The *let-60* Locus Controls the Switch Between Vulval and Nonvulval Cell Fates in *Caenorhabditis elegans*

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ABSTRACT

During induction of the *Caenorhabditis elegans* hermaphrodite vulva by the anchor cell of the gonad, six multipotent vulval precursor cells (VPCs) have two distinct fates: three VPCs generate the vulva and the other three VPCs generate non-specialized hypodermis. Genes that control the fates of the VPCs in response to the anchor cell signal are defined by mutations that cause all six VPCs to generate vulval tissue (Multivulva or Muv) or that cause all six VPCs to generate hypodermis (Vulvaless or Vul). Seven dominant Vul mutations were isolated as dominant suppressors of a *lin-15* Muv mutation. These mutations are dominant alleles of the gene *let-60*, previously identified only by recessive lethal mutations. Our genetic studies of these dominant Vul recessive lethal mutations, recessive lethal mutations, intragenic revertants of the dominant Vul mutations, and the closely mapping semidominant multivulva *lin-34* mutations suggest that: (1) loss-of-function mutations of *let-60* are recessive lethal at a larval stage, but they also cause a Vul phenotype if the lethality is rescued maternally by a *lin-34* gain-of-function mutation. (2) The dominant Vul alleles of *let-60* are dominant negative mutations whose gene products compete with wild-type activity. (3) *lin-34* semidominant Muv alleles are either gain-of-function mutations of *let-60* or gain-of-function mutations of an intimately related gene that elevates *let-60* activity. We propose that *let-60* activity controls VPC fates. In a wild-type animal, reception by a VPC of inductive signal activates *let-60*, and it generates into a vulval cell type; in absence of inductive signal, *let-60* activity is low and the VPC generates hypodermal cells. Our genetic interaction studies suggest that *let-60* acts downstream of *let-23* and *lin-15* and upstream of *lin-1* and *lin-12* in the genetic pathway specifying the switch between vulval and nonvulval cell types.

*Vulval* development in *Caenorhabditis elegans* has been studied intensively as a model system to understand the mechanism by which cell-cell interactions specify the formation of a pattern of cell types during animal development (for recent reviews see Horvitz 1988; Sternberg 1990). During postembryonic development of the *C. elegans* hermaphrodite, each of six vulval precursor cells (VPCs) has the potential to generate either vulval cells or hypodermal cells. During vulval induction, however, only three of the six VPCs are specified to become the two VPC types, 1* and 2*, by a graded signal from the anchor cell of the gonad (Figure 1). 1* and 2* precursor cells divide further to form the vulva. The other three VPCs remain in the ground state (3* cell type) and generate progeny that fuse with a large syncytial hypodermal cell (Sulston and Horvitz 1977; Kimble 1981; Sternberg and Horvitz 1986; Sternberg 1988). The relative positions of the VPCs with respect to the anchor cell determine which of them are induced to 1* or 2* cells (Sternberg and Horvitz 1986). Besides the inductive signal from the anchor cell, a "lateral signal" acts between the VPCs to prevent the immediate neighbors of a presumptive 1* cell from also becoming 1* cells (Sternberg 1988).

Mutations that result in misspecification of VPC fates have defined genes necessary for the normal patterning process (Horvitz and Sulston 1980; Sulston and Horvitz 1981; Greenwald, Sternberg and Horvitz 1983; Ferguson and Horvitz 1985, 1989; Ferguson, Sternberg and Horvitz 1987). Vulvaless (Vul) mutations cause fewer than three VPCs to generate vulval cells, often resulting in an egg-laying defect (Figure 1). Multivulva (Muv) mutations cause more than three VPCs to generate vulval cells and undergo morphogenesis to produce additional vulval-like structures (Figure 1). These mutations define three major classes of genes: (1) "Vul" genes are necessary for 1* and 2* cell fates. (2) "Muv" genes promote the 3* cell fate. (3) *lin-12* is necessary for determining 2* cell fates. Genetic interactions among these three classes of mutants suggest that there are two interacting genetic pathways that specify the fates of VPCs (Sternberg and Horvitz 1989): Vul and Muv genes act in a pathway that determines whether a VPC becomes a 3* (nonvulval) or a non-3* (vulval) cell, and the *lin-12* gene functions in a separate pathway that determines whether a VPC becomes a 2* or non-2* cell. The sites of action of the Muv and Vul genes are not known, but based on...
Given the expected complexity of such a cellular regulatory pathway, we predicted that all essential components were not yet identified. To further dissect this pathway, we have taken the approach of isolating mutations that suppress existing Muv mutations. This approach might not only improve the efficiency at which the mutations that are directly involved in VPC induction are isolated but also might indicate how the new and existing genes interact in the pathway. In this paper we describe the isolation and characterization of dominant extragenic suppressors of the Muv mutation lin-15(n309). These dominant suppressor mutations result in a dominant vulvaless phenotype, and are dominant negative ("antimorphic") alleles of let-60, previously identified only by recessive mutations with a lethal phenotype. We show, by analysis of the dominant and recessive alleles of let-60, that let-60 function is essential for specifying 1° and 2° vulval cell types, since reduction or elimination of the gene activity results in a vulvaless phenotype. We also present suggestive evidence that the lin-34 Muv mutations are gain-of-function alleles of let-60; the Muv phenotype (where more than three VPCs become vulval cell types) might be caused by let-60 hyperactivity. Our study of the genetic interactions of let-60 and other genes in the vulval induction pathway indicates that let-60 acts downstream of let-23 and lin-15 but upstream of lin-1 and lin-12.

MATERIALS AND METHODS

General methods: Methods for culturing, handling, mutagenesis, and genetic manipulation of C. elegans were as described by Brenner (1974). All experiments were performed at 20°C. The standard C. elegans cellular and genetic nomenclature, defined by Sulston and Horvitz (1977) and Horvitz et al. (1979), respectively, is followed in this paper. "VPCs" (vulval precursor cells) are the six cells (P3.p, P4.p, P5.p, P6.p, P7.p and P8.p) that have the potential to participate in vulval development.

Strains: The standard wild type strain N2 and most other strains with various genetic markers were from Brenner (1974) or the Caenorhabditis Genetics Center. The alleles of various mutants used in the paper are listed below. The source of strains other than Brenner (1974) or the Genetics Center are also indicated.

LG I: dpy-5(e61).
LG II: rol-6(e187); unc-1(e120); let-23(mn23) and nnc-1(dpy-10[e128] unc-52[e444])/II (Herman 1978).
LG III: single mutations: unc-36(e251); unc-32(e189).
Linked double mutations: lin-12(n137) dpy-19(e1259) (Ferguson and Horvitz 1985) and unc-32(e189) lin-12(n676) n909) (Greenwald, Sternberg and Horvitz 1983).
LG IV: single mutations: dpy-20(e1282); unc-22(c7) (Moerman and Bailie 1979); nTI(V;V) (Ferguson and Horvitz 1985); lin-34(n1046) (Ferguson and Horvitz 1985); df28 (Moerman and Bailie 1981); n727 (Ellis and Horvitz 1986). lin-1(e1275) (Horvitz and Sulston 1980).
Linked multiple mutations: unc-24(e138) mec-3(e1338)dpy-20(e1282) (provided by M. Chalfie's laboratory), dpy-20(e1362) unc-31(e169), dpy-20(e1282) unc-22(c7) (provided by D. Bailie's laboratory); lin-3(n1059) dpy-20(e1282) (provided by R. Hill); unc-8(e49) dpy-20(e1362); let-60(s59) unc-22(c7) unc-31(e169) and let-63(s254) unc-22(c7) (Rogalski, Moerman and Bailie 1982); let-108(s1160) unc-22(c7) unc-31(e169) and let-60(s1155) unc-22(c7) unc-31(e169) (Clark et al. 1988).
LG V: dpy-11(e224); him-5(e1490) (Hodgkin, Horvitz and Brenner 1979).
LGX: lon-2(e678); unc-3(e151), lin-15(n765) and lin-15(n309) (FERGUSON and HORVITZ 1985).

