Embryonic Morphogenesis in *Caenorhabditis elegans* Integrates the Activity of LET-502 Rho-Binding Kinase, MEL-11 Myosin Phosphatase, DAF-2 Insulin Receptor and FEM-2 PP2c Phosphatase

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ABSTRACT

let-502 rho-binding kinase and *mel-11* myosin phosphatase regulate *Caenorhabditis elegans* embryonic morphogenesis. Genetic analysis presented here establishes the following modes of *let-502* action: (i) loss of only maternal *let-502* results in abnormal early cleavages, (ii) loss of both zygotic and maternal *let-502* causes elongation defects, and (iii) loss of only zygotic *let-502* results in sterility. The morphogenetic function of *let-502* and *mel-11* is apparently redundant with another pathway since elimination of these two genes resulted in progeny that underwent near-normal elongation. Triple mutant analysis indicated that *unc-73* (Rho/Rac guanine exchange factor) and *mlc-4* (myosin light chain) act in parallel to or downstream of *let-502/mel-11*. In contrast *mig-2* (Rho/Rac), *daf-2* (insulin receptor), and *age-1* (PI3 kinase) act within the *let-502/mel-11* pathway. Mutations in the sex-determination gene *fem-2*, which encodes a PP2c phosphatase (unrelated to the MEL-11 phosphatase), enhanced mutations of *let-502* and suppressed those of *mel-11*. *fem-2*'s elongation function appears to be independent of its role in sexual identity since the sex-determination genes *fem-1*, *fem-3*, *tra-1*, and *tra-3* had no effect on *mel-11* or *let-502*. By itself, *fem-2* affects morphogenesis with low penetrance. *fem-2* blocked the near-normal elongation of *let-502*; *mel-11* indicating that *fem-2* acts in a parallel elongation pathway. The action of two redundant pathways likely ensures accurate elongation of the *C. elegans* embryo.

THE Rho family of Ras-like GTPases has been implicated in the regulation of the actin cytoskeleton, resulting in altered cell shapes, movements, and cytokinetic events (for reviews see Van Aelst and D'Souza-Schorey 1997; Hall 1998; Mackay and Hall 1998; Aspenström 1999; Kaibuchi *et al.* 1999). Downstream effectors of Rho-GTPases include the Rho-binding kinases, which have been characterized by binding assays *in vitro*, by *in vivo* cell culture transfection experiments, and by mutational analyses in *Caenorhabditis elegans*. In mammalian fibroblast cells two Rho-binding kinases, ROKα (Rho-associated kinase type α) and $p160^{ROCK}$ (Rho-associated kinase), promote the formation of stress fibers and focal contacts, whereas dominant-negative forms cause disassembly of these structures (Leung *et al.* 1995, 1996; Fujisawa *et al.* 1996; Amano *et al.* 1997; Ishizaki *et al.* 1997).

In addition to regulating actin organization within cells, Rho-binding kinases are involved in smooth muscle contraction. Actin-myosin contractions result when regulatory myosin light chains (MLC) are phosphorylated by myosin light chain kinase (MLCK). Myosin phosphatase PP1c holoenzyme blocks these contractions by dephosphorylating MLC, which thus antagonizes MLCK leading to muscle relaxation (Allen and Walsh 1994; Hartshorne 1998; Hartshorne *et al.* 1998). Phosphorylation of the regulatory targeting subunit of myosin phosphatase (M110, M130, and M133) by ROKα and $p160^{ROCK}$ decreases the activity of myosin phosphatase toward MLC, resulting in the accumulation of MLCK-phosphorylated MLC and a contractile response (Shimizu *et al.* 1994; Ichikawa *et al.* 1996; Kimura *et al.* 1996). The Rho-binding kinases also phosphorylate MLC directly to induce contraction *in vitro* (Amano *et al.* 1996; Kureishi *et al.* 1997; Feng *et al.* 1999).

The ability of Rho-binding kinases to phosphorylate and thereby negatively regulate myosin phosphatase and hence to positively regulate MLC-mediated contraction, suggests a model where Rho-binding kinases can mediate cell shape changes by altering the contractile state of actin/myosin filaments. However, this model is based on evidence from cell culture, smooth muscle preparations, and purified proteins. We previously described a role for the *C. elegans* homologs of Rho-binding kinase, *let-502*, and the regulatory subunit of myosin phosphatase, *mel-11*, in regulating the epidermal cell shape changes that drive elongation of the embryo (Wissmann *et al.* 1997, 1999). This provided *in vivo* evidence that Rho-binding kinases and myosin phosphatases interact to regulate actin-mediated cell shape changes. *C.*

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elegans is thus a useful system to study this pathway, particularly for using genetic methods to identify new components. Genetic methods can identify redundant pathways that would be difficult to find by biochemical approaches.

The *C. elegans* embryo undergoes a fourfold increase in length without additional cell proliferation or increase in cell volume, and this occurs through actin-mediated contractions (Priess and Hirsh 1986). *let-502* mutations result in elongation defects or adult sterility, depending on the allele used (Howell and Rose 1990; Wissmann *et al.* 1997, 1999). Loss of *mel-11* function results in embryonic arrest due to hypercontraction during elongation (Kemphues *et al.* 1988; Wissmann *et al.* 1997, 1999). Mutations of *let-502* and *mel-11* suppress one another's elongation defects, implying that *let-502* and *mel-11* function antagonistically during elongation. *mlc-4*, a *C. elegans* MLC gene, is also expressed in the epidermis at this time, and mutants display elongation defects similar to those of *let-502* mutants (Shelton *et al.* 1999). Our genetic results and the analogies to smooth muscle contraction suggest that MEL-11 may prevent contraction of the epidermal cells prior to elongation by negatively regulating MLC-4. At the appropriate time, LET-502 negatively regulates MEL-11 to relieve the inhibition of MLC-4. This allows contraction of the circumferentially oriented microfilaments in the epidermal cells, causing the cells to change shape, driving elongation of the embryo (Priess and Hirsh 1986; Wissmann *et al.* 1997, 1999).

Several issues were raised by our previous work. In smooth muscle, contraction can be induced by either MLCK or Rho-binding kinase. Therefore, *let-502* Rho-binding kinase (or MLCK) might be redundant for elongation. Indeed, all previously identified *let-502* alleles had gain-of-function (*gf*) properties (Wissmann *et al.* 1997). While these likely represent dominant-negative (antimorphic) mutations, the phenotypes might instead reflect neomorphic activities. In the latter case, the *let-502* loss-of-function (*lf*) phenotype would be wild type (*i.e.*, the gene would be redundant or otherwise nonessential), explaining why only *gf* mutations were found in previous screens for visible phenotypes (lethality or sterility). Alternatively, the high frequency of *gf* mutations could reflect an underlying protein structure that was prone to mutating to *gf* products. In this article, we describe a series of novel *let-502* mutations that were identified using a screen that could isolate *lf* alleles. Analysis of our new mutations, used in conjunction with RNA interference (RNAi), indicates that *let-502* is an essential gene. The zygotic null phenotype is an adult sterile, but hypomorphic and dominant-negative alleles indicate that *let-502* also has essential functions during the early embryonic cleavages and morphogenesis.

Another question left unanswered by previous work was the nature of the cosuppression between *let-502* and *mel-11*. The *let-502; mel-11* double mutants undergo near-normal elongation even though the individual mutations are elongation defective (Wissmann *et al.* 1997). This could imply that the pathway as a whole is redundant; phenotypes arise when only one or the other gene is not functioning. In analogy with vertebrate contractile systems, elongation could be triggered, and properly regulated, entirely through an MLCK-dependent system when the Rho-binding kinase (*let-502*) and myosin phosphatase (*mel-11*) pathway is inoperative. Alternatively, the observed mutual suppression between *let-502* and *mel-11* could result from equally low (but nonzero) levels of the two products. The analysis reported here of *let-502* and *mel-11* double mutants coupled with RNAi indicates that the elongation pathway is redundant. Furthermore, the behavior of other mutations that genetically interact with *mel-11* indicate that two genes (*mig-2* Rho/Rac and *daf-2* insulin receptor) function in the same elongation pathway as *let-502* and *mel-11* while others [*unc-73* Rho/Rac guanine exchange factor (GEF) and *mlc-4* MLC] act either downstream or in parallel to the *let-502/mel-11* system.

A surprising result reported here is that both *mel-11* and *let-502* interact genetically with *fem-2* (PP2c phosphatase). A role for *fem-2* in embryonic elongation has not been previously described, even though its essential function in sex determination is well established. *fem-2*(+) leads to sperm production in both *XX* hermaphrodites and *X0* males and male somatic development in *X0* animals (Kimble *et al.* 1984; Hodgkin 1986; Pilgrim *et al.* 1995; Chin-Sang and Spence 1996; for reviews, see Meyer 1997; Schedl 1997). We found that *fem-2* mutations enhanced alleles of *let-502* and suppressed those of *mel-11*. Our genetic results indicate that *fem-2*'s activity during morphogenesis is independent of its role in sex determination and that *fem-2*'s action during embryonic elongation is redundant with the *let-502/mel-11* elongation pathway.

MATERIALS AND METHODS

Strains and alleles: *C. elegans* (N2 var. Bristol) were maintained under standard conditions (Brenner 1974). Strains were constructed using standard procedures, and *cis*-linked morphological markers were often used to follow mutations of *mel-11* and *let-502* through crosses. Homozygous lethal or sterile mutations were maintained as heterozygous stocks balanced either with appropriate crossover suppressors or normal chromosomes with flanking morphological markers. In those cases where the phenotype being scored might be confused with the homozygous balancer (*e.g.*, *unc-11 unc-40* used to balance *let-502* resembles *let-502; mel-11*), the gene of interest was maintained over a fully wild-type chromosome. To determine hatching rates of different genetic combinations, four or more L4 hermaphrodites were brooded at the appropriate temperatures until they ceased to lay fertilized embryos; a minimum of 400 progeny were scored unless otherwise noted (Mains *et al.* 1990).

To avoid effects on the sexual identity of temperature-sensitive (*ts*) sex-determination mutations, the animals of interest were reared at the permissive temperature of 15° and upshifted

to 20° or 25° as young adults (*i.e.*, after sexual identity was established). These animals were then purged of embryos fertilized prior to upshift by incubation for ≥2 hr (25°) or ≥3 hr (20°) before brood collection commenced.

*fem-3(e2006*ts*)* hermaphrodites quickly stop laying fertilized embryos upon upshift to 25°. To quantify the effects of this mutation on embryonic viability, gravid animals that had been raised at 15° were transferred to a drop of room-temperature water, pregastrulation embryos (<2 hr postfertilization, fewer than 28 cells) were removed from the hermaphrodite by dissection with a scalpel, and embryos were placed on a petri dish preequilibrated to 25°. Since the temperature-sensitive period for *mel-11* begins ~6 hr postfertilization (Wissmann *et al.* 1999), embryos shifted in this manner are likely comparable to those fertilized within hermaphrodites grown at 25°.

