carrying *st549* (*unc-52(st549)*/*unc-52(st549)*/*mnDp34*). Dpy hermaphrodites were selected in the F_2 generation and then outcrossed to determine whether any were homozygous for the *st549* mutation. It was expected that one quarter of these Dpy worms would be homozygous for the lethal mutation if suppression was occurring. None of the alleles tested appeared to suppress the lethal phenotype of *st549* since *unc-52(st549)*;*sup-38 dpy-4* adult animals were never found in the F_2 generation. Similar crosses were also done to test the *ra5* and *ra14* suppressor alleles with the class 3 mutation, *unc-52(ut111)*. In this case heterozygous *unc-52(ut111)*/+ males were mated to homozygous *sup-38dpy-4* hermaphrodites. No *unc-52(ut111)*;*sup-38 dpy-4* progeny were seen in the F_2 generation of these crosses either, indicating lack of suppression.

The same five suppressors were also tested against three other muscle-affecting genes to determine whether they were capable of suppressing different aspects of muscle disorganization. To do this, *sup-38 dpy-4*/++ males were mated with hermaphrodites homozygous for *unc-23(e25)V*, *unc-54(st1008)I*, or *unc-112(r367)V*. The F_2 progeny of these crosses were screened for the presence of DpyUnc hermaphrodites and, in each case, the expected ratio of 1/16 DpyUncs was observed, indicating lack of suppression.

Elimination of suppressor activity: Since the suppressors arose at such a low frequency ($\sim 1.3 \times 10^{-7}$) in our *unc-52* reversion screens, it was not likely that any of these alleles were the null state of the gene. We attempted to isolate loss-of-function alleles in order to determine the normal function of the suppressor locus. We reasoned that the induction of a null

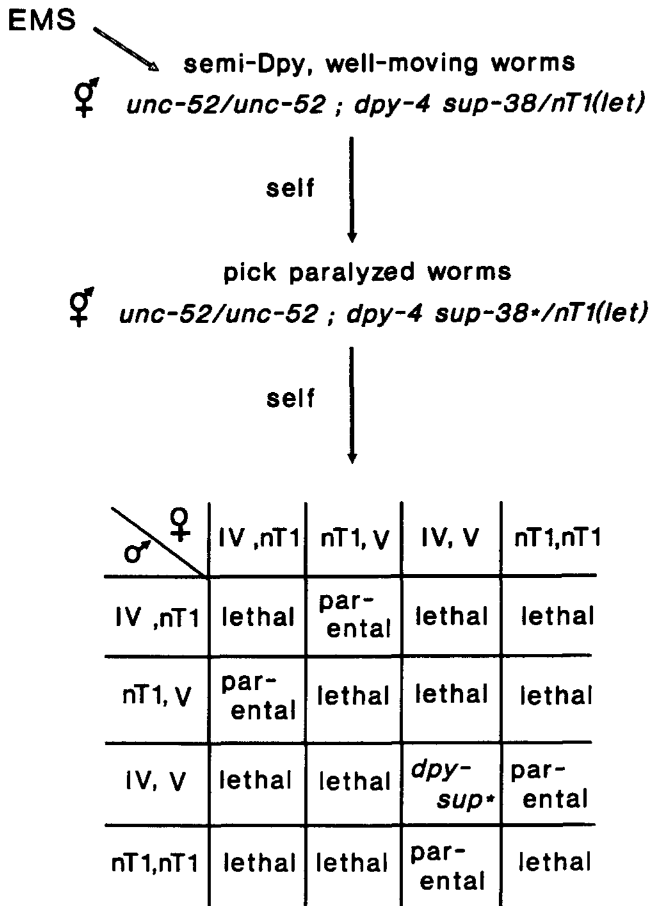

FIGURE 4.—Protocol used to isolate loss-of-function alleles of *sup-38*. Asterisk indicates loss of suppressor activity.

mutation in a gene already carrying a suppressor allele should eliminate the dominant suppressor phenotype. The strain constructed for this experiment was homozygous for an *unc-52* mutation and heterozygous for the suppressor in cis with *dpy-4*, over a recessive lethal balancer chromosome (*unc-52/unc-52;sup-38 dpy-4/nT1[let(m435)IV;V]*) (see Figure 4). Because the suppressor mutations were dominant, these worms were well moving and semi-Dpy, and they segregated both semi-Dpy and Dpy progeny. After mutagenesis, the F_1 progeny of these hermaphrodites were screened for the return of the paralyzed phenotype which indicated loss of the suppressor function. This screen allowed the recovery of putative null *sup-38* alleles regardless of whether the phenotype of these mutations was Wild, Unc or Let.