Analysis of vulval developmental defects: Criteria for recognition of egg-laying defect (Egl) and multivulva (Muv) phenotype were previously described by HORVITZ and SULSTON (1980). For counting percentage Muv, adult animals with one or more pseudovulva (ventral protrusions) in addition to a vulva were classified as Muv. The vulvaless (Vul) phenotype is examined by observing L3 and L4 larvae under Nomarski optics. The percentage of VPC induction is determined as the percentage of VPCs induced to vulval cell type relative to wild type. In a completely vulvaless animal, each of the six VPCs divide once to generate daughters that fuse with the syncytial hypodermis (the 3' fate). The induction in these animals is said to be 0%. In a wild-type hermaphrodite, three of the six VPCs are induced to divide further than the first round of division, producing the progeny characteristic of 1<sup>st</sup> and 2<sup>nd</sup> VPCs (STERNBERG and HORVITZ 1986). The induction of these further divisions is said to be 100%. Animals with fewer than three cells induced to further division have less than 100% induction (Vul); animals with more than three VPCs induced have more than 100% induction (Muv). According to this definition, if only one of the two daughters of a VPC divided further to generate vulval tissue, the induction is one-half-cell. Therefore, an individual animal with 50% VPC induction would have one and "one-half" VPCs induced. See STERNBERG and HORVITZ (1986) for a discussion of such "hybrid" lineages.

To eliminate the signal producing anchor cell, we ablated the somatic gonad precursor cells during the L1 larval stage (SULSTON and WHITE 1980). The laser microbeam used for ablation was described previously (avery and HORVITZ 1987; STERNBERG 1988).

Isolation of lin-15(n309) suppressors: Strain MT309 [lin-15(n309)] was mutagenized with ethylmethane sulfonate (EMS) and the F<sub>2</sub> progeny were screened for non-Muv revertants. In most cases, candidates were picked with an egg-laying defect (Egl) phenotype. Each candidate was transferred to a new plate and those that gave viable non-Muv progeny were further characterized. Ten revertants were isolated after screening about 100,000 F<sub>1</sub> mutagenized gametes. All revertants have an Egl plate phenotype and are defective in VPC induction. The dominant nature of seven alleles was established as follows. For each of these seven revertants, fewer than one-fourth of the healthy progeny of an individual non-Muv animal were Muv (other Muv progeny exploded during adulthood). Muv animals were individually picked to agar plates, and found to segregate only Muv progeny as the original parent lin-15 strain, indicating loss of the suppressor. In addition, any suppressed non-Muv animals always segregate a small portion of Muv animals along with the majority of non-Muv progeny. These results indicate (1) the suppressor mutations in these strains are heterozygous; (2) these mutations are recessive lethal; (3) the suppressor and Egl phenotypes are dominant. All seven revertants were crossed with wild-type males and the suppressor mutations were recovered without the lin-15 mutations in the background. We refer to these alleles as let-60(dn), where dn is dominant negative (see RESULTS).

Genetic mapping of the let-60(dn) mutations: The seven dominant suppressor alleles were mapped by crossing the hermaphrodite mutants with males carrying genetic markers on different linkage groups and following the Egl phenotype (the plate phenotype of vulvaless animals observable under the dissecting microscope) in the F<sub>1</sub> progeny. All of the dominant alleles proved to be linked to linkage group IV. The results of three point mapping with markers on chromosome IV are shown in Table 1. A genetic map with let-60, lin-34 and relevant nearby genes is shown in Figure 2.

Isolation of intragenic revertants of let-60(dn): The dominant Vul phenotype of let-60(dn) was reverted by screening for the appearance of non-Egl F<sub>1</sub> animals following EMS mutagenesis of let-65<sup>+</sup> + unc-22+/+ let-60(s101dr); dpy-20(+) hermaphrodites. F<sub>2</sub> eggs were picked from each plate with nonEgl F<sub>1</sub> animals. Candidate F<sub>2</sub> non-Egl animals were picked and analyzed further by crossing with marker strains. syl101 sy163, isolated by this method, suppresses the dominant Vul phenotype of syl101 completely. The suppressor is tightly linked to syl101d.

The dominant suppression phenotype of let-60(dn) was reverted by screening for the reappearance of the Muv phenotype of lin-15(n309) in a let-60(dn) background. Two let-60(dn) alleles, sy94 and sy101, were used to construct strains with genotypes of the form unc-24 + let-60(dn) +/+ lin-3 + dpy-20(e1282); lin-15(n309)/lin-15(n309). The lin-3 mutation used (n1059) is a recessive lethal allele. These strains were constructed by crossing lin-3 dpy-20/+ with Muv lin-3+ dpy-20/+ males to +/let-60(dn)+/unc-24+dpy-20; lin-15 hermaphrodites. F<sub>1</sub> cross progeny and F<sub>2</sub> progeny were picked and the animals with desired genotype were selected. Hermaphrodites homozygous for lin-15 were identified by the Muv phenotype of the viable Dpy recombinants (resulting from recombination between lin-3 and let-60). The lin-15 Muv phenotype is completely suppressed in these strains, except for the rare Dpy recombinants, which can be easily distinguished from nonrecombinants. These strains were mutagenized with EMS and F<sub>2</sub> progeny were screened for non-Dpy Muv animals resulting from new suppressor mutations. Since the experiment was designed to isolate intragenic loss-of-function mutations, only F<sub>1</sub> animals were screened. sy27 was isolated from a mutagenized culture containing both the strain with sy94dn and the strain with sy101dn, which had

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**TABLE 1**

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<th>Markers</th>
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<td>dpy-20</td>
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<td>s94</td>
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In each mapping experiment, let-60(dn) alleles were placed in trans to two linked markers on chromosome IV. Recombinants resulting from recombination between the two markers were selected and scored for the let-60(dn) phenotype.

<sup>*</sup> Number of recombinant animals that retain the let-60 alleles out of total recombinants homozygous for one marker gene.

<sup>+</sup> Recombinants with genotype homozygous for marker A but not for marker B.

<sup>-</sup> Recombinants with genotype homozygous for marker B but not for marker A.

<sup>0</sup> The recessive lethal allele n1059 is used for lin-3.
been inadvertently mixed. Therefore, the precise genotype of the new strain is not clear, and is designated as \( nXdn \) sy127. A dominant Muv mutation, sy130gf, was also isolated from the strain with sy94dn as the let-60 allele; sy130gf was localized in \( \text{trans} \) on the \( \text{lin-3} \) dpy-20 chromosome. Both \( nXdn \) sy127 and sy130 were characterized further by crossing out the \( \text{lin-15} \) mutation.

Intragenic revertants should be recovered at similar frequency to that of recovering loss-of-function mutations in a wild-type strain (typically, between 1/2000 and 1/5000 EMS-mutagenized gametes; Brenner 1974; Greenwald and Horvitz 1980). The frequency of isolating intragenic suppressors in this screen is tenfold lower: approximately 1/35,000 EMS-mutagenized gametes. One likely explanation is that \( \text{lin-15} \) (n309) animals are slow growing; the intragenic revertant (let-60[ff+]; lin-15) may be less viable or fecund than the parental strain (let-60[dn]+; lin-15). In this screen, we have picked more than 15 Muv animals as candidates for harboring suppressor mutations, but only two gave viable progeny.

**Genetic mapping of let-60[dn] revertants:** All three new mutations, sy127, sy163 and sy130, were mapped with respect to nearby markers. sy127 was mapped relative to unc-8, unc-31 and dpy-20 (see Figure 2). All three Unc-8 nonDpy-20 recombinants from a strain of genotype unc-8 ++ dpy-20(e1362) ++ sy127 sy127 seg sy127. All four Unc-31 nonUnc-22 let-60 recombinants from unc-8 ++ sy127 +/+ let-100 ++ unc-22 unc-31 heterozygotes picked up sy127. Therefore, sy127 is located between unc-8 and unc-31 and close to dpy-20 and unc-22. The distance between unc-8 and unc-31 is approximately 3 map units. sy163 was mapped relative to unc-24 and unc-31. All nine Unc-24 nonUnc-22 recombinants from a strain of genotype unc-24 ++ unc-22/ + sy163 dpy-20 ++ seg sy163 and dpy-20. All ten Unc-31 nonlet-60 recombinants from strains of genotype let-100 +/+ unc-22 unc-31/unc-24 sy163 dpy-20 ++ and let-100 +/+ unc-22 unc-31/+ sy163 dpy-20 ++ seg sy163. Therefore, sy163 is located between unc-24 and unc-31 and close to unc-22.