Nomenclature follows that of Horvitz *et al.* (1979). Genes, alleles, and balancer chromosomes listed below were used; descriptions can be found in Hodgkin (1997) and Edgley *et al.* (1995):

Linkage group I: *let-502(ca201*, *ca201sb54*, *sb93*, *sb95*, *sb103*, *sb106*, *sb107*, *sb108*, *sb109)*, *unc-73(rh40)*, *dpy-5(e61)*, *bli-4(e937)*, *daf-16(mgDf50)*.
Linkage group II: *dpy-10(e128)*, *mel-11(it26*ts, *sb55*ts, *sb56)*, *unc-4(e120)*, *sqt-1(sc13)*, *age-1(mg44)*.
Linkage group III: *fem-2(b245*ts, *e2105)*, *mlc-4(or253)*, *daf-2(e1370*ts, *m212*ts*)*.
Linkage group IV: *fem-1(hc17*ts, *e1965)*, *fem-3(e1996*, *e2006*ts, *q20*gf,ts*)*, *dpy-20(e1282)*, *tra-3(e1107)*.
Linkage group V: *tra-1(e1575*gf*)*.
Linkage group X: *lon-2(e678)*, *mig-2(mu28)*.
Balancer chromosomes: The crossover suppressor *hT2 (I;III)* was sometimes used to balance *let-502* and *mnC1 II* was used to balance *mel-11*.

hDf6 is a deficiency on linkage group I that deletes the *let-502* locus (McKim *et al.* 1988).

Isolation of *mel-11* suppressors: To obtain novel *let-502* alleles, we exploited our observation that a deficiency that deletes the *let-502* locus (*hDf6*) dominantly suppresses the *ts* maternal-effect lethality of *mel-11(it26)*, resulting in 8% hatching at 20° *vs.* 0.4% for controls (Wissmann *et al.* 1999). Therefore, a *mel-11* suppressor screen could identify *let-502(lf)* alleles even if such mutations had no phenotype on their own. *mel-11(it26) unc-4 sqt-1/ mnC1* hermaphrodites were mutagenized with 25 mm ethyl methanesulfonate (Brenner 1974), F_1 progeny were allowed to self at the permissive temperature of 15° for 3–4 days and animals were then upshifted to the restrictive temperature of 20° (where ~1% normally hatch). A total of 8000 haploid genomes were screened for plates with substantial hatching. Twenty-seven independent suppressors were obtained in total. Mutations were outcrossed at least twice and mapped using standard genetic methods. Suppressors of *mel-11(it26)* that mapped to LGI were tested for complementation with *let-502(ca201)*. The seven new *let-502* alleles were cycle sequenced from single worms as outlined previously (Wissmann *et al.* 1997).

RNAi: RNAi was performed as previously described (Fire *et al.* 1998). A full-length 4.3-kb cDNA clone of *let-502* (pAW12.7) in pBluescript SK^+ was used to make *let-502* RNA. Primers with T3 and T7 promoter sequences were designed and used to amplify *mel-11* cDNA, with mRNA collected from gravid hermaphrodites as a template (FastTrack 2.0, Invitrogen, San Diego; Titan, one-tube RT-PCR, Roche Diagnostics). Single-stranded RNA was made from the T3 and T7 promoters, respectively (Ambion system, Ambion, Austin, TX). RNA was precipitated and resuspended in RNase-free TE buffer (10 mm Tris, 1 mm EDTA pH 8.0) according to manufacturer's instructions. Double-stranded RNA was made by mixing equal amounts of the complementary strands together, heating at 70° for 15 min, and then incubating at 37° for 30 min. Injections with 10 ng/μl of *let-502* dsRNA were performed in wild-type and *let-502(sb108)* animals as described by Fire *et al.* (1998), and similar concentrations of *mel-11* dsRNA were injected into wild-type and *mel-11(it26)* animals. *let-502* and *mel-11* dsRNA were mixed with both at concentrations of 10 ng/μl and injected into wild-type and *let-502(sb108); mel-11(it26)* animals. Progeny of injected animals were observed by Nomarski optics on a Zeiss Axioplan microscope and flash-photographed using Kodak Techpan film (Rochester, NY) developed at ASA 100. The *C. elegans* myotonic dystrophy protein kinase *(CeDMPK)* homolog is the *C. elegans* gene most similar to *let-502* at the protein level; however, at the DNA level the longest stretch of identity is only 80% over 50 nucleotides. As a control for possible cross-interference between the genes, dsRNA derived from *CeDMPK* (located on cosmid K08B12) was injected into wild-type animals. The cDNA was made by RT-PCR, according to manufacturer's instructions (FastTrack 2.0, Invitrogen; Titan RT-PCR kit, Roche Diagnostics), using primers specific to the *CeDMPK* gene (*C. elegans* Sequencing Consortium 1998). The RNAi phenotype was viable with what appears to be weak body wall muscle and/or epidermal defects that differed from those seen with *let-502*, indicating that the results obtained from the *let-502(RNAi)* were specific.

Testing of feminizing mutations for interactions with *let-502* and *mel-11*: Since loss of *tra-1* function causes transformation into males, we employed a *gf* allele that acts as a dominant feminizer (Hodgkin 1987) to investigate possible maternal effects on elongation. Because these animals are not self-fertile, we crossed *mel-11(it26) unc-4; tra-1(gf)/+* females to *mel-11(it26) unc-4/+* males. Since *mel-11(it26)* shows near-complete zygotic rescue (Kemphues *et al.* 1988), we can use the *mel-11/+* (phenotypically wild type) outcross progeny as a control to determine the relative viability of their homozygous *mel-11 unc-4* (phenotypically Unc) outcross sibs. Using the cross-assay, we found that *tra-1(gf)* resulted in 5.3% viability of *mel-11* homozygous progeny compared to 2.8% *mel-11* viability in the isogenic control without *tra-1*. In contrast, replacing *tra-1(gf)* with *fem-2(b245)* in the female parent increased the viability of *mel-11(it26)* progeny to 16%. Therefore, *tra-1(gf)* showed little or no interaction with *mel-11*. With similar crosses, we found that maternal homozygosity for null alleles of *fem-1(e1965)* or *fem-3(e1996)* that are also phenotypically female (Doniach and Hodgkin 1984; Hodgkin 1986) also had little effect on *mel-11* viability (data not shown).

Microscopy: Animals were mounted on agarose pads and observed with a Zeiss Axioplan microscope using Nomarski optics. The embryos were flash-photographed with Kodak TechPan film, which was developed at 100 ASA.

RESULTS

Establishing the null phenotype of *let-502* and interactions with *mel-11*: Our previous work on *let-502* and *mel-11* left several questions unanswered (Wissmann *et al.* 1997, 1999). These included determining the *let-502* null phenotype and whether *let-502* and *mel-11* function redundantly with another pathway to regulate embryonic elongation. In the following sections, we will first demonstrate that the *let-502(null)* is an adult sterile, but this gene also acts during the early embryonic cleavages and subsequently during embryonic elongation. We will

TABLE 1

Summary of phenotypes and genetic properties of *let-502* alleles

Allele[a]	Phenotype	Results from Table 3: Maternally contributed	Results from Table 3: Paternally contributed	Results from Table 4: *mel-11* suppression	Gene activity
ca201	L1 arrest, occasional Rol as het	*dn*	*dn*	*dn*	
sb109	L1 arrest, occasional Rol as het	*dn*	*dn*	*dn*	Stronger *dn* ↑
h835, *h783*	L1 arrest, occasional Rol as het	*dn*[b]	ND	ND	
sb93	Early-late larval arrest, Ste	ND	ND	ND	
ca201sb54	Ste, Dpy	w-*dn*/null	Null	Null	~Null
h392, *h509*, *h732*	Ste, Dpy	w-*dn*[b]	ND	ND	
sb103	Dpy, Rol, s-Ste, Mel	Null/hypo	w-*dn*/null/hypo	*dn*	
sb106	Rol, s-Ste, *ts* Mel	w-*dn*/null/hypo[c]	w-*dn*/null/hypo[c]	w-*dn*	More WT activity ↓
sb107	Weak Rol, *ts* s-Ste, Mel	w-*dn*/null/hypo[c]	w-*dn*/null/hypo[c]	w-*dn*	
sb108	Occasional *ts* Rol	Hypo	Hypo	Null	

L1 arrest, arrested with little elongation; early-late, a range of elongation defects from L1 arrest to Dpy, Ste adults; Ste, adult sterile; s-Ste, adult semisterile; Mel, maternal-effect lethal segregating unhatched embryos and arrested L1 unelongated larvae; Rol, rolling adult; het, heterozygote; Dpy, dumpy; *ts*, temperature sensitive at 25°; *dn*, dominant negative; w-*dn*, weak dominant negative; hypo, hypomorphic; ND, not determined. WT, wild type.

[a] Underlined alleles were identified in this study. *ca201* was identified by Wissmann *et al.* (1997). Alleles beginning with *h* were isolated by Howell and Rose (1990).

[b] From Wissmann *et al.* (1997).

[c] The genetic character of these alleles shifted more toward *dn* at 25° compared to 20°.

also show that the *let-502/mel-11* pathway is redundant during embryonic elongation. In later sections, we will examine mutations in genes that interact with *let-502* and *mel-11* and show that several are part of the redundant elongation pathway.

Isolation and phenotypes of new let-502 alleles: All previously identified *let-502* alleles were isolated on the basis of their lethal or sterile phenotypes and they behave as *gf* mutations. When homozygous, genetically strong alleles cause arrest during embryogenesis (*ca201*, *h783*, *h835*) while weak alleles result in adult sterility (*ca201sb54*, *h392*, *h509*, *h732*). Although we use the terms "strong" and "weak," even the strongest *gf* mutations are only weakly dominant, producing only low-penetrance morphological defects in heterozygotes. To ascertain the *let-502* null phenotype, new *let-502* alleles were obtained from a suppressor screen of *mel-11(it26)* that could potentially isolate *lf* alleles, including those with no phenotypes on their own (see materials and methods). The properties and our interpretation of the nature of each mutation (as described below) of the new *let-502* alleles, along with the previously identified mutations, are presented in Table 1 (*sb95* and *sb108* have identical nucleotide changes, and so only *sb108* underwent further genetic testing). While two of the new mutations (*sb93* and *sb109*) are similar to previously identified *let-502* alleles in showing elongation defects and adult sterility (Ste), the other alleles are homozygous viable in the absence of the *mel-11* mutation. The latter alleles show variable penetrance and have associated morphological, Ste, and/or maternal-effect lethal (Mel) phenotypes.

In addition to the previously described elongation and sterile phenotypes, *let-502(sb103)* and *let-502(sb106)* displayed similar incompletely penetrant early cleavage defects. Figure 1 shows a wild-type *C. elegans* embryo at pronuclear fusion (A) and after the first two cell divisions (B and C). At 25°, 58% of the progeny from *let-502(sb106)* homozygotes either failed to complete any cleavages (D) or formed cleavage furrows that would then regress (E and F). All of the *let-502(sb106)* embryos that failed to hatch had cleavage defects and died prior to morphogenesis ($n = 20$). The *let-502(sb106)* larvae that hatched all showed defects ranging from arrest as unelongated larvae to adult morphogenetic phenotypes such as dumpy (Dpy) and rolling (Rol). *let-502(sb103)* behaved similarly, with incompletely penetrant cleavage defects, but all of the hatched embryos failed to elongate.

let-502(sb106) and *let-502(sb103)* homozygous mutants had low brood sizes. At 20°, *let-502(sb106)* mutants produced ~30 progeny/hermaphrodite, but brood size increased to ~150 progeny/hermaphrodite by outcrossing to either wild-type or *let-502(sb106)* males. This suggests that *let-502(sb106)* sterility is due to the homozygous hermaphrodite having limited amounts of available sperm, likely related to our previously observed defects in the spermatheca, the sperm storage organ (Wissmann *et al.* 1999).

