New Genes That Interact With *lin-35 Rb* to Negatively Regulate the *let-60 ras* Pathway in *Caenorhabditis elegans*

Jeffrey H. Thomas,[1,2] Craig J. Ceol,[2] Hillel T. Schwartz and H. Robert Horvitz[3]

Department of Biology, Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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ABSTRACT

Previous studies have shown that a synthetic multivulva phenotype results from mutations in genes that antagonize the *ras*-mediated intercellular signaling system responsible for vulval induction in *Caenorhabditis elegans*. Synthetic multivulva mutations define two classes of genes, A and B, and a mutation in a gene of each class is required to produce the multivulva phenotype. The ectopic vulval tissue in multivulva animals is generated by vulval precursor cells that in the wild type do not generate vulval tissue. One of the class B synthetic multivulva genes, *lin-35*, encodes a protein similar to the retinoblastoma (Rb) protein. In this article, we describe the isolation and characterization of 50 synthetic multivulva mutations, the identification of new components of both the class A and class B *lin-35 Rb* pathways, and the cloning of *lin-52*, a class B gene that may have a conserved role in Rb-mediated signaling.

A receptor tyrosine kinase (RTK) and Ras-mediated signal transduction pathway induces vulval cell fates during the development of the vulva of the *Caenorhabditis elegans* hermaphrodite (Aroian *et al.* 1990; Beitel *et al.* 1990; Han and Sternberg 1990). Little is known about how the activities of such pathways can be negatively regulated. The synthetic multivulva (synMuv) genes act as negative regulators of vulval development (Horvitz and Sulston 1980; Sulston and Horvitz 1981; Ferguson and Horvitz 1985, 1989). One of the synMuv genes, *lin-35*, is a member of the retinoblastoma (Rb) gene family; one member of this family, Rb, acts as a tumor suppressor gene in mammals. Another synMuv gene, *lin-53*, shows similarity to RbAp48, an Rb-binding protein (Lu and Horvitz 1998). Thus, the synMuv genes provide an opportunity to analyze genetically a pathway containing an Rb-like gene, define additional components of this pathway, and elucidate the mechanism by which an Rb-like protein antagonizes a process stimulated by a Ras protein.

The hermaphrodite vulva of *C. elegans* is formed from the descendants of three hypodermal blast cells, P5.p, P6.p, and P7.p (Sulston and Horvitz 1977). These cells are members of the vulval equivalence group, P(3–8).p, a set of six cells with the potential to adopt either of two vulval fates (1° or 2°) or a nonvulval fate (3°; Sulston and White 1980; Kimble 1981; Sternberg and Horvitz 1986). These cell fates are specified by cell interactions. The gonadal anchor cell induces the nearest P(3–8).p cells to adopt vulval fates (Kimble 1981; Sternberg and Horvitz 1986; Thomas *et al.* 1990). Another signal, apparently from the nearby hypodermal syncytium, hyp7, acts to inhibit the adoption of vulval fates (Herman and Hedgecock 1990). It is likely that the anchor cell signal overrides this inhibitory signal to induce the cells nearest to the anchor cell to adopt vulval fates.

Genetic analysis of vulval development has led to the identification and characterization of numerous genes involved in different aspects of this process (for reviews, see Horvitz and Sternberg 1991; Kornfeld 1997; Sternberg and Han 1998). Reduction-of-function mutations in genes encoding signaling proteins reduce the output of the anchor cell signaling pathway and result in a vulvaless (Vul) phenotype in which the vulva is not formed. By contrast, some mutations result in a multivulva (Muv) phenotype in which ectopic vulval tissue is produced. The Muv phenotype of certain mutant strains results from the interaction of two different mutations (Horvitz and Sulston 1980; Sulston and Horvitz 1981; Ferguson and Horvitz 1985, 1989). The mutations that interact to produce such a synMuv phenotype fall into two classes, A and B. Animals carrying both a class A and a class B mutation have a Muv phenotype. Animals that carry mutations in a single class have a wild-type vulval phenotype. Ferguson and Horvitz (1989) proposed that the synMuv genes encode the components of two functionally redundant pathways that negatively regulate vulval development.

Systematic mutagenesis of strains carrying either the class A mutation *lin-8(n111)* or the class B mutation *lin-9 (n112)* as well as the mutagenesis of another strain carrying a previously undetected class A synMuv mutation

[1]*Present address:* Department of Molecular Biology, Princeton University, Princeton, NJ 08544.

[2]These authors contributed equally to this work.