Four-point mapping for sy130 was done by constructing a let-65 ++ unc-22/+ lin-34(sy130gf) dpy-20 heterozygote and screening for Unc nonlet recombinants. lin-34(sy130gf) confers a semidominant Muv phenotype (see Table 2). Among 35 Unc recombinants selected, 23 segregated Dpy and Muv progeny, 12 segregated neither Dpy nor Muv progeny, and none segregated Muv nonDpy progeny. Therefore sy130 maps between let-65 and unc-22 and close to dpy-20. We also isolated two animals of genotype lin-34(n1046gf) dpy-20(let-60[sy100]) dpy-20 as recombinants from lin-34(n1046gf) + unc-22/let-60[sy100] dpy-20 ++ heterozygotes, placing lin-34 to the left of dpy-20 (Figure 2). Similar data placing lin-34 just left of dpy-20 in the region of let-60 have been obtained by G. Beitel and R. Horvitz, and by G. Jongeward (both personal communications).

**Complementation tests:** The following tests were performed.

**let-60[dn] with deficiencies:** For SD8 and ND27, + let-60[sy100] dpy-20/+ lin-34(n1046) ++ unc-22; him-5 males were crossed to hermaphrodites carrying deficiencies in \( \text{trans} \) to \( n71 \) (a chromosomal translocation between linkage groups IV and V that balances the right half of IV). The presence of Unc cross progeny (DF/unc-22) indicated that the mating was successful. Since these deficiencies uncover the dpy-20 mutation, the absence of viable Dpy progeny indicates the failure of let-60 to complement the deficiencies. let-60[dn] with let-60[dn]: For sy92 and sy95, ++ let-60(sy100) dpy-20/unc-24 lin-34(n1046) ++ males were crossed with unc-24 + let-60[dn] + unc-24 mec-3 + dpy-20 hermaphrodites. Only rare nonUnc nonDpy animals are found among the cross progeny (e.g., two nonDpy nonUnc hermaphrodites among more than 20 Dpy nonUnc hermaphrodites from one cross). These rare nonDpy nonUnc F1 animals were recombinants (unc-24 + dpy-20(e1282) ++ lin-34(n1046) ++ ) because they all segregated both Dpy Unc (unc-24 dpy-20) and Muv nonUnc (lin-34(n1046gf)) F2 hermaphrodites. Neither + sy100 dpy-20/unc-24 sy97 ++ nor + sy100 dpy-20/unc-24 sy92 ++ animals were found among the cross progeny, and thus these genotypes were inferred to be lethal. For sy94, a similar result was obtained with unc-24 + let-60(sy100) dpy-20(e1282) ++ lin-34(n1046gf) ++ unc-22 males crossed with unc-24 ++ let-60[sy94dn] +/+ lin-3-34(sy130gf) + dpy-20 hermaphrodites.

**let-60[dn] with let-60[ff]:** let-60(s1124) and let-60(s59), previously isolated in a screen for recessive lethal mutations (Clark et al. 1988), are loss-of-function alleles (see RESULTS). Males of genotype let-60(s1124) ++ unc-22 unc-31/+ dpy-20(e1282) ++ him-5/++ were crossed with ++ let-60(sy100dn) dpy-20/lin-3 lin-34(sy130gf) + dpy-20 hermaphrodites. Phenotypically nonDpy F1 hermaphrodites were examined for vulval induction and further analyzed. Among more than 50 nonDpy F1 progeny examined, half were egg-laying competent (nonEgl) and were determined to be ++ let-60(s1124) ++ unc-22 unc-31/lin-3 lin-34(sy130gf) + dpy-20 ++. (Their progeny were used to examine the vulval induction of s124/s122 progeny). The other half of the F1 progeny were Egl and they segregated only dead larvae as the F2. The genotype of this latter class must be let-60(s1124) ++ unc-22 unc-31/let-60(sy100) dpy-20 ++, and they were rescued by maternal activity of lin-34(sy130gf) (see Figure 5). Therefore, sy100 fails to complement s1124. A similar
Vulval Switch in *C. elegans*

**analysis** was carried out for *let-60(s59): let-60(s59) unc-22/ dpy-20(e1362); him-5* males were crossed with *lin-34(sy130gf)* dpy-20/let-60(sy100) dpy-20. As a control, we crossed N2 males with *let-60(sy100dn) dpy-20/lin-3(sy130gf) dpy-20* hermaphrodites and nonDpy *F₁* hermaphrodites were examined for vulval induction and their genotype inferred by segregation as above. In addition, *si124, s59* and *s155*, the third previously isolated *let-60* allele, were tested for complementation with *sy100* for lethality in separate experiments. *let-60* males and *s59* females were crossed with *let-60(sy100dn) dpy-20 +/lin-3(sy1046gf) + unc-22* hermaphrodites. *F₁* EgI hermaphrodites arising from *let-60* nonDpy nonUnc cross progeny were picked and found to regenerate only dead larvae as *F₂* progeny. *si124* also fails to complement *sy99dn* and *sy101dn* in similar tests. *sy93dn* also failed to complement *let-60* (*si124)*. However, from the cross between *let-60* (*si124*) males and *sy93dn* *F₁* hermaphrodites, a low percentage of *sy93dn/si124* strains (4 out of more than 100) have been found among cross progeny. This observation is not surprising since *sy93dn* homozygotes are viable even though they grow slowly and display uncoordinated movement in addition to being vulvalless.

*let-60* revertants with *let-60*: Both cis-dominant revertants of *let-60* (*sy101dn sy163* and *sydxn sy127*), were tested for complementation for viability with *let-60* (*sy100*). For *sy101dn sy163, let-60* (*sy100*) *dp-20 +/+ lin-3(sy1046gf) unc-22* males were crossed with *let-100 ++ unc-22 unc-31/+ sy163 dpy-20 + unc-31 hermaphrodites; no viable Dpy animals were found among cross progeny. For *sydxn sy127, unc-24* (*let-60(sy100dn) dpy-20 +/+ lin-3(sy1046gf) + unc-22* males were crossed with *unc-24* (*sydxn sy127 +/+ lin-3(sy1046gf) unc-22; lin-15*) hermaphrodites; only rare recombinant *Unc-24* homozygotes were found among the cross progeny. Therefore, *sy101dn sy163* and *sydxn sy127* fail to complement *let-60* (*sy100*) for viability.

*let-60* revertants with deficiency: *sydxn sy127 ++/++ dpy-20 unc-22; *him-5* males were crossed with *sd8*/unc-24 mec-3 dpy-20 hermaphrodites. *F₁* nonDpy animals were picked and tested for a twitching phenotype in 1% nicotine solution (indicating a genotype of *unc-22* or *sd8*/+). It was found among more than 30 *F₁* nonDpy animals tested, only one hermaphrodite shows the twitching phenotype and it is sterile. Therefore, *sydxn sy127* *sd8*/ is lethal.

**trans-Heterozygotes:** The following tests were performed.

*lin-34 with lin-34*: *sy130* was isolated as a dominant suppressor of the dominant suppression phenotype of *let-60* (*dn*). *sy130* was identified as a putative *lin-34gf* allele by crossing *lin-34* (*n1046 unc-22* (*e1282*))/++ males into the revertant hermaphrodites of genotype *unc-24 + let-60(sy94dn+)/lin-3 + sy130 dpy-20(e1282)*. Ninety-eight percent of the *F₁* progeny with genotype *lin-34* (+*unc-24 mec-3 dpy-20*) hermaphrodites, which are Dpy. *NonDpy* F₁ hermaphrodites which should be *lin-3* (*n1046gf*) *unc-31* *sd8* +, were picked for analysis. The percentage of *Muv* animals among nonUnc-31 adult animals was counted under a dissecting microscope. Seven percent (of 512 animals) were *Muv*. A strain of genotype *lin-3* (+*n1046gf*) *unc-22 + unc-24 mec-3 dpy-20* was constructed for a *lin-34* + control; 11% (of 467 heterozygous adult animals) were *Muv.*

*lin-34 and let-60*: *lin-34* (*n1046gf*) was placed in trans to each of six *let-60* alleles (the recessive viable allele *sy93* was not tested). For *let-60* (*sy100dn*) and *let-60* (*sy101dn*), strains with genotype *lin-34* (*n1046gf*) + *unc-22*/+ *let-60* (*sy92dn*), *let-60* (*sy94dn*) and *let-60* (*sy95dn*), strains with genotype + *lin-34* (*n1046 unc-22* *unc-24* *let-60* (*dn*)) + were constructed and analyzed. *lin-34* (*sy130gf*) *dpy-20* was placed in trans to *let-60* (*sy100dn*) *dpy-20, let-60* (*sy99dn*) *unc-31* and *let-60* (*si124*) *unc-22 unc-31*. When each of three *let-60* alleles, *sy100*, *sy95* or *sy92* was placed in trans to *lin-34*, approximately 1/6 to 1/4 the progeny of the heterozygous parents are homozygous *let-60* (*dn*) hermaphrodites. These homozygotes are Vul and segregate only dead larvae as their progeny. We have also constructed similar *lin-34gf* (*sy99dn*) *let-60* heterozygotes with *him-5* in background so that we could examine the mating ability of the male animals (Hodge 1983). Individual L4 males of these strains was placed in a plate containing three to four hermaphrodites with either *Unc* (*unc-24*) or *Dpy Unc* (*dpy-20* and *unc-31*). Heterozygotes. Among the *let-60* (*dn*) males examined, some of the males containing *sy100* (6 of 32), *sy94* (5 of 26), or *sy92* (13 of 22) were able to mate when in *trans to lin-34* (*n1046gf*); none of the *sy101/n1046* heterozygous males were able to mate (none of 20). By contrast, 12 of 38 *lin-34* (+*n1046gf*) + *unc-22 ++ *dpy-20* + *unc-31*; *him-5* males were able to mate.