A strict maternal requirement for *let-502* was found

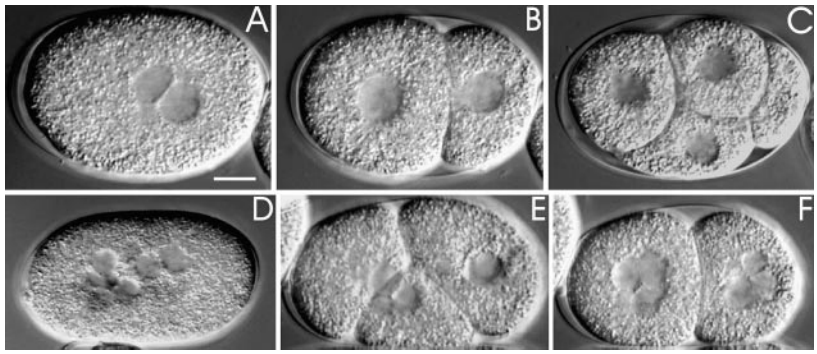

FIGURE 1.—Nomarski photomicrographs of wild-type and mutant embryos at 25°. Shown is a wild-type embryo at (A) pronuclear fusion, (B) the two-cell stage, and (C) the four-cell stage. Nomarski images of *let-502(sb106)* homozygous embryos show (D) an embryo that completely lacks cytokinesis and (E–F) an embryo that formed cleavage furrows, which later regressed. Bar, 10 μm.

in alleles that displayed embryonic lethality. When *let-502(sb103)* or *let-502(sb106)* hermaphrodites were outcrossed to wild-type males, the percentage of unhatched embryos was the same as when hermaphrodites were selfed (Table 2). However, most of the embryos that did hatch grew to morphologically normal adults. Thus, zygotic *let-502*(+) rescued the elongation defects of the hatched animals but not the lethality resulting in unhatched embryos. When *let-502(sb103)*/+ and *let-502 (sb106)*/+ hermaphrodites were selfed, 99% hatching was observed, demonstrating that the failure of embryos to hatch can be also maternally rescued. Taken together, these data indicate that the embryonic viability has a strict maternal requirement for *let-502*(+) while the larval arrest and morphological defects can be rescued by either maternal or zygotic *let-502*(+).

Nature of the new let-502 mutations: We sequenced the new *let-502* alleles and identified their molecular lesions. These mutations are shown in reference to the predicted protein structure in Figure 3, along with the mutations for the previously identified *let-502* alleles. The new *let-502* alleles, including the viable mutations, had missense mutations in conserved amino acids in the kinase domain (Figure 4).

Using genetic tests, we determined whether the new *let-502* alleles behaved as antimorphs (dominant-negatives), nulls, or hypomorphs (MULLER 1932). The classifications were made by examining the progeny that resulted from crossing hermaphrodites heterozygous for a new *let-502* allele to males carrying either a strong *gf* allele (*ca201*), a weak *gf* allele (*ca201sb54*), or a deficiency (*hDf6*) that deletes the *let-502* locus. The most extreme phenotype is early larval arrest, while mid- to late arrest Ste and Mel represent progressively less severe phenotypes. If *b/a* arrests earlier than *b/Df*, then *a* must have *gf* properties (where *a* and *b* are different alleles and *Df* is the deficiency *hDf6*). We will assume that these *gf* properties are dominant negative; this will be confirmed using RNAi in a later section. If *b/a* resembles *b/Df*, then *a* is acting like a null, while a later lethal phase of *b/a* relative to *b/Df* reveals that *a* is hypomorphic since it retains some wild-type activity. If *a/a* is more severe than *a/Df*, then *a* has dominant-negative properties. A less severe *a/a* phenotype compared to *a/Df* indicates that *a* is hypomorphic. Since *let-502* alleles show dominant maternal effects (WISSMANN *et al.* 1997), we performed reciprocal crosses. The results are listed in Table 3 and an overall summary is included in Table 1.

We showed previously that both *ca201* and *ca201sb54* have *gf* (likely dominant-negative) properties, with *ca201* being relatively stronger (WISSMANN *et al.* 1997). This is seen in Table 3 where allelic combinations including *ca201* always showed phenotypes that were equal to, and usually more severe than, those that included the *Df*. This was true whether *ca201* was contributed maternally (column 3 *vs.* 5) or paternally (column 6 *vs.* 8). When maternally inherited (column 4 *vs.* 5),

TABLE 2

Maternal and zygotic rescue of two *let-502* alleles

Temperature	Maternal	Paternal	% hatching	% of hatched that grow to wild-type adults
20°	*sb103*	Self	65	0
20°	*sb103*	N2	81	92
20°	*sb103*/+	Self	99	90
25°	*sb106*[a,b]	Self	41	0
25°	*sb106*[a]	N2	45	93
25°	*sb106*/+	Self	99	93

[a] Shifted to 25° as young adults and then purged for ≥3 hr prior to brooding to remove embryos fertilized at 15°. Upshift prior to adulthood often results in Ste animals.

[b] Total progeny counted were <500 with $n = 252$ for *sb106* selfed at 20° and $n = 312$ for *sb106/hDf6* at 20°.

TABLE 3

Heteroallelic *let-502* phenotypes at 20°

		Maternal[b]			Paternal[b]		
	m/ m[a]	*ca201*	*ca201sb54*	*hDf6*	*ca201*	*ca201sb54*	*hDf6*
ca201	Early	Early	Early-mid	s-Ste	Early	Early	Early
sb109	Early	Early	Early-mid	s-Ste	Early	Early	Early
ca201sb54	Ste	Early	Ste	s-Ste	Early-mid	Ste	s-Ste
sb103	Mel[c]	Early	Mel	Mel	Early-late	Mel	Mel
hDf6	emb Let[d]	Early	s-Ste	emb Let	s-Ste	s-Ste	emb Let
sb106	s-Ste, Rol	Early-mid	Mid-late	s-Ste	Mid-late	s-Ste	s-Ste
sb106 (25°)	Ste, Mel	Early	Early-mid	Early-mid	Early-mid	Mid-late	Mid-late
sb107	Weak Rol	Early-mid	Wild type	Wild type	Mid-late	Wild type	Wild type
sb107 (25°)	s-Ste, Mel	Early	Mid-late	Mid-late	Early-mid	Mid-late	Mid-late
sb108	Wild type	Early-mid	Wild type	Wild type	s-Ste	Wild type	Wild type

Early, L1 arrest; mid, range (L1–L3) of larval arrest; late, Dpy adults.

[a] Phenotypes of homozygotes as listed in Table 1.

[b] Heterozygote hermaphrodites were crossed with heterozygous males and only the heteroallelic progeny were examined.

[c] Embryos arrest prior to morphogenesis or hatch and arrest as unelongated L1.

[d] Deficiency homozygotes arrest as unhatched embryos.

ca201sb54 was more severe than the *Df* when *in trans* to *ca201*, *sb109*, or *sb106* (the latter at 20°), but *ca201sb54* was equivalent to the *Df* for all other alleles. When *ca201sb54* was inherited paternally, it always acted as a null (column 7 *vs.* 8). Thus, *ca201sb54* is similar to a null in most respects, but it behaves as a dominant negative in a few heteroallelic combinations.

sb103, *sb106*, and *sb107* each showed mixtures of dominant-negative, null, and hypomorphic properties depending upon the heteroallelic combination examined. For example, when *sb106/+* males were crossed to *ca201/+* hermaphrodites, the *ca201/sb106* progeny arrested as early to midstage larvae, as compared to *ca201/Df* animals, which consistently arrested as early larvae (line 5 *vs.* 6). Thus, *sb106* retains some wild-type activity and is a hypomorph. In contrast, animals with maternally inherited *ca201sb54* demonstrated that *sb106* has weak dominant-negative characteristics: while *ca201sb54/Df* were semisterile adults, *ca201sb54/sb106* had a slightly more severe phenotype, arresting as mid- to late-stage larvae. Finally, *sb106* behaved as a null when *sb106/sb106* and *sb106/Df* were compared since both led to similar semisterile adult phenotypes. When tests were repeated at 25°, each *sb106* heteroallelic combination showed a slightly earlier arrest (compare lines 6 and 7), indicating that this mutation loses wild-type activity and becomes more dominant negative with increasing temperature. Of the other new mutations, *sb109* consistently acted as a dominant negative in heteroallelic combinations (Table 3, compare lines 2 and 5), while *sb108* was the only allele that clearly behaved as a hypomorph in all tests (Table 3, line 5 *vs.* 10).

RNAi indicates that let-502 is essential: All of the *let-502* alleles have been classified as dominant negatives, nulls, and hypomorphs as summarized in Table 1. The exact null phenotype remains difficult to assign since none of the alleles clearly mimic *hDf6* in all assays. It is possible that removal of zygotic activity results in an adult Ste phenotype like *ca201sb54*, the mutation that most closely approximates the null. Larval arrest would then result from the removal of most maternal and all zygotic *let-502*(+) activity as seen with *ca201*, *i.e.*, the dominant negative *ca201* allele decreases the amount of *let-502*(+) present in the heterozygous hermaphrodite and totally eliminates it from the progeny. Alternatively, the null phenotype could be wild type, similar to *sb108*. If the latter were true, alleles classified as dominant negatives would in fact be neomorphs.

To unambiguously determine the consequences of loss of *let-502* activity we used RNAi, which has been shown to mimic *lf* (often null) phenotypes in *C. elegans* by eliminating maternal and zygotic product (Fire *et al.* 1998). Experiments were performed with double-stranded RNA (dsRNA) obtained from the full-length (4.3-kb) *let-502* cDNA. Embryonic elongation in wild-type animals is shown in Figure 2, A–C, and the *ca201* phenotype is shown in Figure 2D. *let-502* dsRNA injected into wild-type hermaphrodites resulted in embryos that failed to elongate, although a range of phenotypes was observed from early (as shown in Figure 2E) to midlarval arrest, with 10% growing into Ste, Rol, Dpy adults. Therefore, the *let-502(lf)* phenotype is clearly not wild type. The observed phenotypic range could reflect the inefficiency of RNAi or partial *let-502* redundancy. If the RNAi was inefficient, injection of *let-502* dsRNA into hermaphrodites homozygous for the putative hypomorphic *sb108* allele should shift the range of phenotypes to a higher percentage of early larval arrest. This was

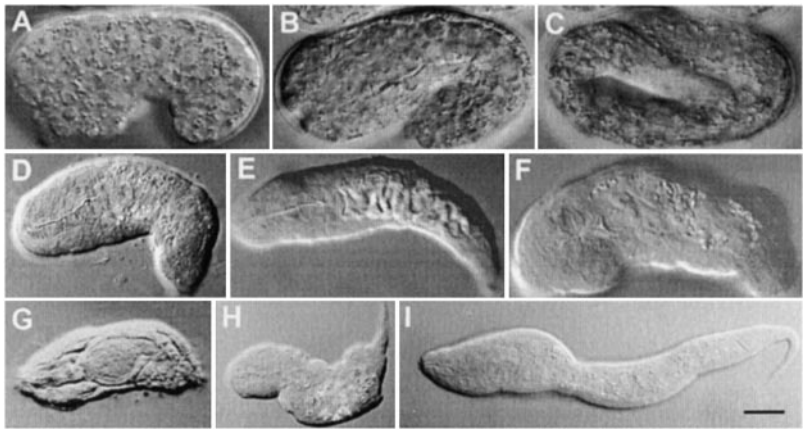

FIGURE 2.—Nomarski photomicrographs of a wild-type embryo in varying stages of embryonic elongation in comparison with *let-502* mutants. A wild-type embryo is shown at (A) early morphogenesis, (B) midmorphogenesis (comma stage), and (C) fully elongated (pretzel stage). (D) Also shown is a Nomarski image of the homozygous *let-502(ca201)* terminal phenotype characterized by the hatched larva arrested with little elongation. *let-502(RNAi)* injected into wild-type hermaphrodites resulted in hatched larva (E) with little elongation. *let-502(RNAi)* injected into *let502(sb108)* hermaphrodites resulted in hatched larva (F) with an elongation defect more severe than that in E. (G) Hatched *let-502(ca201); fem-2(b245)* larva has undergone even less elongation than the *let-502(ca201)* animal shown in D. (I) Hatched *fem-2(e2105)* ceased elongation at a stage similar to that of the *let-502(ca201)* homozygote shown in F. (J) Midstage *fem-2(e2105)* larva shows abnormal elongation in the pharyngeal region. Because of the maternal effect of *fem-2(e2105)*, animals in I and J were the F_2 progeny of heterozygous hermaphrodites. All *fem-2* bearing strains were grown at 25°. Bar, 10 μm.

indeed the case, as dsRNA injected into *let-502(sb108)* animals resulted in all progeny displaying early larval arrest (Figure 2F). Therefore, decreasing maternal and zygotic *let-502*(+) activity results in the larval arrest similar to that seen with the strong dominant-negative alleles. Escapers were Ste and this could represent the zygotic null phenotype.