[3]*Corresponding author:* Department of Biology, Howard Hughes Medical Institute, Rm. 68-425, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, MA 02139. E-mail: horvitz@mit.edu

allowed the identification of additional class A and class B mutations (Ferguson and Horvitz 1989). Both class A and class B alleles, as well as class AB alleles (which are Muv as a consequence of a single mutation), were identified for a locus named *lin-15*, indicating that *lin-15* is a complex locus with distinct class A and class B functions (Ferguson and Horvitz 1985, 1989). Molecular analyses of *lin-15* revealed that it consists of two adjacent genes that encode two nonoverlapping transcripts to control the A and B functions; the class AB alleles affect both genes (Clark *et al.* 1994; Huang *et al.* 1994). These genetic analyses resulted in the identification and characterization of three class A genes (*lin-8*, *lin-15*A, and *lin-38*) and five class B genes (*lin-9*, *lin-15*B, *lin-35*, *lin-36*, and *lin-37*). Three additional class B mutations (*n770*, *n771*, and *n833*) were identified, but were neither further characterized nor given gene names (Ferguson and Horvitz 1989).

SynMuv mutants in which the anchor cell has been ablated nonetheless still display a Muv phenotype (Ferguson *et al.* 1987). This result suggests that in the absence of synMuv gene activity, the P(3–8).p cells do not require the anchor cell signal to adopt vulval cell fates. However, reduction-of-function mutations in genes known to be involved in inductive signal transduction, *let-23 rtk*, *sem-5*, *let-60 ras*, and *lin-45 raf*, are epistatic to synMuv mutations (Ferguson *et al.* 1987; Beitel *et al.* 1990; Han and Sternberg 1990; Clark *et al.* 1992; Han *et al.* 1993; Huang *et al.* 1994; Lu and Horvitz 1998; Thomas and Horvitz 1999). Thus, the activity of the RTK signal transduction cascade, but not the anchor cell-derived RTK ligand itself, is required for the adoption of vulval cell fates by P(3–8).p cells in the absence of inhibitory synMuv gene activity. Genetic mosaic analyses indicate that both *lin-15*AB and *lin-37* act non-cell autonomously and most likely in hyp7 (Herman and Hedgecock 1990; Hedgecock and Herman 1995) while *lin-36* likely acts cell autonomously in the Pn.p cells (Thomas and Horvitz 1999). These observations led to the suggestion that the synMuv genes encode the components of two signaling systems by which hyp7 prevents P(3–8).p cells from adopting vulval fates. When both redundant signaling systems are disabled, P(3–8).p cells adopt vulval fates and produce a Muv phenotype.

The molecular natures of several synMuv genes have been determined. Two class B genes, *lin-15*B and *lin-36*, and one class A gene, *lin-15*A, have been cloned and shown to encode novel proteins (Clark *et al.* 1994; Huang *et al.* 1994; Thomas and Horvitz 1999). The class B gene *lin-9* encodes a protein with sequence similarity to the Drosophila Aly protein, which regulates the meiotic cell cycle and spermatogenesis (Beitel *et al.* 2000; White-Cooper *et al.* 2000). The class B gene *lin-35* encodes a protein with sequence similarity to the Rb protein, and the class B gene *lin-53* encodes a protein with sequence similarity to RbAp48, an Rb-binding protein (Lu and Horvitz 1998). The class B gene *dpl-1*, the discovery of which is described in this manuscript, encodes a protein similar to DP, an Rb-regulated transcription factor that regulates the G1-to-S phase transition of the cell cycle. The class B gene *efl-1* encodes a protein similar to E2F, a component of the DP/E2F heterodimeric transcription factor (Ceol and Horvitz 2001). Another gene with class B activity, *tam-1*, encodes a RING finger and B-box protein involved in modulating gene expression (Hsieh *et al.* 1999). *lin-13*, a gene that has class B and possibly also class A synMuv activity, encodes a protein with an Rb-binding motif (Meléndez and Greenwald 2000). Genetic analysis of *let-418/chd-4*, which encodes a chromodomain helicase protein, indicates that it is a class B synMuv gene (Von Zelewsky *et al.* 2000). *mep-1*, which encodes a zinc-finger protein that interacts with LET-418, has class B synMuv activity (Unhavaithaya *et al.* 2002). RNAi experiments suggest that *hda-1* and *hpl-2*, which encode a protein similar to class I histone deacetylases and a protein similar to heterochromatin protein 1, respectively, may have some synMuv activity (Lu and Horvitz 1998; Couteau *et al.* 2002). Dufourcq *et al.* (2002) reported that *hda-1/gon-10(e1795)* does not have class B synMuv activity but other results using stronger class A synMuv mutations suggest that *hda-1/gon-10(e1795)* does have class B synMuv activity (C. J. Ceol, E. C. Andersen and H. R. Horvitz, unpublished results). Many of these genes are known as components of a nucleosome-remodeling and histone deacetylase (NuRD) complex, and the class B synMuv genes have been proposed to remodel chromatin and repress transcription of genes important for vulval cell fate specification (Lu and Horvitz 1998; Von Zelewsky *et al.* 2000). Some of the class B synMuv genes are involved in the promotion of early larval P3.p fusion and G1-to-S phase progression of the cell cycle (Chen and Han 2001; Boxem and Van Den Heuvel 2002).