**Construction and analysis of double mutants:** The following methods were used.

*let-60* (*si124*) with *lin-1*: *Lin-5* (*n309*): Heterozygous + *let-60* (*si124*) + *unc-22* (*e7190*/*n1046*/*e49*) + *dpy-20* (*e1362*) +++; *him-5*+/+ males were mated with *dpy-20* (*e1282*); *lin-1* (*n309*)/*lin-1* (*n309*). NonDpy *F₁* hermaphrodites were picked and tested for a twitching phenotype in 1% nicotine solution, indicating a genotype of *unc-22* or *sd8*/*. It was found among more than 30 *F₁* nonDpy animals tested, only one hermaphrodite shows the twitching phenotype and it is sterile. Therefore, *sy101dn sy124* is lethal.

**trans-Heterozygotes:** The following tests were performed.

*lin-34 with lin-34*: *sy130* was isolated as a dominant suppressor of the dominant suppression phenotype of *let-60* (*dn*). *sy130* was identified as a putative *lin-34gf* alleleby crossing *lin-34* (*n1046 unc-22* (*e1282*))/++ males into the revertant hermaphrodites of genotype *unc-24 + let-60(sy94dn+)/lin-3 + sy130 dpy-20(e1282)*. Ninety-eight percent of the *F₁* progeny with genotype *lin-34* (+*unc-24 mec-3 dpy-20*) hermaphrodites, which are Dpy. *NonDpy* F₁ hermaphrodites which should be *lin-3* (*n1046gf*) *unc-31* *sd8* +, were picked for analysis. The percentage of *Muv* animals among nonUnc-31 adult animals was counted under a dissecting microscope. Seven percent (of 512 animals) were *Muv*. A strain of genotype *lin-3* (+*n1046gf*) *unc-22 + unc-24 mec-3 dpy-20* was constructed for a *lin-34* + control; 11% (of 467 heterozygous adult animals) were *Muv.*

*lin-34 and let-60*: *lin-34* (*n1046gf*) was placed in trans to each of six *let-60* alleles (the recessive viable allele *sy93* was not tested). For *let-60* (*sy100dn*) and *let-60* (*sy101dn*), strains with genotype *lin-34* (*n1046gf*) + *unc-22*/+ *let-60* (*sy92dn*), *let-60* (*sy94dn*) and *let-60* (*sy95dn*), strains with
60(s1124) homozygotes from heterozygous mothers often yield occasional survivors, but these survivors are Vul.

let-60(sy100dn) with lin-12(n137); A strain with genotype dpy-19 + lin-12(n137)/+ unc-32 lin-12(n676 n909); lin-3 (MT2375; P. STERNBERG and R. HORVITZ, unpublished) was used for the construction. n137 is a dominant allele of lin-12. n676 n909 is a lin-12(d) mutant plus an intragenic revertant resulting in loss of lin-12 function (GREENWALD, STERNBERG and HORVITZ 1983). MT2375 males were mated to ++ let-65 + unc-22/unc-24 mec-3 + dpy-20+ hermaphrodites. The male cross progeny (showing Lin-12(d) phenotype) were picked and mated to unc-36; + let-60(sy100dn) dpy-20/lin-34(n1046gf)+ hermaphrodites. Hermaphrodite progeny heterozygous for the unc-22 mutation were selected with 1% nicotine (MOERMAN and BAILEY 1979). Hermaphrodites with the Lin-12(d) phenotype (Egl with five small ventral protrusions) were picked. Those broods segregating unc-36; let-60(sy100dn)/+; let-65 + unc-22 animals were identified, and their genotype was determined to be +dpy-19 lin-12(n137)/unc-36 ++; +let-60(sy100) dpy-20 /+let-65 ++ unc-22. Animals with this genotype display the Lin-12(d) phenotype. Analogous experiments were done with let-60(sy99dn) and let-60(sy94dn) with similar results.

let-60(s1124)/+ with lin-12(n137); MT2375 males (see above) were crossed with + let-60(s1124) + unc-22 unc-31/unc-8 + dpy-20 ++ hermaphrodites. F1 hermaphrodites heterozygous for unc-22 (show twitching phenotype in 1% nicotine) were picked at the L4 stage. Egl adults with lin-12(d) phenotype (with five small ventral protrusions and Egl) were present. The Muv phe tolerable phenotype (with five small ventral protrusions and Egl) were present. These mutations by mutagenizing EMS and screening for phenotypically non-Muv revertants resulted in loss ofLin-12 function (GREENWALD, STERNBERG and HORVITZ 1983). MT2375 males were mated to ++ let-65 + unc-22/unc-24 mec-3 + dpy-20+ hermaphrodites. The male cross progeny (showing Lin-12(d) phenotype) were picked and mated to unc-36; + let-60(sy100dn) dpy-20/lin-34(n1046gf)+ hermaphrodites. Hermaphrodite progeny heterozygous for the unc-22 mutation were selected with 1% nicotine (MOERMAN and BAILEY 1979). Hermaphrodites with the Lin-12(d) phenotype (Egl with five small ventral protrusions and Egl) were picked. Those broods segregating unc-36; let-60(sy100dn)/+; let-65 + unc-22 animals were identified, and their genotype was determined to be +dpy-19 lin-12(n137)/unc-36 ++; +let-60(sy100) dpy-20 /+let-65 ++ unc-22. Animals with this genotype display the Lin-12(d) phenotype. Analogous experiments were done with let-60(sy99dn) and let-60(sy94dn) with similar results.

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RESULTS

Isolation of dominant Vul mutations as suppressors of a lin-15 Muv mutation: lin-15 mutations cause all six VPCs to become 1° or 2° (multivulva, or Muv, see Figure 4B) regardless of whether the inductive signal is present (FERGUSON and HORVITZ 1985; FERGUSON, STERNBERG and HORVITZ 1987; STERNBERG 1988; Sternberg and HORVITZ 1989). Mutations of genes whose products interact with lin-15 gene product or of genes acting downstream of lin-15 in the pathway might be expected to suppress the Muv phenotype of lin-15. We have isolated such suppressor mutations by mutagenizing lin-15(n309) animals with EMS and screening for phenotypically non-Muv revertants in the F2 (Figure 3).

After screening approximately 100,000 EMS-mu-
The mutations isolated and studied in this paper are divided into three different classes. The two gain-of-function mutations (n1046 and sy130) are alleles of lin-34 and the rest of the mutations are alleles of let-60.