We occasionally observed injected hermaphrodites that laid unhatched embryos, but these were not examined for the cleavage defects observed in *let-502(sb106)* and *let-502(sb103)*. The lack of consistent embryonic lethality likely indicates that RNAi does not eliminate all maternal *let-502* activity. Embryos with sufficient maternal *let-502*(+) to prevent the early cleavage defects would later fail to elongate properly.

let-502 suppression of mel-11: The genetic properties of the *let-502* alleles were tested further by their suppression of homozygous *mel-11*(*it26)* worms. At 20° *hDf6/+; mel-11(it26)* worms had a small percentage (8%) of progeny that survived compared to 0.4% for the control, indicating partial suppression of the elongation defects. However, as shown in Table 4, most *let-502* alleles were better at suppressing *mel-11(it26)* than the deficiency, as expected for dominant-negative alleles. *sb109*, *ca201*, and *sb103* had the highest levels of suppression as heterozygotes (63–68%). *sb107*, *sb108*, *sb106*, and *ca201sb54* had lower levels of suppression as heterozygotes, but still had higher levels of suppression than *hDf6* (26–37% *vs.* 8%, respectively). However, the comparison of *let-502/+; mel-11* to *hDf6/+; mel-11* is deceptive because while the *let-502* homozygous segregants hatch, *hDf6* homozygotes do not. Therefore, to directly compare the suppression of *mel-11* by *let-502/+* to *hDf6/+*, only the percentage of heterozygous progeny should be considered. Using this metric, *sb108* (11%) and *ca201sb54* (6.8%) are similar to *hDf6* (8.0%) in their ability to dominantly suppress *mel-11(it26)*. The interpretations of these results are summarized in Table 1.

In contrast to the elongation phenotypes, the *let-502* adult sterile phenotype was not suppressed by *mel-11(it26)*. *let-502(ca201); mel-11(it26)* and *let-502(sb109); mel-11(it26)* grew to adulthood due to *mel-11*'s suppression of elongation defects, but the animals were sterile. Similarly, *let-502(sb106); mel-11(it26)* had low brood sizes similar to those seen with *let-502(sb106)* alone at both 20° and 25° (data not shown).

mel-11 can suppress the early cleavage defects of *let-502(sb106)*. This is most clearly seen using the hypomorphic allele *mel-11(sb55)* (WISSMANN *et al.* 1999). By itself, *let-502(sb106)* showed 65% hatching at 20°, but inclusion of *mel-11(sb55)* increased the hatching rate to 91% (Table 4).

RNAi indicates that let-502/mel-11 are nonessential for elongation: The mutual suppression of *mel-11* and *let-502* elongation defects could be due to a balance of equally low residual amounts of *let-502*(+) and *mel-11*(+) activity. Alternatively, the *let-502/mel-11* pathway could be redundant, and a parallel pathway might regulate embryonic elongation in the absence of both *let-502* and *mel-11*. To distinguish between these possibilities, *mel-11* and *let-502* dsRNAs were coinjected into *let-502(sb108); mel-11(it26)* animals, which were then brooded at the nonpermissive temperature of 25°. Since *let-502(sb108)* is hypomorphic and *mel-11(it26)* behaves as a genetic null at 25° (WISSMANN *et al.* 1999), RNAi should further decrease the already limiting amounts of the two gene products. The hatching rate was the same as for uninjected animals (90%), suggesting that normal elongation in *let-502; mel-11* is the double null elongation phenotype.

Characterization of the genetic pathways that contribute to embryonic elongation: Based on analogies with vertebrate systems, there are a number of *C. elegans* candidate genes that are likely to mediate embryonic elongation in concert with *let-502* and *mel-11*. We had previously demonstrated that mutations in *unc-73* and

TABLE 4

***let-502* suppression of *mel-11(it26)* at 20°**

Maternal genotype	% hatching	% *let-502/+* progeny[a]
mel-11(it26)	0.4	—[b]
hDf6/+; mel-11(it26)	8.0	—
let-502(ca201)/+; mel-11(it26)	64	51
let-502(sb109)/+; mel-11(it26)	68	58
let-502(ca201sb54)/+; mel-11(it26)	26	6.8
let-502(sb103)/+; mel-11(it26)	63	38
let-502(sb106)/+; mel-11(it26)	33	14
let-502(sb107)/+; mel-11(it26)	37	18
let-502(sb108)/+; mel-11(it26)	36	11
let-502(ca201); mel-11(it26)	Ste	—
let-502(sb109); mel-11(it26)	Ste	—
let-502(sb103); mel-11(it26)	65[c]	—
let-502(sb103)	65[d]	—
let-502(sb106); mel-11(it26)	52[e]	—
let-502(sb106)	66[d]	—
let-502(sb107); mel-11(it26)	87	—
let-502(sb108); mel-11(it26)	90	—
mel-11(sb55)	65	—
let-502(sb106); mel-11(sb55)	91	—

[a] Inferred using linked morphological markers *in cis* or *trans* that allowed homozygotes and heterozygotes to be distinguished. The heterozygotes are being used for direct comparison to *hDf6* since for the majority of the alleles, except *ca201* and *sb109*, there was an excess of *let-502* homozygotes segregated from heterozygous parents (*i.e.*, assuming that the +/+ sibs die, homozygotes were greater than the expected 1/3 of the survivors).

[b] Not relevant. Strains either lacked or were homozygous for *let-502*.

[c] Of those that hatch, 20% grow to adulthood, compared to 0% for *sb103* single mutant.

[d] From Table 2.

[e] Of those that hatch, all of the progeny grow to adulthood, compared to 88% for the *let-502(sb106)* single mutant.

TABLE 5

Interactions of *let-502* and *mel-11* with *mlc-4* at 20°

Paternal genotype[a]	% hatching	% Mlc-4 progeny
mel-11	0.4[b]	—[c]
mel-11; mlc-4/+	23	23
let-502; mlc-4/+	90	25
let-502; mel-11	90[b]	—
let-502; mel-11; mlc-4/+	90	29

[a] The following alleles were used: *let-502(sb108)*, which is hypomorphic, and *mel-11(it26)*, which is *ts*.

[b] From Table 4.

[c] Not relevant since *mlc-4* was not present.

mig-2 genetically interact with *mel-11* (Wissmann *et al.* 1999). Recently, Shelton *et al.* (1999) showed that *mlc-4* has elongation defects similar to those of strong *let-502* alleles. In the following sections we will determine how these genes precisely fit into the elongation pathway. We will also identify additional genes that genetically interact with *let-502* and/or *mel-11*.

Enhancers of *mel-11* could mediate elongation by acting in the *let-502/mel-11* pathway, either as an activator of *mel-11*(+) or as an inhibitor of *let-502*(+). Alternatively, these genes could act in a parallel elongation pathway. We distinguished these possibilities by building triple mutants with *let-502(ca201)* and *mel-11(it26)*. Addition of a mutation in a gene acting upstream of *let-502* or *mel-11* should not affect the near-normal elongation of the *let-502; mel-11* double. That is, in the absence of both *let-502* and *mel-11*, the additional loss of an upstream gene would be irrelevant. In contrast, mutations acting downstream of the *let-502/mel-11* pathway would block elongation while mutations in genes acting in parallel would prevent morphogenesis because both pathways would be compromised.

mlc-4 is the likely downstream target of let-502 and mel-11: The similar epidermal expression patterns and unelongated phenotypes of *let-502* and *mlc-4* suggest that MLC-4 is the target for the contraction regulated by the LET-502/MEL-11 pathway during elongation (Shelton *et al.* 1999). *mlc-4/+* hermaphrodites segregated one-fourth the unelongated progeny. While few *mel-11(it26)* progeny hatched at 20° due to hypercontraction, the viability was increased to 23% when the hermaphrodite parent was also heterozygous for *mlc-4* (Table 5). All of the survivors exhibited the Mlc-4 unelongated larval arrest phenotype, indicating that the *mlc-4* failure to elongate was epistatic to the *mel-11* hypercontraction. That is, *mlc-4*(+) is required for *mel-11*(−) embryos to hypercontract. Furthermore, *mlc-4* blocked the near-normal elongation characteristic of *let-502; mel-11*, which indicates that *mlc-4* acts in parallel to or downstream of *let-502* and *mel-11*. Based on analogies with other systems, *mlc-4* is likely downstream.

unc-73 functions in a pathway parallel to let502/mel-11: We previously reported (Wissmann *et al.* 1999) that *unc-73* (Rho/Rac GEF, Steven *et al.* 1998) enhances the maternal-effect lethality of *mel-11*. Table 6 shows that *unc-73* appears to act downstream or on a branch of the elongation pathway different from *let-502* and *mel-11*. Even at the semipermissive temperature, *unc-73/+; mel-11* segregated only 1.4% Unc progeny, 18-fold less than the expected 25%. Similarly, at the restrictive temperature, the viability of *unc-73 let-502; mel-11* was only 0.4%, 30-fold less than the *let-502; mel-11* control. In this case, the enhancement of *mel-11* by *unc-73* was epistatic to *let-502*'s suppression of *mel-11*. Similar results were obtained using the hypomorphic allele *let-502(sb108)* where the hatching rate decreased over 25-fold when *unc-73* was included with *mel-11*, with the few survivors arresting as early larvae (Table 6). Together, these data suggest that *unc-73* acts downstream or in a pathway parallel to *let-502/mel-11*.

mig-2 acts upstream of let-502 and/or mel-11: We also previously reported (Wissmann *et al.* 1999) that *mig-2*

TABLE 6

Interaction of *unc-73* with *let-502; mel-11*

	Temperature			
	15°		25°	
Parental genotype[a]	% hatch	% Unc F_1	% hatch	% Unc or suppressed *let-502* F_1
unc73/+	98	24	—[b]	—
mel-11	7.3	—	0	—
unc-73/+; mel-11	5.1	1.4	0	0
let-502(ca201)/+; mel-11	—	—	55	12
unc-73 let-502(ca201)/+; mel-11[c]	—	—	28	0.4
let-502(sb108); mel-11	—	—	71	—
unc-73 let-502(sb108); mel-11[c]	—	—	2.9[d]	—

[a] The following alleles were used: *unc-73(rh40)* and *mel-11(it26)*. *let-502(ca201)* is a strong dominant negative while *let-502(sb108)* is hypomorphic.

[b] Not determined or not relevant since the strain was homozygous for or lacked the marker in question.