In this article, we identify and characterize 50 new synMuv mutations. Some of these mutations define new class A and class B loci. *lin-52*, one of the new class B loci, encodes a protein that is similar to mammalian and Drosophila proteins of unknown function. Because *lin-52* has genetic properties similar to *lin-35* Rb, LIN-52 homologs may act in an Rb pathway in mammals.

MATERIALS AND METHODS

Strains and general techniques: *Caenorhabditis elegans* var. Bristol strain N2 was the wild-type strain used in this study. To map *lin-52*, we used the strain RW7000, which contains the polymorphism *stP127* (Williams *et al.* 1992). Mutations were described by Hodgkin *et al.* (1988) unless otherwise noted.

LGI: *bli-3(e767)*, *sup-11(n403)*, *dpy-5(e61)*, *lin-35(n745)*, *unc-29 (e1072)*, *dpy-14(e188)*, *unc-13(e1091)*, *lin-11(n566)*, *unc-75 (e950)*, *unc-101(m1)*, *unc-54(e1092)* (Waterston *et al.* 1980).

LGII: *lin-8(n111)*, *lin-31(n301)*, *unc-85(e1414)*, *bli-2(e768)*, *dpy-10(e128)*, *rol-6(e187)*, *let-23(n1045, mn23, mn216)* (Herman 1978; Sigurdson *et al.* 1984; Ferguson and Horvitz 1985),

let-240(mn209), *unc-4(e120)*, *unc-53(e569)*, *rol-1(e91)*, *lin-38 (n751)*, *unc-52(e444)*, *mnDf67* (Sigurdson *et al.* 1984), *mnDf85* (Sigurdson *et al.* 1984), *mnDf46* (Sigurdson *et al.* 1984), *mnC1[dpy-10(e128) unc-52(e444)]*.

LGIII: *dpy-1(e1)*, *unc-93(e1500)*, *dpy-27(y57)* (Plenefisch *et al.* 1989), *unc-79(e1068)*, *dpy-17(e164)*, *lon-1(e185)*, *sma-3(e491)*, *lin-37(n758)*, *egl-5(n945)*, *lin-36(n766)*, *nDf40* (Hengartner *et al.* 1992), *unc-36(e251)*, *dpy-19(e1259)*, *lin-9(n112)*, *sqv-3 (n2842)*, *unc-32(e189)*, *unc-16(e109)*, *unc-47(e307)*, *unc-69 (e587)*, *unc-25(e156)*, *unc-49(e382)*, *dpy-18(e364)*, *qC1[dpy-19 (e1259) glp-1(q339)]* (Austin and Kimble 1989; Graham and Kimble 1993).

LGIV: *dpy-9(e12)*, *egl-18(n162)*, *lin-1(e1275)*, *unc-17(e245)*, *unc-5 (e53)*, *dpy-20(e1282)*, *unc-22(e66)*, *unc-30(e191)*, *lev-1(x22)*, *ced-3(n717)*, *unc-26(e205)*, *dpy-4(e1166)*.

LGV: *unc-34(e566)*, *dpy-11(e224)*, *unc-51(e369)*.

LGX: *lon-2(e678)*, *unc-3(e151)*, *lin-15(n433, n744, n765, n767)* (Ferguson and Horvitz 1989).

In addition, we used strains containing the chromosomal aberration *eT1(III;V)* (Rosenbluth and Baillie 1981). Methods for the culture and genetic manipulation of *C. elegans* have been described by Brenner (1974). Genetic nomenclature was described by Horvitz *et al.* (1979). Nomenclature for synMuv strains is as used by Ferguson and Horvitz (1989).