- The phenotype of each let-60 allele is described as "Vul" for vulvalless, "Muv" for multivulva, or "Let" for lethal. ND, not determined.
- Genotypes of let-60, lin-34 and lin-15. "m" indicates the mutation in let-60 or lin-34; "+" indicates the wild type allele. n309 is an allele of lin-15. m/m; m/+ indicates the strain is homozygous for the mutation in chromosome IV and homozygous for the lin-15 mutation on chromosome X.
- The percentage of hermaphrodites that fail to lay eggs (only tested for m/+ strains). To score %Egl for the let-60(dn) alleles, strains with genotype unc-24+/+ let-60+/+ lin-15(n309) were used except sy93, which was not linked to unc-24 and was tested in a strain with wild type unc-24. For each of the let-60(dn) alleles, more than 200 F1 progeny were scored. Fewer than 1% of the hermaphrodites were sterile.
- Male mating indicates the capability of males of m/+ genotype to mate with hermaphrodites. More than 30 L4 or young adult males were used in tests for each let-60(dn) allele. "-" indicates no cross-progeny were found in a mating test. Defects in male spicules were found in let-60(dn)+/+ animals for all the let-60(dn) alleles.
- The references for previously isolated alleles: (1) MOERMAN and BAILLIE (1981); (2) CLARK et al. (1988); (3) ROGALSKI, MOERMAN and BAILLIE (1982); (4) FERGUSON and HORVITZ (1985).
- Animals that escape the early larval lethality are Vul, and die as young adults.
- "wMuv" indicates a weakly penetrant Muv phenotype. For lin-34(n1046) and lin-34(sy130), about 10–20% of the heterogeneous animals are Muv, compared to greater than 90% Muv among homozygotes.

Phenotypes of let-60 and lin-34

<table>
<thead>
<tr>
<th>Class</th>
<th>Allele (m)</th>
<th>Helmaphrodite phenotype</th>
<th>%Egl</th>
<th>Male mating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dominant negative [let-60(dn)]</td>
<td>sy93</td>
<td>Vul</td>
<td>Vul</td>
<td>Vul</td>
</tr>
<tr>
<td></td>
<td>sy99</td>
<td>Let</td>
<td>Let</td>
<td>Vul</td>
</tr>
<tr>
<td></td>
<td>sy101</td>
<td>Let</td>
<td>Let</td>
<td>Vul</td>
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<td></td>
<td>sy94</td>
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</tr>
<tr>
<td></td>
<td>sy112</td>
<td>Let</td>
<td>Let</td>
<td>Vul</td>
</tr>
<tr>
<td></td>
<td>sy95</td>
<td>Let</td>
<td>Let</td>
<td>Vul</td>
</tr>
<tr>
<td></td>
<td>sy92</td>
<td>Let</td>
<td>Let</td>
<td>Vul</td>
</tr>
</tbody>
</table>

| Gain-of-function [lin-34gf] | n1046     | Muv         | Muv  | wMuv        | ND  | +          | (4) |
|                            | sy130     | Muv         | Muv  | Muv         | ND  | +          |   |

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Isolation of intragenic revertants and determinants of the dominant Vul mutations as let-60 alleles: To ascertain the wild-type function of the gene identified by the dominant Vul mutations, it was necessary to obtain and characterize intragenic revertants. A let-60(dn) heterozygote is expected to exhibit a phenotype similar to that of a deficiency/+ animal and should not exhibit any dominant phenotype. Adding a loss-of-function mutation in cis to a dominant let-60 allele should thus suppress the dominant phenotype caused by the allele.

To revert the dominant phenotype of the let-60(dn) alleles, we carried out two different screens. In one screen, we sought to isolate revertants of the Vul phenotype of let-60(dn) (Figure 3B). One tightly
linked dominant suppressor of let-60(sy101dn), sy163 (Table 2), was isolated after screening approximately 9000 EMS-mutagenized gametes. sy163 suppresses the let-60(dn) dominant phenotypes completely, and the double mutant alleles (sy101dn sy163) remain recessive lethal at a young larval stage. sy101dn sy163/+ hermaphrodites have a wild-type level of vulval induction.

In another screen, we sought to restore the Muv phenotype to a lin-15(n309) strain which is dominantly suppressed by a let-60(dn) and hence Vul (Figure 3C). From a screen of approximately 35,000 mutagenized gametes, we isolated two new mutations that suppress the suppressor phenotype of let-60(dn). In both isolates, the Muv phenotype of lin-15(n309) reappears [being no longer suppressed by the let-60(dn) mutation]. The dominant Vul phenotype of let-60(dn) is also completely suppressed by two new alleles in the absence of the lin-15 mutation. In one of the revertants, syXdn sy127 (syX is either sy94dn or sy101dn, see MATERIALS AND METHODS for explanation), the new mutation is also tightly linked to the dominant negative allele. syXdn sy127 is also recessive lethal at an early larval stage (L1-L2) and fails to complement both sy100dn and a deficiency for its lethal phenotype. The syXdn sy127/+ heterozygous strain has a wild-type level of vulval induction (see Figure 4, D and H), and males of this genotype mate normally.

The two linked revertants, sy101dn sy163 and syXdn sy127, are most likely intragenic revertants and loss-of-function mutations of the let-60 gene (also see Table 2 for comparison). Both revertants behave like deficiencies uncovering the region and the recessive lethal let-60 alleles [loss-of-function mutations (l); discussed below] isolated independently of let-60(dn)
A

\begin{align*}
\text{P} & \quad \text{let-60}(dn) \quad + \quad \times \quad \text{let-60}(dn) \quad + \\
\text{F}_1 & \quad \text{let-60}(dn) \quad \times \quad \text{let-60}(dn) \quad + \\
& \quad 0\% \text{ vulval induction \text{[If = s1124]} \\
& \quad 4\% \text{ vulval induction \text{[If = a59]}} \\
& \quad \text{F}_2 \text{ progeny all die}
\end{align*}

B

\begin{align*}
\text{P} & \quad \pm \quad \times \quad \text{let-60}(dn) \quad + \\
\text{F} & \quad \text{let-60}(dn) \quad + \\
& \quad 65\% \text{ vulval induction}
\end{align*}

Figure 5.—Genetic interactions between let-60(dn) and let-60(If). The complete genotypes of the parent strains are described in MATERIALS AND METHODS. dn, dominant negative; If, loss-of-function; gf, gain-of-function. The allele for let-60(dn) is sy100, and the allele for lin-34(gf) is sy130. s1124 and a59 were used as loss-of-function mutations. See Figure 6 for the maternal effect of lin-34(gf). Only hermaphrodite F₁ and F₂ progeny were analyzed. Compared to 65% vulval induction in +/let-60(dn) animals (B), the 0% and 4% vulval induction phenotype of let-60(If)/let-60(dn) animals (A) indicates the let-60(If) alleles fail to provide function in vulval induction.

(CLARK et al. 1988). It is unlikely that our “cis” revertants (rev) and the dominant Vul alleles (dn) are in different, nearby genes: the phenotype of let-60(dn rev)/+ is wild type and thus is distinct from the lethal phenotype of let-60(dn)/let-60(If).

Previously, three let-60 larval lethal mutations were isolated by screening recessive lethal alleles in the region on chromosome IV (ROGALSKI, MOERMAN and BAILLIE 1982; CLARK et al. 1988) (Table 2). As described above, we carried out complementation tests between these previously isolated alleles and our dominant Vul recessive lethal mutations, and found they fail to complement for viability (see MATERIALS AND METHODS, also Figure 5). The three previously isolated lethal alleles as well as our two cis revertant alleles, sy101dn sy163 and syXdn sy127, are recessive and behave in complementation tests similar to a deficiency uncovering the let-60 locus. The three alleles were isolated in relatively high frequency (3 out of an equivalent of 6500 EMS-mutagenized gametes, ROGALSKI, MOERMAN and BAILLIE 1982; CLARK et al. 1988) [see MATERIALS AND METHODS for discussion of relative frequencies of obtaining let-60(If)]. Based on all these results, we believe that the five recessive lethal alleles, including the two tightly linked let-60(dn) revertants and the three previously isolated alleles, represent loss-of-function mutations of let-60.

let-60 function in vulval development and the nature of the dominant Vul mutations: To determine the function of the let-60 gene in vulval development, it is critical to know the phenotype of a loss-of-function mutation. We have already discussed above that the loss-of-function mutations are recessive lethal prior to the L3 stage and have no dominant phenotype. It is thus difficult to study the phenotype of loss-of-function mutations in vulval development, which occurs during the L3 stage. However, some of the s1124If/s1124If progeny from a s1124If/+ heterozygote can surpass the larval lethal stage to survive to the early adult stage. We have examined the vulval induction in such “survivor” animals of genotype let-60(s1124If)/let-60(s1124If) from a let-60(s1124If)/lin-34(sy130gf) mother [see below for analysis of lin-34(sy130gf)]. Under Nomarski optics, we found that these survivors have 0% VPC induction (14 animals examined). Failure of vulval induction in these animals is not due to the fact that the animals are sick or dying, since the Vul phenotype of the surviving let-60(s1124If) animals can be completely suppressed by lin-1 (see below). This result indicates that Vul is also a loss-of-function phenotype.