[c] *unc-73* shows no genetic interactions with *let-502* (Wissmann *et al.* 1997; P. E. Mains, unpublished observations).

[d] All hatched embryos arrested as early larvae.

(Rho/Rac-like, Zipkin *et al.* 1997) enhances the maternal-effect lethality of *mel-11*. Table 7 demonstrates that *mig-2* acts in the same branch of the elongation pathway as do *let-502* and *mel-11*. At the semipermissive temperature *mig-2* causes a 20-fold enhancement of *mel-11(it26)*. At the restrictive temperature, no progeny survived. However, even at the nonpermissive temperature, the viability of *let-502(ca201); mel-11; mig-2* was decreased <2-fold compared to *let-502(ca201); mel-11*. Therefore, the suppression of *mel-11* by *let-502* was epistatic to the enhancement of *mel-11* by *mig-2*, consistent with *mig-2* acting in the *let-502/mel-11* pathway. We performed similar experiments with the hypomorphic allele *let-502(sb108)*. As shown in Table 7, *let-502(sb108); mel-11; mig-2* showed only a small decrease in hatching compared to the corresponding strain lacking *mig-2*. Although the interpretation is clearer using the elongation null *let-502(ca201)*, use of the hypomorphic mutation *sb108* leads to the same conclusion that *mig-2* is acting upstream of *let-502* and/or *mel-11*.

daf-2 acts in the let-502/mel-11 pathway: In other systems, the insulin receptor pathway acts upstream of the Rac (Nobes *et al.* 1995) and Rho (Ohan *et al.* 1999) signal transduction cascades. To determine the effects of the *C. elegans* homologs of these genes on embryonic elongation, we built double mutants between *mel-11* and the *daf-2* gene that encodes a *C. elegans* insulin-like receptor (Kimura *et al.* 1997). *daf-2* acts in genetic pathways affecting dauer formation and life span (see Kenyon 1997 and Riddle and Albert 1997 for reviews; Gems *et al.* 1998). While *daf-2(e1370)* had modest effects on *mel-11(it26)*, it strongly enhanced *mel-11(sb55)* hypomorphic mutations, decreasing the hatching rate nearly 50-fold at 25° (Table 8). *daf-2(m212)* strongly enhanced *mel-11(it26)*, decreasing hatching nearly 30-fold at 15° (Table 8). *daf-2* mutations did not genetically interact with *let-502* alleles (data not shown).

To determine if *daf-2* is functioning in the context of other parts of the dauer/longevity pathways when it enhances *mel-11*, we examined the effects of mutations of other genes in the *daf* pathway. The gene *age-1*, which encodes a phosphatidylinositol-3-OH kinase (PI3 kinase, Morris *et al.* 1996), ordinarily acts downstream of *daf-2*. As shown in Table 8, *age-1* strongly enhanced *mel-11(it26)*. *daf-16* (Ogg *et al.* 1997) suppresses the dauer and longevity phenotypes of *daf-2*, but showed only a weak genetic interaction with *mel-11* alone (Table 8). However, *daf-16* partially suppressed the enhancement of *mel-11* by *daf-2(m212)*. Therefore, *mel-11* genetically interacts with several members of the dauer/longevity pathways.

To determine if the *daf-2* genetic interaction occurs upstream of *let-502/mel-11* or in a parallel pathway, we examined the *let-502(ca201); mel-11; daf-2* triple. As shown in Table 8, *daf-2* did not affect the viability of this genotype relative to *let-502; mel-11*, indicating that *daf-2* acts upstream of *let-502/mel-11*. Similar results were obtained using the *let-502(sb108)* hypomorph, where viability showed a modest (twofold) decrease in the presence of *daf-2* relative to *let-502(sb108); mel-11*.

fem-2 functions in a pathway parallel to let-502/mel-11: While constructing self-sterile strains for other purposes, we noted that *fem-2* enhanced both dominant and recessive phenotypes of the strong dominant-negative allele *let-502(ca201)*: all *ca201/+; fem-2* progeny showed elongation defects, ranging from early arrest to Dpy, lumpy larva and adults, while *let-502(ca201); fem-2* homozygotes underwent even less elongation than *let-*

TABLE 7

Interaction of *mig-2* with *let-502; mel-11*

	Temperature		
	15°	25°	
Parental genotype[a]	% hatch	% hatch	% suppressed *let-502* segregants
mig-2	99[b]	90	—[c]
mel-11	21	0	—
mig-2; mel-11	1.1	0	—
let-502(ca201)/+; mel-11	—	52	14
let-502(ca201)/+; mel-11; mig-2	—	50	8.2
let-502(sb108); mel-11	—	71	—
let-502(sb108); mel-11; mig-2	—	53	—

[a] The following alleles were used: *mig-2(mu28)* and *mel-11(it26)*. *let-502(ca201)* is a strong dominant negative while *let-502(sb108)* is hypomorphic.
[b] From Wissmann *et al.* (1999).
[c] Not determined or not relevant since the strains either lacked or were homozygous for *let-502*.

502(ca201) (Figure 2G). This was true for both a *ts* allele, *fem-2(b245)*, and a genetic null allele, *fem-2(e2105)* (Table 9, lines 1–3; Hodgkin 1986; Pilgrim *et al.* 1995).

Other mutations of *let-502* were similarly enhanced by *fem-2*, indicating a lack of allele specificity. In the presence of *fem-2*, the weak/null allele *let-502(ca201sb54)* arrest was shifted from adult Ste to early to midstage larvae (Table 9, lines 4–5). When the phenotypically wild-type hypomorph *let-502(sb108)* was combined with *fem-2*, all larvae arrested with elongation defects (lines 6–8).

The enhancement of *let-502* by *fem-2* involved both maternal and zygotic activities of the two genes. Mating *let-502(sb108); fem-2(b245)* hermaphrodites to either *let-502(−); fem-2(+)* or *let-502(+); fem-2(−)* males resulted in partial rescue of the elongation defects in the resulting progeny (Table 9, lines 9–10); complete rescue was seen upon mating to wild-type males (line 11).

Although sexual phenotypes of *fem-2* are well characterized, defects in morphogenesis have not been described. We found that both of the *fem-2* alleles examined resulted in low penetrant elongation defects. At 25°, 17% of *fem-2(b245*ts*)* larvae appeared slightly small (Sma), with rounded heads (Table 9, line 12), but this phenotype became less apparent as the animals matured. With the null allele *fem-2(e2105)*, we observed 4% of progeny arresting with a *let-502(ca201)*-like phenotype (Figure 2H) and another 20% of animals arrested from L2 onward, often with a Sma-like phenotype (Figure 2I) or grew to Rol adults (line 13).

Since *let-502* and *mel-11* suppress one another's elongation defects, a strong enhancer of *let-502* would decrease *let-502* activity and should suppress *mel-11*. This was indeed the case. At 20°, *fem-2(b245)* suppressed the *ts* maternal-effect lethality caused by *mel-11(it26)* by over 50-fold (Table 10, lines 1–3). At 25°, 0/1777 *mel-11(it26)* embryos hatched, but the presence of *fem-2* increased this value to 3.1% (61/1955). The genetic null allele *fem-2(e2105)* also suppressed *mel-11* (lines 4–5). *fem-2* suppression of *mel-11* is maternal since there was little

TABLE 8

Interaction of *daf* mutations with *let-502; mel-11*

	% hatching		
Parental genotype	15°	20°	25°
mel-11(it26)	20	—[a]	—
daf-2(e1370)	97	99	98
mel-11(it26); daf-2(e1370)	9.8	—	—
mel-11(sb55)	—	61	18
mel-11(sb55); daf-2(e1370)	—	20	0.4
daf-2(m212)	99	97	100
mel-11(it26); daf-2(m212)	0.7	—	0
age-1[b]	100	—	—
mel-11(it26) age-1[b]	0.4	—	—
daf-16	98	—	—
mel-11(it26); daf-16	9.8	—	—
mel-11(it26); daf-2(m212); daf-16	6.6	—	—
let-502(ca201)/+; mel-11(it26)	—	64[c]	—
let-502(ca201)/+; mel-11(it26); daf-2(m212)	—	—	23 (16)[d]
let-502(sb108); mel-11(it26)	—	—	71[e]
let-502(sb108); mel-11(it26); daf-2(m212)	—	—	35

[a] Not determined.
[b] Because the *age-1* phenotype shows a maternal effect, the F_1 progeny of first generation homozygotes were scored for hatching.
[c] From Table 4.
[d] The hatching rate of 23% is for all progeny; the viability of homozygous *let-502* segregants was 16%. The control values of an isogenic strain lacking *daf-2* were 52% and 14%, respectively (Table 7).
[e] From Table 6.

TABLE 9

Interactions of *let-502* and sex-determination mutations at 25°

Parental genotype[a]	Progeny phenotypes[b]
1. *let-502(ca201)/+*	22% *ca201*-like, occasional (<10%) Rol adult
2. *let-502(ca201)/+; fem-2(b245)*	26% extreme-*ca201*, 50% slightly larger than *ca201* to Dpy adult
3. *let-502(ca201)/+; fem-2(e2105)*[c]	25% extreme-*ca201*, 50% slightly larger than *ca201* to Dpy adult
4. *let-502(ca201sb54)/+*	28% sterile Dpy adult
5. *let-502(ca201sb54)/+; fem-2(b245)*	28% *ca201* to Dpy Lumpy L2/L3
6. *let-502(sb108)*	Wild-type adult, occasional (<10%) Rol
7. *let-502(sb108); fem-2(b245)*	Most *ca201*-like, some Dpy Lumpy L2/L3
8. *let-502(sb108); fem-2(e2105)*[c]	All *ca201*-like
9. *let-502(sb108); fem-2(b245)* × *let-502(sb108)* ♂	All *ca201*-like to Lumpy-Dpy adult (13% adult)
10. *let-502(sb108); fem-2(b245)* × *fem-2(b245)* ♂	All *ca201*-like to Lumpy-Dpy adult (84% adult)
11. *let-502(sb108); fem-2(b245)* × *+/+* ♂	97% wild-type adult
12. *fem-2(b245)*	17% Sma adults
13. *fem-2(e2105)*	4% *ca201*-like,[d] 20% Sma,[d] larval arrest, Rol adult
14. *let-502(ca201)/+; fem-1(hc17*ts*)*	~1/4 *ca201*-like
15. *let-502(ca201)/+; fem-1(e1965)/+*[e]	~1/4 *ca201*-like
16. *let-502(sb108); fem-1(e1965)/+*[e]	All wild-type adult
17. *let-502(ca201)/+; fem-3(e1996)/+*[e]	~1/4 *ca201*-like
18. *let-502(ca201)/+; fem-3(e2006*ts*)*	~1/4 *ca201*-like
19. *let-502(sb108); fem-3(e2006*ts*)*	All wild-type adult
20. *let-502(ca201)/+; fem-3(q20*gf,ts*)*	~1/4 *ca201*-like
21. *let-502(sb108); fem-2(b245); fem-3(q20*gf,ts*)*	All *ca201*-like
22. *let-502(ca201)/+; tra-3(e1107)*[f]	~1/4 *ca201*-like
23. *let-502(sb108); tra-3(e1107)*[f]	All wild-type adult

[a] *ca201*, *dn*; *ca201sb54*, weak *dn*; *sb108*, partial *lf*. For *ts* alleles of sex-determination genes, adult animals were shifted to the nonpermissive 25° as adults to avoid effects of the mutation on parental sexual phenotype.

[b] *ca201*-like and extreme-*ca201* refer to the animals like those shown in Figure 2, D and G, respectively. Lines 14–23 were assessed qualitatively to see if the *ca201* homozygotes were arresting with different phenotypes from controls.