Mutagenesis of class A and class B mutants: Screens for new synMuv strains were conducted essentially as described by Ferguson and Horvitz (1989), using EMS as a mutagen, according to Brenner (1974). Only one Muv strain was selected from each group of mutagenized P_0 hermaphrodites for subsequent analysis to ensure that each mutation was independently derived. N2 males were crossed with the Muv strains, and the F_2 progeny were scored for the Muv phenotype. The segregation of 1/16 or fewer Muv F_2 progeny indicated candidate synMuv strains. In no case did the Muv phenotype of a strain in which the original mutation was autosomal result from a synthetic interaction with a second mutation on the same linkage group, on the basis of either of two criteria: (1) failure of the new Muv mutation to complement a known Muv mutation or (2) linkage of the Muv phenotype to a chromosome in addition to the chromosome containing the parental mutation. In mutagenesis experiments in which the original mutation was a *lin-15* allele, strains that segregated 1/4 or fewer Muv F_2 progeny were also retained for further analysis, since new *lin-15* mutations would be tightly linked to the parental mutation. All candidate synMuv strains were backcrossed to their strain of origin two to five times.

To isolate class B mutations, a *lin-8(n111)* homozygous strain and a *lin-15(n433)* homozygous strain were mutagenized. Ferguson and Horvitz (1989) previously mutagenized *lin-8 (n111)* and *lin-15(n767)* animals to isolate class B mutations. After the mutagenesis of *lin-8(n111)* animals, we screened ~6000 haploid genomes and isolated 15 synMuv strains. After the mutagenesis of *lin-15(n433)* animals, we screened ~10,000 haploid genomes and isolated 15 synMuv strains. To isolate class A mutations, a *lin-36(n766)* homozygous strain and a *lin-15(n744)* homozygous strain were mutagenized. Ferguson and Horvitz (1989) previously mutagenized *lin-9(n112)* animals to isolate class A mutations. After the mutagenesis of *lin-36(n766)* animals, we screened ~10,000 haploid genomes and isolated 5 synMuv strains. After the mutagenesis of *lin-15 (n744)* animals, we screened ~13,000 haploid genomes and isolated 14 synMuv strains.

The *lin-52* mutation *n3718* was isolated following mutagenesis of a *lin-15(n767)* homozygous mutant strain (C. J. Ceol, F. Stegmeier, M. M. Harrison and H. R. Horvitz, unpublished results). Other results of this screen are described elsewhere.

Molecular analysis of *lin-15*AB lesions: Genomic DNA was purified, essentially using standard methods, from *lin-15*AB strains isolated after the mutageneses of *lin-15(n744)* and *lin-15(n433)* animals (Sulston and Hodgkin 1988). DNA was digested by *Eco*RI, separated by agarose gel electrophoresis, and probed with ^{32}P-labeled *lin-15* plasmid DNA (Sambrook *et al.* 1989; Clark *et al.* 1994). Some samples of genomic DNA that showed a lesion were digested with *Eco*RI and *Eag*I, *Sac*I, *Msc*I, *Bgl*I, *Mlu*I, or *Nru*I and probed with ^{32}P-labeled *lin-15* plasmid DNA.

Molecular analysis of *lin-13* and *lin-52* lesions: N2, *lin-13*, and *lin-52* strains were lysed and the coding regions and adjacent noncoding regions of the *lin-13* and *lin-52* genes were amplified using the polymerase chain reaction (PCR). The sequences of the PCR products were determined using an automated ABI 373A cycle sequencer (Applied Biosystems, Foster City, CA). The sequence of each mutation was confirmed using an independently derived PCR product.

Nomarski observation and P(3–8).p cell lineage analysis of *lin-54* animals: P(3–8).p cells and their descendants in *lin-8 (n111); lin-54(n2231)* animals were observed using Nomarski optics at different times during vulval development as described by Sulston and Horvitz (1977). The nomenclature and criteria of Sternberg and Horvitz (1986, 1989) were used to describe and assign 1°, 2°, and 3° cell fates.

Construction of strains homozygous for newly isolated synMuv mutations: Strains carrying a single homozygous synMuv mutation were constructed and their genotypes confirmed essentially as described by Ferguson and Horvitz (1989). In these experiments, *unc-79 dpy-27* balanced *lin-13(n770)*, *unc-69* balanced *lin-52(n771)*, *unc-29* balanced *lin-53(n833)*, *unc-22 ced-3 unc-26* balanced *lin-54(n2231)*, and *rol-6 unc-4* balanced both *dpl-1(n2994)* and *lin-56(n2728)*.

Construction of unlinked synMuv double mutants: Class A; class A or class B; class B double mutants carrying a new mutation and a *lin-15* mutation of the same class were constructed essentially as described by Ferguson and Horvitz (1989). Class B; class B double mutants carrying a new mutation and an autosomal mutation of the same class were also constructed essentially as described by Ferguson and Horvitz (1989). To ensure that mutations were not lost by recombination, several independent lines were isolated for each strain.