Approximately 240,000 animals were screened as described above, and nine strains of paralyzed worms were isolated (Table 4). The initial analysis was complicated by the fact that most of the strains isolated carried more than one new mutation, presumably because the nT1 translocation allowed the recovery of mutations anywhere in the balanced region (covering approximately 50 map units). Mutations that did not affect *sup-38* were removed from the strains as described in MATERIALS AND METHODS, and the *sup-38 dpy-4* chromosomes carrying new *sup-38* mutations were then rebalanced over nT1.

Four of the nine strains regained suppressor activity after outcrossing and were not analyzed further (see MATERIALS AND METHODS and Table 4). Four strains appeared to have lost suppressor activity completely, suggesting that second-site mutations had been induced in the *sup-38* locus. Suppressor activity in the remaining strain (*ra5ra55*) appeared to have been reduced, but not eliminated since suppression became recessive instead of dominant (see Table 4). The phenotypes of these five putative *sup-38* alleles were very similar. Homozygous *sup-38 dpy-4* hermaphrodites produced by heterozygous parents were well moving, large Dpys. These animals produced only very small, Dpy progeny which died at the L4 stage or, if they reached adulthood, produced few or no progeny. Thus, these strains exhibited a maternal effect lethal (Mel) phenotype. Strains carrying *ra5ra60* or *ra5ra61* mutations were quite strict Mels, while the *ra5ra55*, *ra14ra65* and *ra14ra66* mutations were leaky and caused only a significant reduction in brood size. Dpy progeny obtained from *dpy-4 sup-38*(*ra5ra61*)/nT1 or *dpy-4 sup-38*(*ra14ra65*)/nT1 hermaphrodites were mated to wild-type males to determine whether the lethal phenotype exhibited by these mutants was a strict maternal-effect. The heterozygous progeny of these animals were not Mel, indicating that a wild-type copy of *sup-38* in the zygote rescued the maternal phenotype. Another characteristic common to all of the *sup-38*(Mel) alleles was the maternal effect enhancement of the Dpy-4 phenotype. Animals carrying a *sup-38* mutation in cis with *dpy-4* were much shorter than mutants carrying the *dpy-4* mutation on its own. Sup-38 Mel mutants displayed no gross abnormalities when observed using Nomarski microscopy, and had completely wild-type muscle structure when examined under polarized light.

Based on the reduced brood sizes of these strains, complementation tests were done as described in MATERIALS AND METHODS, and the results obtained indicated that all five mutations were alleles of a single locus. Animals heteroallelic for any of the putative null alleles were very small Dpys which exhibited the Mel phenotype typical of homozygous loss-of-function mutants.

The maternal effect lethal phenotype of the null suppressor mutations was used to determine their map position relative to *dpy-4*. Homozygous DpyUnc-22 recombinants obtained from + *sup-38*(Mel) *dpy-4/unc-22*++ hermaphrodites were tested to determine whether any of them exhibited a maternal effect lethal phenotype. If the suppressor locus was to the right of *dpy-4* then all of the DpyUnc recombinants should have been Mel. However, five out of the 29 recom-

TABLE 4

Screen for Sup-38 loss-of-function mutants

Suppressor allele	No. of chromosomes screened	No. of Uncs isolated	Phenotype	No. of Sup-38 nulls or hypomorphs
ra5	80,000	1	Carries lethal near *dpy-4* still has suppressor	3
		1	Suppressor is now recessive (and Mel)	
		2	Carries lethal on LGV lost suppressor (now Mel)	
ra14	40,000	1	Carries lethal on LGV still has suppressor	2
		2	Carries lethal near *dpy-4* lost suppressor (now Mel)	
ra18	40,000	1	Carries lethal on LGV still has suppressor	0
ra21	80,000	1	Carries lethal near *dpy-4* still has suppressor	0

binants were not, confirming that the Mel mutations lie to the left of *dpy-4*, in the same region as the Sup mutations.