[c] *fem-2(e2105)* shows a maternal effect, so the homozygous *fem-2* progeny of *fem-2/+* animals are self-fertile hermaphrodites, and their progeny display the indicated phenotypes.

[d] See Figure 2, H and I.

[e] These are null alleles of the loci (Doniach and Hodgkin 1984; Hodgkin 1986).

[f] *tra-3* shows a maternal effect, so the homozygous *tra-3* progeny of *tra-3/+* animals are self-fertile hermaphrodites, which produce all pseudomale broods, which were scored.

or no rescue when the hermaphrodite parent was heterozygous for *fem-2* (line 6). Suppression of maternal-effect lethality was also observed for the hypomorphic *mel-11(sb55)* mutation (lines 7–8). The putative null allele *mel-11(sb56)* is an adult sterile due to spermathecal defects (Wissmann *et al.* 1999). This phenotype was not suppressed by *fem-2* (data not shown).

In the triple mutant *let-502(ca201)/+; mel-11; fem-2* animals, we could assess whether *let-502*'s suppression by *mel-11* or the enhancement by *fem-2* predominated. Among the progeny of these hermaphrodites, 27% arrested as unelongated larvae (Table 11). These likely represented all of the *let-502(ca201)* homozygotes, and most of these animals arrested with the characteristic very short *let-502(ca201); fem-2* phenotype seen in Figure 2G. Thus, the enhancement of *let-502* by *fem-2* overcomes the suppression of *let-502* by *mel-11*, suggesting *fem-2* acts in parallel to the *let-502/mel-11* pathway.

We also examined *unc-73/+; mel-11; fem-2* triple mutants to determine if *fem-2*'s suppression of *mel-11* would overcome the enhancement of *mel-11* by *unc-73*. While *unc-73/+; mel-11* hermaphrodites segregated few Unc progeny at 15° (1.4%, 3/219), this value increased over fourfold, to 6.2% (12/192), when *fem-2* was included (Table 12). (This was less than the theoretical maximum of 25%, likely because the *fem-2* allele is non-null at the temperature used. There are insufficient surviving progeny at higher temperatures to conduct the experiment.) Thus, the ability of *fem-2* to block the enhancement of *mel-11* by *unc-73* indicates that *fem-2* probably acts downstream or in parallel to *unc-73*.

fem-2's role during elongation is independent of other sex-determination genes: fem-2 acts in concert with a number of genes to determine the sexual identity of the *C. elegans* soma and germline (reviewed in Schedl 1997; Meyer 1997). Because interactions between *fem-2* and *let-502* showed a strong maternal component, we focused mainly on feminizing (rather than masculinizing) mutations so that we could assess the effects of mutant product contributed by the oocyte. The genes *fem-1* and *fem-3*, which act at the same step of sex determination as *fem-2* (Kimble *et al.* 1984), showed little or no genetic

TABLE 10

Interaction of *mel-11* and *fem* mutations

Parental genotype[a]	% hatching 15°	20°	25°
1. *fem-2(b245*ts)	93	94	75
2. *mel-11(it26)*	19	0.4	0[b]
3. *mel-11(it26); fem-2(b245*ts)	—[c]	22	3.1[d]
4. *fem-2(e2105)*	97	98	95
5. *mel-11(it26); fem-2(e2105)*[e]	76	15	—
6. *mel-11(it26); fem-2(b245)/+*	—	2.3[f]	0[g]
7. *mel-11(sb55)*	—	—	12
8. *mel-11(sb55); fem-2(b245*ts)	—	—	52
9. *mel-11(it26); fem-1(hc17*ts)[h]	17	1.1	0
10. *mel-11(sb55); fem-1(hc17*ts)	—	—	13
11. *mel-11(it26); fem-2(b245*ts); *fem-1(hc17*ts)	—	21	—
12. *mel-11(it26); fem-3(e2006*ts)[h]	26	2.2	—
13. *mel-11(sb55); fem-3(e2006*ts)[i]	—	—	10
14. *mel-11(it26); fem-3(q20*gf,ts)	14	1.0	0.1
15. *mel-11(it26); fem-2(b245*ts); *fem-3(q20*gf,ts)	—	19	—
16. *mel-11(it26); tra-3(e1107)*[j]	13	—	—

[a] The following proportions were self-fertile for each of the *ts fem* mutations by themselves when reared at 20°: *fem-1(hc17)* 4/16; *fem-2(b245)* 9/16; *fem-3(e2006)* 13/22; *fem-3(q20)* 8/16. These data indicate that the *fem* gene products were partially limiting for potential *mel-11* interactions at the intermediate temperature. Control hatching frequencies for animals upshifted as young adults (*i.e.*, after the temperature-sensitive period for fertility) ranged from 90 to 99% for *fem-1(hc17)*, *fem-3(e2006* or *q20)*, and *tra-3*.

[b] 0/1777 embryos hatched.

[c] Not determined.

[d] 61/1955 embryos hatched.

[e] *fem-2(e2105)*, although a null, shows a maternal effect, so the homozygous *fem-2* progeny of *fem-2/+* animals are self-fertile hermaphrodites and can be tested.

[f] The hatching frequency is increased from the *mel-11(it26)* control, but this likely stems from interexperiment variation. Zygotic suppression would have resulted in an enrichment of *fem-2* homozygotes among the survivors. This was not the case as approximately one-quarter (4/13) of the survivors were Fem.

[g] 0/1733 embryos hatched.

[h] Null alleles of *fem-1* and *fem-3* also were tested using crosses described in MATERIALS AND METHODS, but these alleles had little effect on *mel-11(it26)* viability.

[i] *mel-11(sb55); fem-3(e2006)* adults quickly stopped laying eggs upon upshift to 25°. To overcome this, early embryos were dissected from gravid adults and then upshifted (see MATERIALS AND METHODS).

[j] *tra-3* shows a maternal effect, so the homozygous *tra-3* progeny of *tra-3/+* animals are self-fertile hermaphrodites, which yield all pseudomale broods, which were scored.

TABLE 11

Interactions between *let-502*, *mel-11*, and *fem-2* at 25°

Parental genotype[a]	% hatching	% with *ca201*-like among survivors
let-502(ca201)/+	96	22
let-502(ca201)/+; fem-2	96	23[b]
let-502(ca201)/+; mel-11	60	5.4
let-502(ca201)/+; mel-11; fem-2	61	27[b]

[a] *mel-11(it26)* and *fem-2(b245)* used in all cases.

[b] Most arrested with the extreme-ca201 phenotype as shown in Figure 2G.

interactions with *let-502* or *mel-11*, nor did they modify interactions between *fem-2* and elongation genes (Table 9, lines 14–21; Table 10, lines 9–15). Significantly, a *fem-3(gf)* mutation that suppresses the germline sexual phenotypes of *fem-2* (BARTON *et al.* 1987) did not alter the *fem-2* interactions with *let-502* or *mel-11* (Table 9, line 21; Table 10, line 15). *tra-3*, which acts upstream of *fem-2* in the sex-determination pathway (HODGKIN and BRENNER 1977), showed no genetic interactions with *let-502* or *mel-11* (Table 9, lines 22–23; Table 10, line 16). We also tested a *gf* allele of the downstream sex-determination gene *tra-1* (HODGKIN 1987), but again no genetic interactions were seen with *mel-11* (data is in MATERIALS AND METHODS). Thus, *fem-2* apparently acts independently of other sex-determination genes during embryonic elongation.

DISCUSSION

C. elegans elongation, the transformation of the embryonic ball of cells into a vermiform larva, involves dramatic epidermal cell shape changes. Microfilaments become circumferentially aligned within epidermal cells just prior to elongation, and contraction of these microfilaments results in the dramatic cell shape changes that drive elongation of the embryo (PRIESS and HIRSH 1986). *let-502* (Rho-binding kinase), *mel-11* (regulatory subunit of myosin phosphatase), and *mlc-4* (nonmuscle regulatory myosin light chain) encode proteins predicted to function in a biochemical pathway that regulates contractile and relaxation events (WISSMANN *et al.* 1997, 1999; SHELTON *et al.* 1999). In this report, we further examined *let-502*'s role in elongation by analyzing mutants with genetic and phenotypic properties distinct from the previously identified *let-502* mutants. This analysis uncovered a new phenotype, early cleavage defects. We also used the new *let-502* alleles to investigate the nature of their interactions with *mel-11* and a number of other genes thought to be involved in morphogenesis. A surprising result was that the *fem-2* sex-determination gene has a previously unknown role in the process.

In vertebrate smooth muscle, contraction can be triggered by phosphorylation of MLC by either MLCK or Rho-binding kinase (SOMLYO and SOMLYO 1994; AMANO *et al.* 1996; KLEMKE *et al.* 1997; KUREISHI *et al.* 1997; STULL *et al.* 1998; FENG *et al.* 1999). Therefore, it is possible that embryonic elongation in *C. elegans* could be redundant at two levels. First, *let-502* Rho-binding

TABLE 12

Interactions between *mel-11*, *unc-73*, and *fem-2* at 15°

Parental genotype[a]	% hatch	% Unc F_1
unc73/+	98[b]	24[b]
mel-11	7.3[b]	—
unc-73/+; mel-11	5.1[b]	1.4[b]
mel-11; fem-2	53	—
unc-73/+ mel-11; fem-2	29	6.2

[a] *mel-11(it26)*, *unc-73(rh40)*, and *fem-2(b245)* used in all cases.
[b] From Table 6.

kinase might not be necessary for elongation if the process can be triggered solely by MLCK. Second, the entire *let-502/mel-11* pathway might be redundant if appropriate regulation of MLCK can elongate the embryo. In that case, *let-502* and *mel-11* would be necessary only in the presence of the others' wild-type activity. In this work we show that *let-502* is essential, but only in the presence of wild-type *mel-11* activity.

***let-502* is an essential gene:** The results obtained from different assays for genetic properties (summarized in Table 1) indicated that *let-502* alleles often do not fall into discrete categories. Instead, many show mixtures of dominant-negative, null, and hypomorphic properties depending on the heteroallelic combination examined. A caveat of this analysis is that the deficiency used as a benchmark for the null could delete other genes that interact with either *let-502* or *mel-11*. *unc-73* is uncovered by *hDf6* and is known to interact with *mel-11* (but not *let-502*, Wissmann *et al.* 1999). However, this was unlikely to have influenced our results since *unc-73* shows no dominant interactions with either *let-502* or *mel-11*. *hDf6* is a reasonably small deficiency (McKim *et al.* 1988), so dominant interactions with other, unidentified genes are also unlikely.