In these constructions, *lin-15(n767)* and *lin-15(n744)* were used as the class A and class B *lin-15* alleles, respectively. The autosomal class B mutation used was *lin-36(n766)*. This allele was marked *in cis* by *unc-32*. The following markers were linked *in cis* to the new mutations: *unc-32* to *lin-13(n770)*, *unc-32* to *lin-52(n771)*, *dpy-5* to *lin-53(n833)*, *dpy-20* to *lin-54(n2231)*, *rol-6* to *dpl-1(n2994)*, and *rol-6* to *lin-56(n2728)*.

Construction of linked synMuv double mutants: To construct a class B class B double mutant between *lin-13(n770)* and *lin-36*, hermaphrodites of genotype *lin-13 + + unc-32/+ egl-5 lin-36 +; lin-15*A were generated. The frequency of recombination between *lin-13* and *egl-5* is greater than that between *lin-36* and *unc-32* and much greater than that between *egl-5* and *lin-36*. Muv non-Unc non-Egl recombinant progeny were isolated; from these animals, Egl Muv progeny were selected and the *lin-15*A mutation was crossed out, yielding animals of putative homozygous genotype *lin-13 egl-5 lin-36*. This genotype was confirmed by crossing with another class A mutation and performing complementation tests with *lin-13* and with *lin-36* to show that the strain contained both class B mutations.

A class B class B double mutant between *lin-36* and *lin-52 (n771)* was constructed in a manner similar to that for the construction of the double between *lin-13* and *lin-36*. Hermaphrodites of genotype *+ lin-36 unc-36 +/sma-3 + + lin-52; lin-15*A were generated. The frequency of recombination between *unc-36* and *lin-52* is greater than that between *sma-3* and *lin-36*

and much greater than that between *lin-36* and *unc-36*. Muv non-Sma recombinant progeny were isolated and used to generate Unc Muv progeny of putative genotype *lin-36 unc-36 lin-52; lin-15*A. *lin-15*A was removed to generate animals of homozygous genotype *lin-36 unc-36 lin-52*. This genotype was confirmed by crossing with another class A mutation and performing complementation tests with *lin-52* and with *lin-36* to show that the strain contained both class B mutations.

To construct a class A class A double mutant between *lin-8* and *lin-56(n2728)*, animals of genotype *lin-8 unc-85 dpy-10 + +/+ + + rol-6 lin-56; lin-15*B/+ were constructed. From these animals, Unc non-Dpy non-Muv recombinant animals that did not segregate Muv progeny were isolated; these animals segregated Rol Unc animals of putative genotype *lin-8 unc-85 rol-6 lin-56*. This genotype was confirmed by conducting complementation tests, in the presence of a class B mutation, with *lin-8* and *lin-56* to show that the strain was homozygous for both class A mutations.

Transgenic animals: Germline transformation was performed as described by Mello *et al.* (1991) by injecting cosmid (5–10 ng/μl) or plasmid (50–80 ng/μl) DNA into *lin-52 (n771); lin-15(n767)* animals. pRF4, which causes a dominant Rol phenotype, was used as a co-injection marker.

***lin-52* cDNA isolation and RNA-mediated interference:** We obtained a partial *lin-52* cDNA clone (kindly provided by Yuji Kohara), yk253b12, that included 249 nucleotides of the *lin-52* open reading frame and also included the 3′ untranslated region and a poly(A) tail. We used the 5′ rapid amplification of cDNA ends (RACE) system v2.0 (GIBCO-BRL, Gaithersburg, MD) to determine the 5′ end of the *lin-52* transcript. *lin-52* 5′ RACE products were *trans*-spliced to the SL2 leader sequence. Consistent with our observations, Zorio *et al.* (1994) previously found that an SL2 oligonucleotide could be used to PCR amplify the gene sequence corresponding to ZK632.13, the predicted gene we have identified as *lin-52*. RNA-mediated interference (RNAi) was conducted as described by Fire *et al.* (1998), using double-stranded RNA corresponding to the full-length *lin-52* cDNA.

RESULTS

Isolation of new synMuv strains: To identify new class A mutations, we used EMS to mutagenize class B *lin-36 (n766)* or class B *lin-15(n744)* homozygotes, which display wild-type vulval development. The vulval morphology of synMuv strains can be readily distinguished from that of Muv strains mutant in *lin-1* or *lin-31*. SynMuv animals usually have a vulva with wild-type morphology and have a few regularly spaced pseudovulvae. By contrast, *lin-1* animals frequently have abnormal and distinctively protruding vulvae, and *lin-31* animals are often egg-laying defective, have incomplete vulvae, and have a variable number of small pseudovulval protrusions that are distinctive in number and morphology. A Muv phenotype that segregated as 1/16 or less in the F_2 generation after crossing with wild-type males was considered a candidate for being synMuv, as was a strain obtained in a *lin-15* background that segregated as either 1/16 or 1/4 because new *lin-15* mutations would be linked to the parental *lin-15* mutation. We obtained five synMuv strains from the mutagenesis of *lin-36(n766)* animals and 14 synMuv strains from the mutagenesis of *lin-15(n744)* animals. A total of 19 new class A mutations were identified (Table 1).