The ability of *ra61* and *ra65* to complement two essential genes in this region, *let-323* and *let-324* indicated that the *sup-38* alleles represent a newly defined genetic locus (see MATERIALS AND METHODS).

DISCUSSION

Mutations in the *unc-52* locus of *C. elegans* have been divided into three groups based on their mutant phenotypes and complementation patterns. Class 1 mutations result in animals which become progressively paralyzed as they mature (BRENNER 1974). The degree of final paralysis and the stage of its onset vary depending upon the allele and are correlated with a disorganization of the muscle sarcomeres at the cellular level. We have observed that worms carrying class 1 alleles also exhibit variably disorganized somatic gonads and have greatly reduced brood sizes. This may be due to the fact that the sheath cells which surround the proximal arm of the gonad are myoepithelial cells (HIRSH, OPPENHEIM and KLASS 1976) which contain some of the same components as the body wall muscle cells (FRANCIS and WATERSTON 1991). The *unc-52* gene product may, therefore, be important to both of these types of contractile cells.

A second class of mutation at the *unc-52* locus, represented by the allele *st549*, causes homozygous animals to arrest at the twofold stage of embryogenesis before elongation is complete. Mutants of this class show an almost complete lack of movement even before elongation ceases (B. WILLIAMS, personal communication). A class 3 mutation, *ut111*, also results in embryonic lethality, and embryos homozygous for this mutation also fail to elongate beyond the twofold stage, although they do hatch and are capable of some movement (K. KONDO and I. KATSURA, personal communication). PRIESS and HIRSH (1986) have shown that cytoskeletal elements of the hypodermis are responsible for generating the circumferential tension necessary for elongation of the *C. elegans* embryo. The phenotype exhibited by animals homozygous for lethal mutations in many muscle-affecting genes is consistent with the idea that muscle function is also necessary for elongation beyond the twofold stage (BARSTEAD and WATERSTON 1991; B. WILLIAMS and R. H. WATERSTON, personal communication). It is not surprising, therefore, that mutants homozygous for lethal mutations in *unc-52* fail to elongate since the product of this gene appears to be involved in the interaction between muscle cells and the extracellular matrix which connects them to the hypodermis.

The results of complementation tests with the various *unc-52* mutations have revealed that class 1 and class 3 alleles complement each other almost fully. Class 1/*ut111* animals exhibited a slight disorganization of the body-wall muscles, but young animals were essentially wild-type in size and ability to move. Analysis of *unc-52* cDNAs indicates that several different transcripts are produced from this gene (T. ROGALSKI and D. MOERMAN, unpublished results) and this may explain the complementation pattern we have observed. Since the *st549* allele produced the most severe phenotype and failed to complement all other alleles, it may represent the null state of *unc-52*. The class 1 and class 3 mutations, on the other hand, may affect either different, specific gene transcripts or different domains within a single Unc-52 polypeptide. We have identified over 20 intragenic revertants of the class 1 alleles in screens for suppressors of these mutations. Further analysis of these revertants should contribute to our understanding of the *unc-52* locus.

The *e1421* allele behaved differently from other class 1 alleles in complementation tests with the *st549* and *ut111* alleles. When *e1421* was maternally inherited in these complementation tests, the results were similar to those obtained using other class 1 alleles. However, when *e1421* was inherited from the male, *st549/e1421* animals were early larval lethals which resembled *ut111* homozygotes in phenotype. The *ut111/e1421* heteroallelic animals from analogous crosses were also lethal, but these animals did not die until the late larval stages or early in adulthood. This paternal effect was not seen with any of the other alleles and the cause of it is unclear at this point. The

results of reversion studied revealed that there are also other differences between *e1421* and the other class 1 alleles (see below).