Our collection of different *let-502* alleles demonstrates that the gene has at least three essential and genetically distinct functions during the *C. elegans* life cycle. These functions differ in their requirements for maternal and zygotic gene activity. Animals homozygous for the weak dominant-negative/null mutation *ca201sb54* are sterile, demonstrating a strict zygotic requirement for *let-502*(+), likely in the somatic gonad where *let-502* reporters are expressed (Wissmann *et al.* 1999). The alleles *sb103* and *sb106* retain sufficient wild-type zygotic activity for partial fertility, but the low levels of maternal *let-502*(+) activity in the embryos that they produce are often insufficient for the early cleavages. Some *sb103* and *sb106* embryos have adequate maternal *let-502*(+) function to escape these early defects, but they often arrest soon after hatching or show morphological defects. This reveals a second embryonic requirement for

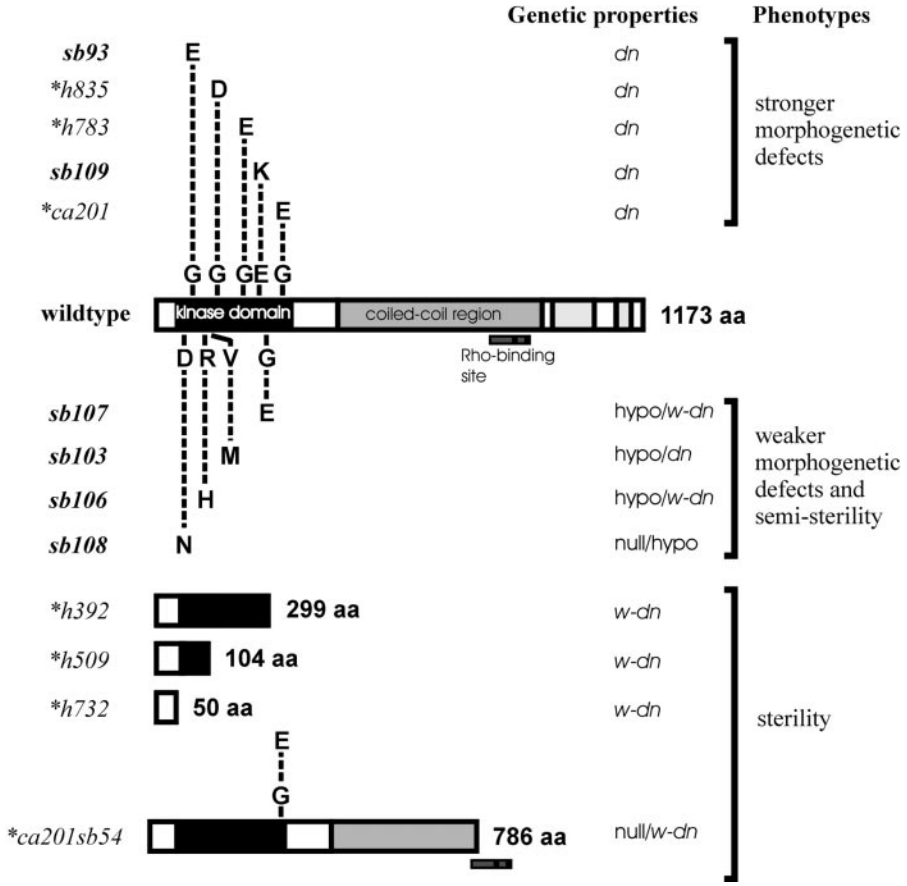

FIGURE 3.—A schematic representation of the *let-502* mutations. The domains are indicated with the exception of the PH domain and Cys-rich region in the C terminus of the protein. The nonsense mutants (which lead to predicted truncations) are shown as truncated proteins. Also shown are the genetic properties of the alleles: null, hypomorph (hypo), dominant negative (*dn*) or weak dominant negative (*w-dn*), and their corresponding phenotypes. Alleles isolated in this study are designated in boldface type. The other alleles were identified by Howell and Rose (1990) and Wissmann *et al.* (1997).

let-502 during elongation. This elongation function can be provided either maternally or zygotically since embryos can be rescued by either maternal heterozygosity for *let-502*(+) or by mating homozygous mothers to wild-type males. The elongation phenotype is also apparent for the homozygous progeny of hermaphrodites heterozygous for the stronger dominant-negative alleles like *ca201*. The maternal *let-502*(+) contribution from *ca201*/+ mothers, although high enough to rescue the early cleavage defects, is insufficient (or by midembryogenesis has decayed to levels that are too low) to provide the elongation function to their homozygous progeny [which, unlike *sb106* embryos, totally lack zygotic *let-502*(+)]. In addition to varying in their maternal *vs.* zygotic requirements for *let-502* expression, the elongation and cleavage phenotypes, but not the adult sterility, are suppressed by *mel-11*.

In higher eukaryotes, Rho-binding kinase and the related Citron kinase are implicated in cytokinesis (Madaule *et al.* 1998; Yasui *et al.* 1998). In Drosophila, recent evidence supports roles for Rho and Rho GEF in cytokinesis and cleavage furrow formation, and in mammalian cell culture elevated levels of myosin-binding subunit phosphorylated by Rho-binding kinase are found at the cleavage furrow (Kawano *et al.* 1999; Prokopenko *et al.* 1999). In *C. elegans*, *let-502* could be the Rho-binding kinase that acts in early cleavage events. We are currently investigating the *let-502* cleavage defects in more detail.

Structure of LET-502: As shown in Figures 3 and 4, the newly identified *let-502* alleles have no obvious correlation between the molecular nature of their mutations (conservation or proximity to residues known to be critical for kinase function; Johnson *et al.* 1996) and their genetic properties and phenotypes. All of the mutations are within conserved residues. Four of the new mutations *sb103*, *sb106*, *sb107*, and *sb108* have mixtures of dominant-negative and *lf* properties. These mutations likely decrease but do not eliminate wild-type activity, explaining their hypomorphic properties. However, multimerization of these products with LET-502(+) or other proteins (likely through the extensive LET-502 coiled-coil domain) could decrease the activity of the resulting complexes, explaining their partial dominant-negative behavior. Our different genetic assays could emphasize either the hypomorphic or dominant-negative aspects of a mutation because each test presents the mutant protein with different interaction partners and/or has different tissue-specific requirements for LET-502(+) activity. We likely failed to isolate true null alleles because if a mutation does not completely abolish both enzymatic activity and the ability to interact with itself or other proteins, it will have dominant-negative properties. Further interpretation of these results awaits more information on the three-dimensional structure of Rho-binding kinase.

The *let-502* and *mel-11* elongation pathway is redundant: Coinjecting both *let-502* and *mel-11* dsRNAs into *let-502(sb108); mel-11(it26)* animals, which already have low levels of both gene activities, resulted in progeny that still elongated. This argues that the function of each gene is required only when the other is present and that there is another pathway that can compensate for the combined loss of *let-502*(+) and *mel-11*(+) activities. This parallel pathway likely involves MLCK, which is known to trigger actin-myosin contractile events independently of Rho-binding kinase (Somlyo and Somlyo 1994; Amano *et al.* 1996; Klemke *et al.* 1997; Kureishi *et al.* 1997; Stull *et al.* 1998; Feng *et al.* 1999).

Recently Sanders *et al.* (1999) showed that p21-activated kinase (PAK) can inhibit MLCK, which could alleviate the need for myosin phosphatase (MEL-11) to negatively regulate MLCK-induced contraction. Chen *et al.* (1996) showed that a *C. elegans* PAK localizes to the epidermal cell borders during elongation. However, others have reported that PAK can induce rather than inhibit contraction (Van Eyk *et al.* 1998; Sells *et al.* 1999; see Bagrodia and Cerione 1999 for discussion). In addition, a number of other protein kinases inhibit MLCK by phosphorylation, but the *in vivo* significance of these results is not clear (Stull *et al.* 1993; Verin *et al.* 1998).

Consistent with the idea of redundancy, we found that mutations that enhance *mel-11* fall into two groups on the basis of their interactions with the *let-502; mel-11* double. Since elongation is nearly normal in the double mutant, addition of a third mutation compromising the redundant elongation pathway would be lethal. This is what we observed with *unc-73* and *fem-2*. In contrast, genes acting upstream of *let-502* and/or *mel-11* should not affect the double mutant phenotype; this was observed for *mig-2* and *daf-2*. As expected, the gene predicted to act at the convergence of the two elongation pathways, *mlc-4*, blocked elongation when combined with *let-502; mel-11*.

Figure 5 shows a summary of our genetic interactions placed in the context of vertebrate smooth muscle contraction. One might have predicted that UNC-73, a Rho/Rac GEF (Steven *et al.* 1998), and MIG-2, a Rho/Rac-like protein (Zipkin *et al.* 1997), would function in a linear pathway. However, the results reported here place them on branches of different elongation pathways. Previous results also indicate that *unc-73* and *mig-2* do not act exclusively in a linear pathway. Zipkin *et al.* (1997) reported that *unc-73; mig-2* has cell and nuclear migration phenotypes and fertility defects not seen in the two single mutants indicating that the genes function in different (redundant) pathways. We previously reported that *unc-73(null)* more strongly enhanced *mel-11* mutations than did *mig-2(null)*, which again implies that *unc-73* cannot act solely through *mig-2* (Wissmann *et al.* 1999). The UNC-73 Rho/Rac GEF could interact with another Rac to activate PAK, as shown in Figure 5.