To identify new class B mutations, we used EMS to mutagenize animals homozygous for the class A mutations *lin-8(n111)* or *lin-15(n433)*. Muv strains were tested to determine if their phenotypes depended upon two unlinked loci as described for the isolation of class A mutations. We obtained 15 synMuv strains from the mutagenesis of *lin-8(n111)* animals and 15 synMuv strains from the mutagenesis of *lin-15(n433)* animals. A total of 30 new class B mutations were identified in these screens (Table 1).

An additional class B mutation, *n3718*, was obtained in a screen for synMuv mutants following the mutagenesis of *lin-15(n767)* animals (C. J. Ceol, F. Stegmeier, M. M. Harrison and H. R. Horvitz, unpublished results).

Linkage and complementation: SynMuv mutations already shown to segregate as two unlinked loci were expected to display linkage to two loci: the parental locus and the new locus (Ferguson and Horvitz 1989). Mutations caused by most of the candidate synMuv strains displayed linkage to the parental locus and to another linkage group. A few *lin-15* strains, discussed below, did not display linkage to a new location (Table 1).

Newly isolated synMuv mutations were tested for complementation with alleles of the then-known synMuv genes, *lin-8*, *lin-9*, *lin-15*A, *lin-15*B, *lin-35*, *lin-36*, *lin-37*, *lin-38*, and *lin(n770)*, *lin(n771)*, and *lin(n833)*, three previously identified but not extensively characterized synMuv mutations (Ferguson and Horvitz 1989). Mutations that complemented all known synMuv complementation groups were tested against each other after strains carrying identical parental mutations of the opposite class were constructed.

Mutations were assigned to the same complementation group only if hermaphrodites of genotype *a; b1/b2*, where *a* is the background mutation required for the synthetic interaction and *b1* and *b2* are the two mutations being tested, were Muv and segregated only Muv progeny. As described by Ferguson and Horvitz (1989), this approach was necessary to distinguish intragenic noncomplementation from the intergenic noncomplementation observed in some doubly heterozygous synMuv strains. Several combinations of genotype *a; b1/+; b2/+* displayed intergenic noncomplementation; in most cases, the penetrance and expressivity of the Muv phenotype produced by intergenic noncomplementation was lower than that produced by homozygosity at either of the two loci that displayed intergenic noncomplementation. Thus, animals lacking the activity of a synMuv gene of one class and having reduced doses of two genes of the other class as well as no maternal activity from one of these genes were occasionally Muv. This observation suggests that the synMuv genes are dose sensitive. *lin-53 (n833)* (see below) was notable in that it showed very strong intergenic noncomplementation with other class B mutations, consistent with the observation that this

TABLE 1

Origins, chromosomal linkages, and phenotypes of new synthetic Multivulva strains