The *unc-52* protein must play an essential role in development since some mutations at this locus result in embryonic lethality. It must also be involved in maintaining the normal structure of the muscles and the somatic gonad in adult worms since class 1 mutations in the gene result in disruption of these tissues. Sequence analysis (T. ROGALSKI and D. MOERMAN, unpublished results) indicates that *unc-52* encodes a protein with considerable similarity to the mouse basement membrane heparan sulfate proteoglycan, perlecan (NOONAN *et al.* 1991). The basement membrane appears very early during embryogenesis in all higher organisms, and is known to be important in the development and maintenance of most tissues (MARTIN and TIMPL 1987). The fact that the Unc-52 protein in *C. elegans* is localized to the basement membrane but affects the integrity of the internal structure of the muscle cells allows us to examine the role of the basement membrane *in vivo* in determining and maintaining the internal structure of the cells or tissues it underlies.

The conservation of extracellular matrix proteins between higher organisms and a genetically manipulable system like *C. elegans* has provided a system for identifying other molecules which are involved in the formation and maintenance of this structure. Mutations in a previously unidentified locus, *sup-38*, have been isolated and shown to suppress the paralysis typical of class 1 mutations in *unc-52*. The *sup-38* mutations suppressed the disorganized muscle phenotype of the class 1 mutations, but did not suppress the gonad disorganization associated with these alleles, nor did they suppress the lethal alleles of *unc-52*. A total of nine suppressor mutations have been mapped to LG IV(R). Three of these, *ra5*, *ra14* and *ra21*, have been shown to be allelic, and two other mutations, *ra18* and *ra20* are most likely alleles of *sup-38* as well. Three of the five intergenic suppressors, *ra18*, *ra20* and *ra21*, are allele-specific and will only suppress *unc-52(e1421)*, whereas *ra5* and *ra14* are able to suppress all of the class 1 alleles. The specificity of the *e1421*-specific suppressors is not just a reflection of the fact that *e1421* is one of the mildest *unc-52* alleles because they failed to suppress *e669su250* which is even milder. All five suppressor alleles confer a similar phenotype, with mutant hermaphrodites exhibiting a slightly fragile muscle structure and variable gonad disorganization. The *ra18*, *ra20* and *ra21* alleles result in a milder phenotype than the other two alleles and are dominant suppressors, whereas *ra5* and *ra14* are semidominant. Mutants of genotype *ra5*/+ or *ra14*/+ are healthier and have larger brood sizes than homozygous mutant worms (Table 3), but still exhibit some gonad and muscle disorganization, and have a more severe phenotype than *ra18*, *ra20* or *ra21* homozygous animals (data not shown).

The suppressor alleles of *sup-38* are not likely to represent the null state of the gene because they arose at an extremely low frequency (1.3×10^{-7}). We have isolated four loss-of-function alleles of *sup-38* by selecting for loss of suppressor activity. The frequency of this type of mutation was approximately one in 30,000 (excluding the *e1421*-specific suppressor screens), indicating either that *sup-38* is a relatively small target for EMS mutagenesis (BRENNER 1974), or that these are not null alleles of the locus. Alleles of this type show a maternal effect since the late larval lethal phenotype was only seen in progeny from homozygous *sup-38* hermaphrodites. A fifth mutation isolated in these screens results in loss of the dominant suppressor function, so that worms carrying this new mutation are now only capable of suppressing the class 1 *unc-52* mutant phenotype when they are homozygous. Mutants homozygous for this recessive suppressor mutation also display the Mel phenotype typical of the putative *sup-38* null mutants, but occasionally homozygous worms mature and produce offspring. Two of the other strains also exhibited this leaky Mel phenotype and may, therefore, also be hypomorphs rather than complete loss-of-function mutants. The lack of stable deficiencies for this region has prevented a thorough genetic analysis of these mutations. Surprisingly, the body wall muscles of worms homozygous for the null, or loss-of-function mutations were completely wild type, indicating that the *sup-38* gene product is not essential for normal muscle structure. The fragile sarcomere organization seen in the suppressor mutants may, therefore, be due to abnormal expression of *sup-38*. It is unclear, at this point, whether suppression is due to a *sup-38* gene product interacting with, or simply replacing a mutant *unc-52* gene product. Molecular analysis of the suppressor locus should provide insight into its normal function and its mechanism of suppression of *unc-52*.

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