We also performed a genetic interaction analysis to overcome the recessive lethal problem of let-60(If) and determine the phenotype of loss-of-function alleles in vulval induction. Two previously isolated recessive lethal alleles, s1124 (CLARK et al. 1988) and s59 (ROGALSKI, MOERMAN and BAILLIE 1982), were placed in trans to our dominant Vul, recessive lethal allele let-60(sy100dn). A let-60(sy100dn) homozygote from a lin-34(gf)/let-60(dn) mother is viable for one generation (it normally would be larval lethal from a let-60(dn)/+ mother) (see below and Figure 6). We took advantage of this maternal effect of lin-34(gf) Muv mutations to examine interactions between let-60(sy100dn) and let-60(If). A let-60(dn)/let-60(If) heterozygote from a let-60(dn)/lin-34(gf) mother is also expected to live for one generation and hence allows us to examine the phenotype in vulval induction (Figure 5). We crossed let-60(If)/+ males with let-60(sy100dn)/lin-34(sy130gf) hermaphrodites, we found that let-60(If) fails to provide any function in vulval induction when lethality is rescued. Examined with Nomarski optics, let-60(If)/let-60(sy100dn) animals from a let-60(sy100dn)/lin-34(sy130gf) mother have nearly no vulval induction (0% VPC induction among 16 s1124If/sy100dn heterozygous hermaphrodites and 4% VPC induction among eight s59If/sy100dn heterozygous hermaphrodites.) By contrast, sy100dn/+ animals (18 examined) from a cross between wild-type (N2) males and hermaphrodites let-60(sy100dn)/lin-34(sy130gf) display about 65% VPC induction (Figure 5). This result confirms that loss-of-function results in a vulvalless phenotype. We thus conclude that let-60 is necessary for vulval development.

Since the let-60(dn) mutations act in the same phenotypic direction (Vul and Let) as let-60(If), these dominant Vul mutations of let-60 are “dominant negative” (dn) mutations (“antimorphic mutations”). In a
**Figure 6.**—Dominant suppression of let-60(dn) by semidominant Muv mutations of lin-34(gf). (A) \(\text{let-60(sy100dn)} +\) heterozygote, there is less wild-type gene activity than that in a \(\text{let-60(lf)/+}\) heterozygote. The dominant Vul phenotype of \(\text{let-60(dn)}\) is the result of this reduction of gene activity.

**lin-34 Muv mutations, tightly linked to let-60, suppress let-60(dn) phenotypes:** lin-34 was previously defined by the semidominant Muv mutation \(n1046\) (Ferguson and Horvitz 1985). This mutation confers a "strong Muv" phenotype (defined here as greater than 80% penetrance) in homozygotes and a "weak Muv" phenotype (defined here as less than 30% penetrance) in heterozygotes. Additional semidominant Muv alleles of lin-34 have been isolated as suppressors of mutations in lin-10 (D. Parry, S. Kim and R. Horvitz, personal communication), let-23 (G. Jongeward and P. W. Sternberg, unpublished results) and let-341 (S. Clark and R. Horvitz, personal communication). We have also isolated a semidominant Muv allele \((sy130)\), as a dominant suppressor of the dominant suppressor phenotype of \(\text{let-60(dn)}\) (see above and Figure 3C). \(sy130/sy130\) animals are Muv (about 95%) (Figure 4G and Table 2). \(sy130/+\) animals have a weak Muv phenotype (about 10%). \(sy130\) interacts in \(\text{trans}\) with \(\text{lin-34(n1046)}\) to produce a highly penetrant (strong) Muv phenotype (95% penetrance) as homozygotes and as weak Muv phenotype (less than 40%) as heterozygotes. (D) A maternally rescued hermaphrodite ("bag of larvae") described in (B). The genotype of the hermaphrodite is \(\text{let-60(sy100dn) dpy-20(let-60(sy100dn) dpy-20 and the genotype of its parent is let-60(sy100dn) dpy-20+/lin-34(n1046gf) + unc-22. The photomicrograph was taken under Nomarski optics as in Figure 4 (same scale as in Figure 4).
a trans-dominant suppressor of let-60(dn), indicating a close relationship between these two classes of mutations. We have further examined the interactions of let-60 alleles with other lin-34 Muv alleles. Three types of results demonstrate that the lin-34 Muv mutations strongly suppress the let-60 mutations (Figure 6). (1) The lin-34 mutations dominantly suppress the dominant Vul phenotype of let-60(dn): let-60(dn)/lin-34 animals show the weakly penetrant Muv phenotype of lin-34/+ rather than the Vul phenotype of let-60(dn)/+. Specifically, between 5% and 20% of animals of genotypes lin-34(n1046) or lin-34(sy130) in trans to each of six let-60(dn) alleles are Muv (the remaining 80–95% are wild type, data not shown). The suppression of the Vul phenotype of let-60(dn) by lin-34 mutations is complete, even though the majority (80–90%) of lin-34/+ animals are not Muv. (2) lin-34 mutations suppress maternally the lethality of some let-60(dn) alleles (sy100, sy92 and sy95). This maternal effect is also dominant. For example, homozygous let-60(sy100dn) F1 progeny from a let-60(sy100dn)/+ mother are normally lethal at a larval stage. However, the let-60(sy100dn)/let-60(sy100dn) F1 progeny from a let-60(sy100dn)/lin-34(n1046) parent are viable for one more generation; the F2 progeny are all dead larvae (Figure 6). lin-34 mutations do not rescue maternally the defect in vulval induction in let-60(dn)/let-60(dn) animals. The sy100dn homozygotes rescued by the lin-34 maternal effect have 0% VPC induction (none of 10 animals examined under Nomarski optics had any VPCs induced to vulval cell types). (3) lin-34 Muv mutations can partially overcome the male mating defect of some let-60(dn)/+ animals; sy92dn, sy95dn, sy100dn and sy94dn males can mate at low efficiency if placed in trans to lin-34(n1046) (see MATERIALS AND METHODS).

A lin-34 mutation in trans to a deficiency (e.g., lin-34(n1046)/sdF8) displays a weak Muv phenotype (about 8% of animals are Muv), similar to lin-34/+(about 10–20%, see MATERIAL AND METHODS; also see Ferguson and Horvitz 1985). This observation suggests that the lin-34 mutations are not loss-of-function mutations, because otherwise, the lin-34/Df hemizygotes should display a Muv phenotype of equal or greater penetrance than lin-34/lin-34 homozygotes (above 90% Muv). Moreover, lin-34(gf), which are most likely alleles of let-60, have a phenotype (Muv) opposite to that of let-60(k)/ (Vul). Therefore, all the lin-34 Muv alleles are likely to be gain-of-function mutations. A simple explanation for our results is that the activity of let-60 is elevated by the presence of a lin-34(gf) mutation, either because lin-34(gf) mutations are gain-of-function alleles of let-60, or that lin-34(gf) mutations are gain-of-function alleles of another gene that acts positively in the same signaling pathway as let-60.

### TABLE 3

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>let-60</td>
<td>lin-15</td>
</tr>
<tr>
<td>+/+;</td>
<td>+/+</td>
</tr>
<tr>
<td>+/+;</td>
<td>n309/n309</td>
</tr>
<tr>
<td>sy100/+;</td>
<td>+/+</td>
</tr>
<tr>
<td>sy100/+;</td>
<td>n309/n309</td>
</tr>
</tbody>
</table>

* The complete genotype on chromosome IV is + + let-60(sy100dn) + /unc-24 mec-3 + dpv-20.

**Egl** stands for egg-laying defective, which, in this case, results from an animal being vulvaless. More than 200 animals were scored.

Percentage VPCs induced to vulval cells relative to wild type, scored with Nomarski optics (see MATERIALS AND METHODS).

The signal is eliminated by ablation of gonad cells during the first larval stage (MATERIALS AND METHODS). Data for wild type are from Sulston and White (1980), and for lin-15(n309) from Sternberg and Horvitz (1989) and Sternberg (1988).

### Genetic interactions of let-60 with other genes in the vulval induction pathway

To understand the role of let-60 in the genetic pathway specifying the VPC fates, we constructed and analyzed several double mutant strains carrying a let-60(dn) mutation and Muv mutations in lin-1, lin-12 and lin-15. In addition, we examined the interaction between let-23 and lin-34(gf). Our results suggest that let-60 acts downstream of let-23 and lin-15 but upstream of lin-1 and lin-12 in the pathway specifying the VPC fate.