Genotype	Penetrance of Muv phenotype (%)		Strain growth		
	15°	20°	15°	20°	25°
lin-8(n111) II; lin-13(n2238) III	65 (n = 391)	100 (n = 74)	Very slow	Slow	Inviable
lin-8(n111) II; lin-15(n2230) X	20 (n = 254)	93 (n = 376)	Slow	WT	Slow
lin-8(n111) II; lin-15(n2233) X	100 (n = 235)	100 (n = 385)	Slow	WT	Slow
lin-8(n111) II; lin-15(n2241) X	100 (n = 130)	100 (n = 238)	Slow	WT	Inviable
lin-8(n111) II; lin-15(n2244) X	91 (n = 128)	99.6 (n = 280)	WT	WT	Slow
lin-8(n111) II; lin-15(n2245) X	6 (n = 202)	98 (n = 306)	WT	WT	WT
lin-35(n2232) I; lin-8(n111) II	100 (n = 200)	99.9 (n = 942)	Inviable	WT	Inviable
lin-35(n2236) I; lin-8(n111) II	99 (n = 96)	100 (n = 97)	Inviable	Slow	Inviable
lin-35(n2239) I; lin-8(n111) II	100 (n = 197)	100 (n = 290)	Inviable	WT	Slow
lin-35(n2242) I; lin-8(n111) II	ND	100 (n = 278)	Inviable	Slow	Very slow
lin-8(n111) II; lin-36(n2235) III	13 (n = 279)	79 (n = 313)	WT	WT	WT
lin-8(n111) II; lin-36(n2240) III	7 (n = 189)	79 (n = 373)	WT	WT	WT
lin-8(n111) II; lin-36(n2243) III	51 (n = 115)	94 (n = 593)	WT	WT	WT
lin-8(n111) II; lin-37(n2234) III	96 (n = 192)	100 (n = 278)	WT	WT	Inviable
lin-8(n111) II; lin-54(n2231) IV	39 (n = 334)	99 (n = 164)	Very slow	WT	Slow
lin-13(n2981) III; lin-15(n433) X	0 (n = 237)	84 (n = 241)	WT	WT	Inviable
lin-13(n2984) III; lin-15(n433) X	1 (n = 217)	97 (n = 239)	WT	WT	Inviable
lin-13(n2985) III; lin-15(n433) X	2 (n = 229)	94 (n = 213)	WT	WT	Slow
lin-13(n2988) III; lin-15(n433) X	33 (n = 247)	97 (n = 261)	Slow	Slow	Inviable
lin-15(n2980 n433) X[a]	2 (n = 209)	99 (n = 252)	WT	WT	Slow
lin-15(n2983 n433) X[a]	2 (n = 232)	99 (n = 227)	WT	WT	Slow
lin-15(n2987 n433) X[a]	0 (n = 207)	91 (n = 267)	WT	WT	WT
lin-15(n2989 n433) X[a]	0 (n = 238)	100 (n = 235)	WT	WT	WT
lin-15(n2991 n433) X[a]	2 (n = 226)	100 (n = 216)	WT	WT	WT
lin-15(n2993 n433) X[a]	0 (n = 205)	79 (n = 201)	WT	WT	WT
lin-35(n2977) I; lin-15(n433) X	8 (n = 257)	100 (n = 234)	Slow	WT	Slow
lin-35(n2996) I; lin-15(n433) X	18 (n = 216)	100 (n = 202)	Slow	Slow	Slow
lin-53(n2978) I; lin-15(n433) X	0 (n = 211)	59 (n = 203)	WT	WT	WT
lin-54(n2990) IV; lin-15(n433) X	4 (n = 199)	95 (n = 216)	WT	WT	Slow
dpl-1(n2994) II; lin-15(n433) X	4 (n = 246)	78 (n = 234)	WT	WT	Slow
lin-52(n3718) III; lin-15(n767) X	100 (n = 41)	100 (n = 82)	—[b]	—[b]	—[b]
lin-8(n2376) II; lin-36(n766) III	18 (n = 382)	96 (n = 189)	WT	WT	WT
lin-8(n2378) II; lin-36(n766) III	19 (n = 456)	100 (n = 125)	WT	WT	WT
lin-8(n2403) II; lin-36(n766) III	48 (n = 402)	99 (n = 549)	WT	WT	WT
lin-36(n766) III; lin-15(n2375) X	0 (n = 544)	82 (n = 675)	WT	WT	Slow
lin-38(n2402) II; lin-36(n766) III	28 (n = 643)	99.7 (n = 667)	WT	WT	WT
lin-8(n2724) II; lin-15(n744) X	100 (n = 211)	100 (n = 126)	Slow	Slow	Slow
lin-8(n2731) II; lin-15(n744) X	100 (n = 217)	100 (n = 167)	Slow	Slow	Slow
lin-8(n2738) II; lin-15(n744) X	100 (n = 128)	100 (n = 140)	Slow	Slow	Very slow
lin-8(n2739) II; lin-15(n744) X	100 (n = 158)	100 (n = 97)	Slow	Slow	Slow
lin-8(n2741) II; lin-15(n744) X	100 (n = 157)	100 (n = 155)	WT	Slow	Slow
lin-15(n744 n2725) X[a]	100 (n = 152)	100 (n = 177)	Slow	Slow	Very slow
lin-15(n744 n2726) X[a]	100 (n = 141)	100 (n = 145)	Slow	Slow	Very slow
lin-15(n744 n2733) X[a]	100 (n = 193)	100 (n = 140)	Slow	Slow	Inviable
lin-15(n744 n2734) X[a]	100 (n = 124)	100 (n = 159)	Slow	Slow	Slow
lin-15(n744 n2735) X[a]	100 (n = 132)	100 (n = 176)	Slow	Slow	Very slow
lin-15(n744 n2737) X[a]	99 (n = 199)	100 (n = 121)	Slow	Slow	Inviable
lin-15(n744 n2742) X[a]	100 (n = 121)	100 (n = 173)	Slow	Slow	Very slow
lin-38(n2727) II; lin-15(n744) X	100 (n = 201)	100 (n = 165)	Slow	Slow	Slow
lin-56(n2728) II; lin-15(n744) X	100 (n = 214)	100 (n = 163)	Slow	Slow	Slow