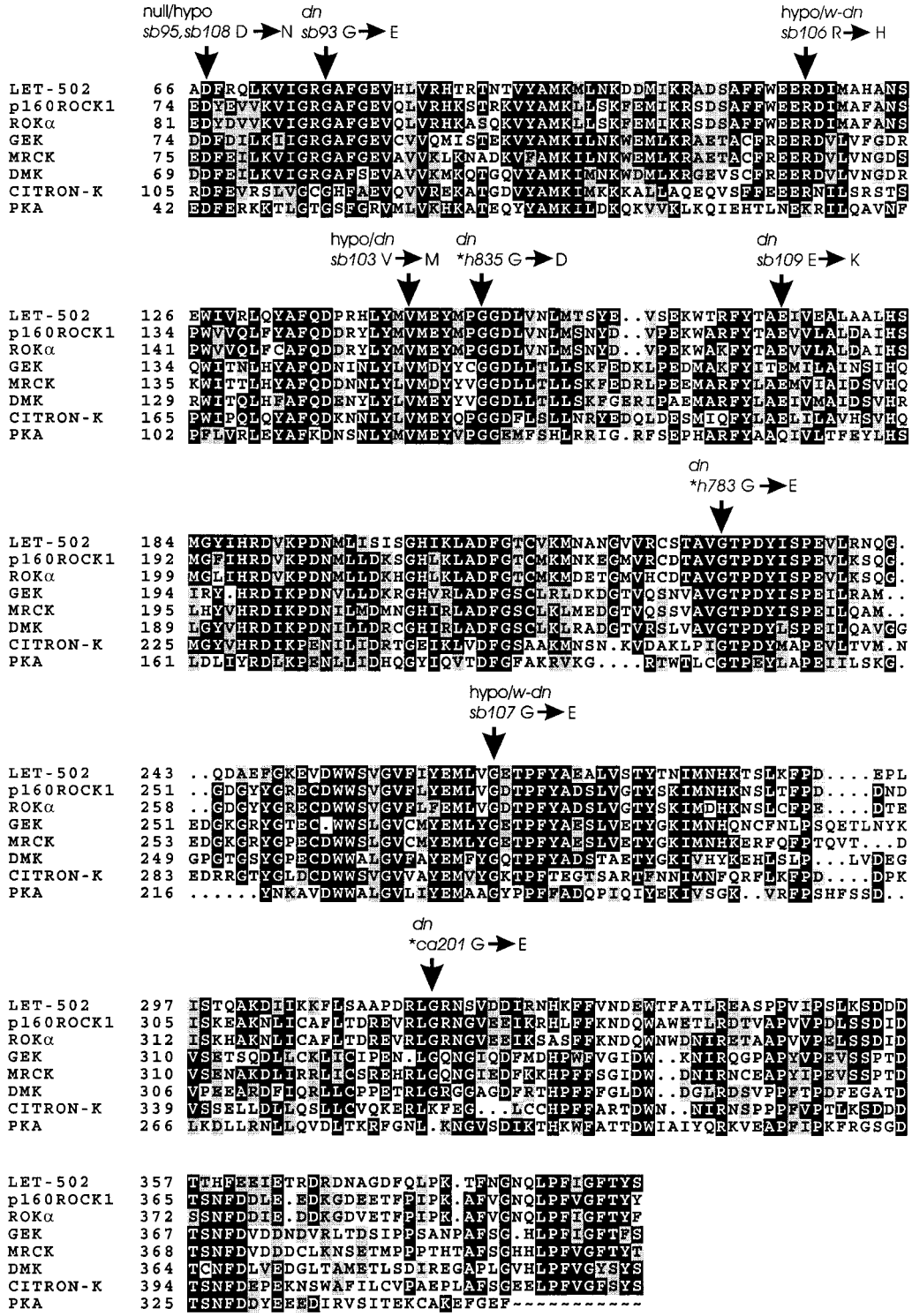

FIGURE 4.—Alignment of the protein kinase domain from LET-502 in comparison with the kinase domain from p160[ROCK] (human Rho-associated kinase; FUJISAWA *et al.* 1996), ROKα (rat Rho-associated kinase type α; LEUNG *et al.* 1995), GEK (*D. melanogaster* Genghis Khan; LUO *et al.* 1997), MRCK (rat myotonic dystrophy kinase-related Cdc42-binding kinase; LEUNG *et al.* 1998), DMPK (human myotonic dystrophy kinase; BROOK *et al.* 1992; FU *et al.* 1992), Citron-K (mouse Citron kinase; DI CUNTO *et al.* 1998; MADAULE *et al.* 1998), and PKA (human cAMP-dependent kinase β; BEEBE *et al.* 1990). Genetics Computer Group, Inc. (GCG) software, PileUp, and freeware Boxshade were used to perform the sequence analysis. Identical residues are shaded in black and conserved residues in gray. The location of mutations in LET-502 and the corresponding amino acid changes are shown. Also indicated is whether the allele behaves genetically as a hypomorph (hypo), dominant negative (*dn*), or weak dominant negative (*w-dn*). Previously identified alleles are designated with asterisks (HOWELL and ROSE 1990; WISSMANN *et al.* 1997). *ca201sb54*, *h392*, *h509*, and *h732* are truncations due to nonsense or splice donor/acceptor mutations and are not shown.

In mammalian systems, the insulin receptor acts upstream of Rac, likely through PI3 kinase (NOBES *et al.* 1995). It was possible that the enhancement of *mel-11* by mutations of the *C. elegans* insulin receptor (*daf-2*, KIMURA *et al.* 1997) and PI3 kinase (*age-1*, MORRIS *et al.* 1996) would influence *mel-11* through *mig-2* (a Rac-related molecule)-mediated changes in the actin cytoskeleton. However, the *daf-2* enhancement of *mel-11* is suppressed by *daf-16*, a forkhead-like transcription factor acting downstream of *daf-2* in the dauer and longevity pathways (OGG *et al.* 1997). This implies that the *daf-2* enhancement of *mel-11* is not due to an immediate effect on *mig-2* Rac and the actin cytoskeleton, but it instead results from changes in gene expression. We found that *daf-2(m212); mig-2(mu28)* is slightly Unc, although each individual mutation is not (P. E. MAINS,

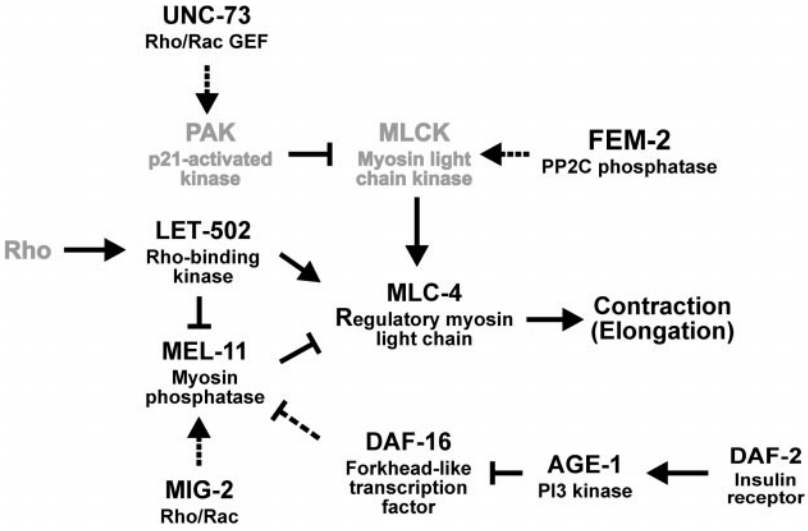

FIGURE 5.—Pathway of *C. elegans* elongation, based on our genetic results and analogies with other systems. Positive and negative interactions are indicated by pointed and barred arrows, respectively. Dashed arrows indicate that precise relationships are not yet clear. In smooth muscle, the demonstrated biochemical activities of Rho, Rho-binding kinase, myosin phosphatase, regulatory myosin light chain, PAK, and MLCK place them in the pathway as shown. The genetic interactions of corresponding *C. elegans* genes (*let-502*, *mel-11*, and *mlc-4*) are consistent with placing them in the same positions of the pathway as their smooth muscle homologs. Rho, PAK, and MLCK have not been tested for their roles in *C. elegans* elongation and this is indicated by the lighter text. Genetic interactions of *unc-73*, *fem-2*, *daf-2*, *age-1*, *daf-16*, and *mig-2* suggest placements in the pathway as shown (see text for rationale).

unpublished results). This is another example showing that *daf-2* and *mig-2* must act in redundant pathways, rather than *daf-2* always acting upstream of *mig-2*.

It should be noted that while *mig-2* and *daf-2* enhance *mel-11*, they do not interact genetically with *let-502*. This suggests that although mutations in *mig-2* and *daf-2* can compromise the limited *mel-11* activity present in a *mel-11*(−) background, they cannot decrease wild-type *mel-11* sufficiently to bring about suppression of *let-502* (as is seen when *mel-11* is mutant). This suggests that there are multiple inputs regulating *mel-11*, each with a relatively modest effect.

The sex-determination gene *fem-2* acts in parallel to the *let-502*/*mel-11* elongation pathway: We found that *fem-2(null)* results in a low penetrant unelongated larval arrest phenotype similar to that caused by strong *let-502* alleles. Substantial numbers of the progeny of both the null and a ts *fem-2* allele show a Sma phenotype, similar to that seen with *sma-1*, which encodes a β-spectrin that is also involved in embryonic elongation (McKeown *et al.* 1998). Furthermore, *fem-2* mutations enhance *let-502* defects and suppress those of *mel-11*. *fem-2*'s role both in spermatogenesis and for male somatic development is well documented (Kimble *et al.* 1984; Hodgkin 1986). However, *fem-2*'s role during embryonic elongation was not previously noted.

fem-1 and *fem-3* were likely candidates for interactions with *fem-2* during elongation since the three *fem* genes act at the same genetic step in sex determination (reviewed in Meyer 1997; Schedl 1997). In addition, FEM-2 and FEM-3 physically interact (Chin-Sang and Spence 1996) and FEM-3 appears to be the limiting *fem* for sex determination (Mehra *et al.* 1999). However, neither *fem-1* nor *fem-3* genetically interacted with *let-502* or *mel-11* nor did *fem-1* or *fem-3* alter the interactions of *fem-2* with *let-502* or *mel-11*. Mutations in upstream (*tra-3*) and downstream (*tra-1*) sex-determination genes also had little or no effect on elongation when combined with *let-502* or *mel-11*. Thus *fem-2*'s affect on morphogenesis appears to be independent of its well-known role in sex determination. Likewise, mutations of *let-502* and *mel-11* have no obvious effects on sexual differentiation. Males and hermaphrodites for all alleles appear sexually normal under the dissecting microscope, and *let-502(ca201)* heterozygotes and *let-502(sb108)* or *mel-11(it26)* homozygotes mate efficiently as both males and hermaphrodites. Furthermore, all of the sexual phenotypes of the sex-determination genes tested appeared unaltered in combination with *mel-11* and *let-502*.

How does *fem-2* fit into the pathway of embryonic elongation? *fem-2* and *mel-11* encode subunits of unrelated types of phosphatases (Pilgrim *et al.* 1995; Chin-Sang and Spence 1996; Wissmann *et al.* 1997). These phosphatases cannot be interchangeable because while *fem-2*(+) potentiates morphogenesis, *mel-11*(+) inhibits it. Analysis of *let-502; mel-11; fem-2* suggested that *fem-2* is part of a redundant system that can elongate the embryo in the absence of the *let-502*/*mel-11* pathway.

What are possible substrates for FEM-2 PP2c phosphatase during embryonic elongation? As described above, PAK could inhibit MLCK by phosphorylation. Postulating that *fem-2* encodes a phosphatase that reactivates MLCK to allow contraction is consistent with our genetic results indicating that *fem-2* acts redundantly with the *let-502*/*mel-11* pathway. The convergence of the effects of *fem-2* and *unc-73* at the level of MLCK is also compatible with the partial epistasis of *fem-2*'s suppression of *mel-11* over *unc-73*'s enhancement of *mel-11*.

While the model we describe assumes that *let-502*, *mel-11*, and *fem-2* influence the contraction of an actin/myosin lattice, another (not mutually exclusive) model postulates roles for these genes in organizing microfilament-membrane attachments and/or in mediating attachments between neighboring cells, processes that likely occur during elongation. Rho regulates the formation of cadherin-based cell-cell contacts (Braga *et al.* 1997). Rho, Rho-binding kinases, and PP1c phosphatase are implicated in forming stress fibers and focal adhe-

sions, which link the actin cytoskeleton to surface structures such as adjacent cells or the external substrate (Kimura *et al.* 1996; Leung *et al.* 1996; Amano *et al.* 1997). Adducin and members of the ERM family, which connect actin filaments to the membrane and spectrin, respectively, colocalize with both Rho-binding kinase and the regulatory subunit of PP1c, and ERM and adducin are substrates for both (Fukata *et al.* 1998; Kimura *et al.* 1998). Recently Hishiya *et al.* (1999) demonstrated that a PP2c phosphatase dephosphorylated moesin (an ERM family member and a Rho-binding kinase substrate) decreasing moesin's ability to organize actin microfilaments. Such an activity would not account for the *fem-2* genetic interactions with *let-502*, since the two genes are not acting antagonistically, as are the PP2c and Rho-binding kinase in the situation described by Hishiya *et al.* (1999).

Redundancy in Rho-binding kinase contractile systems: Rho-binding kinases have been implicated in a variety of cytoskeletal and cell shape changes (for reviews see Van Aelst and D'Souza-Schorey 1997; Aspenström 1999; Kaibuchi *et al.* 1999), including smooth muscle contraction (Uehata *et al.* 1997; Feng *et* al. 1999), the formation of stress fibers (Kimura *et al.* 1996; Leung *et al.* 1996), focal adhesions (Amano *et al.* 1997), and tumor invasion (Itoh *et al.* 1999). It will be interesting to see if PP2c also functions (possibly redundantly) in these other contractile and morphogenetic systems as *fem-2* does during *C. elegans* elongation.

Why is it necessary to have two independent pathways to elongate the *C. elegans* embryo? Parallel pathways involving different Rho family members also appear to act redundantly during dorsal closure during Drosophila embryogenesis (Harden *et al.* 1999; Lu and Settleman 1999). Redundancy, which appears to be the rule rather than the exception in eukaryotes, greatly increases the fidelity of a developmental process (Nowak *et al.* 1997; McAdams and Arkin 1999). Morphogenesis is not perfect in either *fem-2* mutants or in the *let-502; mel-11* double mutant; having both pathways operating simultaneously ensures accurate elongation of the *C. elegans* embryo.

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