**lin-15 acts upstream of let-60:** The seven dominant negative let-60 Vul mutations were isolated as suppressors of lin-15(n309). This suppression is not specific to the n309 allele because another lin-15 allele, n763, can also be dominantly suppressed by let-60(dn) mutations. We have also examined the interaction between lin-15(n309) and a loss-of-function mutation of let-60, s1124. This analysis was possible, because as mentioned above, a small percentage of animals homozygous for the recessive lethal allele let-60(s1124df) can grow to an early adult stage. While the Muv phenotype of lin-15(n309) is fully displayed in a let-60(s1124df)+ background, the Muv phenotype is changed to a completely Vul phenotype in "survivors" of genotype let-60(s1124df); lin-15(n309). This suppression itself suggests that the let-60 gene acts downstream of lin-15 in the genetic pathway that specifies VPC types. Furthermore, we have observed that the Vul and Muv phenotypes are mutually suppressed in a let-60(sy100dn)/+; lin-15(n309) double mutant; not only is the Muv phenotype of lin-15 suppressed by let-60(sy100dn), but the Vul phenotype of let-60(sy100dn)/+ is also partially suppressed by the presence of the lin-15 mutation. The level of VPC induction is close to wild type in a sy100/++; n309/ n309 double mutant (88% VPC induction; 21% Egl) in contrast to 57% VPC induction (87% Egl) in the strain with sy100dn/+ only, Table 3). More impor-
tanty, although the Muv phenotype of lin-15(n309) is independent of the inductive signal from the gonad anchor cell (FERGUSON, STERNBERG and HORVITZ 1987; STERNBERG 1988), the induction of VPCs depends absolutely on the inductive signal in the mutually suppressed let-60(sy100dn)/+; lin-15(n309) double mutant. We have ablated all the gonad cells and hence the signal-producing anchor cell of ten let-60(sy100dn)/+; lin-15(n309) double mutants at the L1 larval stage; all VPCs generated hypodermal cells in these animals (Table 3). These results suggest that let-60 and lin-15 may function antagonistically in the pathway specifying VPC fates, and that the let-60(sy100dn) mutation can compensate to some degree for the lin-15(n309) defect and restore the relative normal output of the signal response pathway. One possibility is that lin-15 is a negative regulator of let-60 activity. Reduction of lin-15 activity could then result in a higher level of let-60, which is no longer subject to the regulation by the upstream signal. This view is supported by the fact that the gain-of-function lin-34 mutations also display a signal-independent Muv phenotype. Specifically, an average of 120% VPC induction was found among five lin-34(gf) animals whose gonad primordia were ablated at an early larval stage (100% is wild type, 200% is maximal for Muv; see MATERIALS and METHODS). lin-34(gf) animals with intact gonads display an average of 165% induction (13 animals).

lin-1 acts after let-60: lin-1 is another Muv gene that acts in the genetic pathway specifying VPC fates (HORVITZ and SULSTON 1980; SULSTON and HORVITZ 1981; FERGUSON and HORVITZ 1985; FERGUSON, STERNBERG and HORVITZ 1987). The Muv phenotype of lin-1 is epistatic to the Vul phenotype of many Vul genes in the pathway (FERGUSON, STERNBERG and HORVITZ 1987), and the lin-1 phenotype is coexpressed with lin-12 phenotypes in double mutants (P. W. STERNBERG, unpublished observation). These results lead to a hypothesis that lin-1 acts downstream of Vul genes (e.g., let-23) and other Muv genes (e.g., lin-15) and, as a negative regulator of 1°- and 2°-specific functions. To further characterize the position of let-60 in the pathway, we constructed double mutants with lin-1(e1275) and the loss-of-function mutation of let-60, s1124. We found that lin-1(e1275) does not rescue the lethality of let-60(s1124)/let-60(s1124f); the typical double homozygous animals are larval lethal, but a small percentage of them survive to reach adulthood stage and are sick and sterile. However, those small number of surviving adult animals are all Muv, indicating the Vul phenotype of the s1124 mutation is suppressed by the lin-1 mutation. We have also found that the Vul phenotype of let-60(sy100dn) is suppressed by the lin-1 Muv mutation. The lin-1 Muv phenotype is fully expressed even in a double homozygote. We could observe this phenotype because the lethality of let-60(sy100dn)/let-60(sy100dn) is suppressed by lin-1(e1275). The homozygous double mutant is viable and can be continuously propagated. These results suggest that lin-1 acts downstream of let-60 in the vulval induction pathway, and that lin-1 interacts with let-60 in a pathway required for larval growth.

lin-12 acts after let-60 in 2° fate specification: One of many lin-12 functions is to distinguish between 2° and non-2° (1° or 3°) VPC types during vulval induction (GREENWALD, STERNBERG and HORVITZ 1983; STERNBERG and HORVITZ 1989). lin-12 dominant mutations (lin-12(d)) cause all six VPCs to be 2°, while lin-12 loss-of-function mutations cause all six VPCs to be non-2°. It has been proposed that lin-12 is involved in the lateral signaling which prevents the neighbors of a presumptive 1° from also becoming 1°, and that lin-12 acts downstream of most Muv and Vul genes whose function is to specify the choice between 3° and non-3° cell fates (STERNBERG and HORVITZ 1989). For example, a let-23 Vul mutation causes all six VPCs to adopt the 3° cell type. In a lin-12(d); let-23 double mutant, all six VPCs are 2°. To order the action of let-60 with respect to lin-12, we constructed and examined a double mutant with a lin-12(d) allele n137 and each of four let-60 alleles (dominant negative alleles sy100dn, sy99dn, sy94dn and a loss-of-function allele s1124) (see MATERIALS and METHODS). We found that the lin-12(d) phenotype (five ventral protrusions and egg-laying defective) is fully expressed in all lin-12(d)/+; let-60(dn/+) strains, and in survivors of genotype lin-12(d)/+; let-60(s1124)/let-60(s1124). In other words, all six VPCs are 2° in the double mutants. Therefore, lin-12 hyperactivity bypasses the need for let-60 function for promoting 2° fate, suggesting that lin-12 acts after let-60 in 2° fate specification.

lin-34 acts after let-23: let-23 is another essential gene with a function in vulval induction (FERGUSON and HORVITZ 1985; FERGUSON, STERNBERG and HORVITZ 1987). Some recessive mutations of let-23 cause a Vul phenotype. However, loss-of-function of let-23 results in a larval lethal phenotype (R. V. AROIAN and P. W. STERNBERG, manuscript in preparation). A lin-34(gf) Vul mutation has been isolated as a suppressor of the let-23 Vul phenotype (G. JONGEWARD and P. STERNBERG, unpublished results), suggesting that lin-34 acts downstream of let-23 during vulval induction. We constructed a double mutant with lin-34(n1046gf) and a loss-of-function, recessive lethal mutation of let-23, mn23 (HERMAN 1978). We found that let-23(mn23); lin-34(n1046gf) hermaphrodites were sterile adults and showed a Muv phenotype (88% of the animals are Muv). Sterility is another phenotype associated with some let-23 muta-
tions (R. V. AROIAN and P. W. STERNBERG, manuscript in preparation) and was not suppressed by lin-34(n1046gf). However, the let-23 lethal and Vul phenotypes were clearly suppressed by the lin-34(gf). Therefore, we conclude that lin-34 acts after let-23 in the genetic pathways involved both in vulval induction and larval growth.

DISCUSSION

Dominant negative mutations of let-60: We have exploited the properties of dominant vulvaless (Vul) mutations in the let-60 gene to analyze its role in vulval induction. Loss of let-60 activity results in death at an early larval stage, prior to vulval induction. These dominant Vul mutations were isolated as extragenic suppressors of a lin-13 multivula mutation, in effect selecting for vulvalessness and viability. These mutations thus allowed us to conclude that let-60 plays an important role in vulval induction. We used the dominant Vul mutations to obtain both recessive lethal loss-of-function alleles of let-60 as well as semi-dominant multivula lin-34 mutations that behave as gain-of-function alleles of let-60 (see below). Analysis of these mutations has allowed us to understand the role of let-60 in the switch between vulval and non-vulval VPC fates during vulval induction, as detailed below.

We have found that these dominant Vul mutations are dominant negative, i.e., they result in a let-60 product that appears to compete with the wild-type product (“antimorphic,” MULLER 1932). In let-60(dn)/+ heterozygotes, let-60 activity is reduced more than in a heterozygote carrying one copy of a loss-of-function mutation (Jf/+), indicating that its function in vulval induction is disrupted. There are many possible ways that a mutant gene product can compete with a wild-type gene product and cause the dominant negative effects (reviewed by HERSKOWITZ 1987). For example, a let-60 gene product may normally form multimers, and the multimeric complex containing wild-type and mutant products could be defective in vulval induction.

A key component of a developmental switch: let-60 has the properties of a component of a developmental switch because its activity determines which of two alternative fates the six VPCs have. We propose that, in wild-type animals, let-60 activity is increased by the inductive signal. Mutations with opposite effects on let-60 activity have opposite consequences for VPC fates (Table 4). Loss or significant reduction of let-60 activity causes the VPCs to become the non-vulval cell type (C) even in the presence of inductive signal. In contrast, in lin-34 Muv mutants, all six VPCs become vulval cell types. Based on mapping results and genetic interactions between mutations of let-60 and lin-34, lin-34 Muv mutations appear to be either gain-of-function mutations of let-60 or gain-of-function mutations of an intimately related gene that elevates let-60 activity. In either case, lin-34(gf) mutations apparently result in elevation of let-60 activity. Thus, an increase of let-60 activity causes all six VPCs to become vulval cell types compared to the three in wild type, even in the absence of the inductive signal (Table 4). The site of let-60 action is unknown; however, we hypothesize that let-60 acts in the VPCs in the pathway of response to inductive signal because this is the simplest interpretation of existing data.

If let-60 and lin-34 are the same gene, changes of the gene activity caused by dominant negative (“antimorphic”) mutations let-60(dn) and gain-of-function (“hypermorphic”) mutations lin-34(gf) may be the consequence of qualitatively different changes in protein structure. For example, the let-60 product might contain a functional domain and a regulatory domain. The let-60(dn) Vul phenotype may result from defects in the functional (e.g., catalytic) domain, while the lin-34(gf) Muv phenotype may be caused by defects in the regulatory domain. The regulatory domain could be a site for interacting with a negative regulator, which would keep let-60 inactive until the VPC receives inductive signal.

let-60 appears to act in more than one aspect of C. elegans development. We have described that all the putative loss-of-function mutations and most of the dominant negative mutations are recessive lethal at an early larval stage. We have also described that the let-60(dn) mutations result in defects in male spicules and mating. The spicule defect of let-60(dn) males is due to at least one alteration in cell fate (H. CHAMBERLIN and P. W. STERNBERG, unpublished results). These observations suggest that let-60 acts in multiple cells during development.

let-60 function is regulated by let-23 and lin-15: Vulval induction is a complicated and multistep process. Along with other Muv and Vul genes, let-60 functions in one of the key steps in distinguishing

<table>
<thead>
<tr>
<th>let-60 genotype</th>
<th>+/- signal</th>
<th>let-60 activity</th>
<th>VPC fate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>+</td>
<td>High</td>
<td>Vulval [1+ or 2+]</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>Low</td>
<td>Nonvulval [3+</td>
</tr>
<tr>
<td>Mutants</td>
<td>+ or -</td>
<td>Always high</td>
<td>Vulval [1+ or 2+]</td>
</tr>
<tr>
<td></td>
<td>+ or -</td>
<td>Always low</td>
<td>Nonvulval [3+]</td>
</tr>
</tbody>
</table>

We propose that in each of the six VPCs, inductive signal indirectly regulates the let-60 activity which controls VPC fates. In the column marked "+/- signal," "+" means the individual VPC receives the signal from anchor cell, "-" means the individual VPC does not receive the signal either due to the position of the cell or due to elimination of the signal source by ablation of gonad cells (MATERIALS AND METHODS). let-60 activity levels are defined genetically: lin-34(gf) causes "high" activity (hyperactive), and let-60(dn) or let-60(dn) cause "low" activity.
Figure 7.—Functional relationship between let-60 and some other genes in the genetic pathway of vulval cell specification. Based on known genetic interactions (Ferguson, Sternberg and Horvitz 1987; Sternberg and Horvitz 1989) we propose the functional relationships between let-60 and some other genes in the pathway. The arrows indicate the positive regulation of one gene by another. “1°” bars indicate the negative regulation of one gene by another. The arrows and bars do not necessarily indicate a direct interaction. We propose that let-60 activity is positively regulated by inductive signal through let-23 and negatively controlled by lin-15 via let-23: let-60 controls the 1°- and 2°-specific functions through inhibition of lin-1: let-12 could act either in combination with lin-1 or downstream of lin-1 to specify 2° functions. The interaction between let-12 pathway and let-60 pathway might involve intercellular or autocrine signals (Sternberg and Horvitz 1989).

whether a VPC becomes a vulval cell type (1°, 2°) or a nonvulval cell type (3°) in response to an inductive signal. By studying genetic interactions between let-60 and other Muv or Vul genes, we can start to elucidate the functional relationship between these genes. The relationship between let-60 and other Vul and Muv genes is proposed as shown in Figure 7. Since the ordering of gene action is based on dominant mutations [lin-12(d), lin-34(gf), let-60(dn)] and possibly non-null recessive mutations (lin-15, lin-1), we regard these conclusions, which represent the simplest interpretations of our data, as tentative.

We propose that let-60 activity is positively controlled by let-23 activity. Again, this is based on our conclusion that lin-34(gf) are either gain-of-function alleles of let-60 or gain-of-function mutations of an intimately related gene that activates let-60. Both the lethal and vulvalless phenotypes of let-23 are suppressed by lin-34(gf) mutations (G. Jongeward and P.W. Sternberg, unpublished results; this study), and lin-34(gf) mutations result in a signal-independent Muv phenotype. In other words, a lin-34(gf) mutation bypasses the need for either inductive signal or let-23.

lin-15 is proposed to be a negative regulator of the vulval induction pathway acting before let-60, since a decrease in let-60 activity suppresses the Muv phenotype of lin-15. However, lin-15 could exert its negative effect on let-60 via let-23, since the lin-15 Muv phenotype is also suppressed by let-23 Vul mutations. If lin-15 interacts with let-60 via let-23 as proposed in Figure 7, the mutual suppression between lin-15(n309) and let-60(sy100dn) (Table 3) could be explained by an increase in let-23 activity in the lin-15(n309) background which compensates for the reduction in let-60 activity. It is known that to some extent, there is also mutual suppression between particular lin-15 and let-23 mutations (Sternberg and Horvitz 1989). This mutual suppression could result from partial defects in the lin-15 and let-23 gene products, which either have antagonistic regulatory effects on let-60 gene activity, or directly interact with each other. We do not believe that the controlling effect of the inductive signal on let-60 is exerted via lin-15, because the dependence on inductive signal is not relieved by the lin-15 mutation in a lin-15(n309); let-60(sy100dn)+ double mutant. Moreover, although a lin-15 mutation alone causes a signal-independent Muv phenotype, the exact pattern of VPC fates in a lin-15 mutant can be responsive to the inductive signal (Sternberg 1988). Furthermore, lin-15 most likely acts in cells other than the VPCs (R. Herman and E. Hedgecock, personal communication).

let-60 controls VPC fates via lin-1 and lin-12: lin-1 is proposed to act downstream of the let-60 gene because lin-1 mutations are epistatic to let-60 mutations (Figure 7). lin-1 mutations cause a Muv phenotype, and lin-1 might act as a negative regulator of the expression of 1°- and 2°-specific functions. lin-12 is proposed to act downstream of the let-60 gene in promoting the 2°-specific functions because dominant lin-12 mutations are epistatic to let-60 mutations with respect to the 2° cell fate. lin-12 is a component of a developmental switch specifying 2° vs. non-2° (1° or 3°) VPC fates (Greenwald, Sternberg and Horvitz 1983; Sternberg and Horvitz 1989). In contrast, let-60 is a component of a developmental switch specifying 3° vs. non-3° VPC fates (1° or 2°). The Vul/Muv pathway is likely to control, at least in part, the activity of lin-12 (Sternberg and Horvitz 1989).

It is not known whether the interaction of these pathways occurs within the same VPC or via intercellular signals. The precise pattern of VPC fates is established by the combined action of these two pathways. The activity states of let-60 and lin-12 define the action of each pathway.

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