New synMuv mutations were mapped to linkage groups using strains carrying the markers *bli-3 I*, *dpy-5 I*, *unc-54 I*, *unc-85 II*, *bli-2 II*, *mnC1 dpy-10 unc-52 II*, *unc-52 II*, *dpy-1 III*, *unc-32 III*, *unc-25 III*, *dpy-9 IV*, *egl-18 IV*, *unc-5 IV*, *dpy-4 IV*, *unc-34 V*, *dpy-11 V*, *unc-51 V*, *lon-2 X*, and *unc-3 X* in a manner similar to that described previously (Trent *et al.* 1983; Ferguson and Horvitz 1989). The penetrance of the Muv phenotype of each synMuv strain was determined at 15° and 20° after growth at the indicated temperature for two or more generations. Several strains displayed a temperature-dependent reduction in viability. This reduction in viability was tested in an assay similar to that of Ferguson and Horvitz (1989) but differing in that exactly 10 eggs laid by hermaphrodites of the indicated genotype grown at 20° were placed on each of the four assay plates (each with a 2-cm diameter lawn of bacteria) used at each temperature. The plates were checked daily to determine when the bacterial lawn was consumed. The data are presented according to the following criteria: at 15°, wild type (WT), 8.5–14 days; slow, 14–24 days; very slow, 24–28 days; inviable, lethal or >28 days. At 20°, WT, 5.5–9 days; slow, 9–17 days; very slow, 17–28 days; inviable, lethal or >28 days. At 25°, WT, 5–7.5 days; slow, 7.5–15 days; very slow, 15–28 days; inviable, lethal or >28 days. The last value of the range described was included in that category. The data obtained from the WT strain N2 were 15°, 10 days; 20°, 6 days; 25°, 5 days. ND, not determined because the strain was inviable at the listed temperature.

[a] The mutations in these strains displayed linkage only to *unc-3 X*. Linkage of the new mutation to *unc-3 X* is assumed since the mutations in these strains segregated as single-locus Muv mutations and failed to complement *lin-15(n765) X*.

[b] As the *lin-52(n3718)* mutation causes recessive sterility, the growth rate of *lin-52(n3718)* mutants derived from *lin-52(n3718)* homozygous parents could not be measured.

mutation causes dominant-negative activity (Lu and Horvitz 1998).

A total of 38 mutations failed to complement alleles of known synMuv genes. These mutations included 8 *lin-8* alleles, 8 *lin-15*A alleles, 10 *lin-15*B alleles, 6 *lin-35* alleles, 3 *lin-36* alleles, 1 *lin-37* allele, and 2 *lin-38* alleles. Another six mutations failed to complement *n770* or *n833*, mutations that had previously been isolated but not extensively characterized. The mutations that defined the *n770* complementation group failed to complement *lin-13* for class B activity. We named the other gene, defined by *n833*, *lin-53*. Our new mutations included 5 *lin-13* class B alleles and 1 *lin-53* allele. We obtained no new alleles of *lin-9*. *n771*, another mutation that had been previously isolated but not extensively characterized, defined the gene we named *lin-52*. We obtained one new *lin-52* mutation in a separate screen. Another four mutations defined three new complementation groups, which we named *lin-54*, *dpl-1*, and *lin-56*. [The name *dpl-1* was assigned after studies by Ceol and Horvitz (2001) showed the DPL-1 protein to be similar to mammalian DP.] There were 2 *lin-54* alleles, 1 *dpl-1* allele, and 1 *lin-56* allele (Table 1).

Identification of *lin-15*AB double mutants: The mutations of several Muv strains isolated in a *lin-15*A or *lin-15*B background did not segregate as two loci yet displayed a Muv phenotype similar to that of synMuv strains. These strains included five isolated in a *lin-15(n433)* background and seven isolated in a *lin-15(n744)* background. The Muv phenotype of these strains showed linkage only to *unc-3 X*, which marked the parental *lin-15* mutation, and failed to complement *lin-15(n765)*, a *lin-15* allele defective in both class A and class B activities. Thus, the new strains are defective in both *lin-15*A and *lin-15*B activities and they carry new *lin-15* alleles.

Six of the seven *lin-15* Muv mutants that have been isolated as single mutants and analyzed—all but *lin-15 (n765)*—have gross mutations that disrupt both *lin-15* mRNAs (Clark *et al.* 1994; Huang *et al.* 1994). *lin-15 (n765)* is a deletion in the class B gene and presumably also has a point mutation in the class A gene. Several *lin-15*A and *lin-15*B mutations have been analyzed molecularly; each specifically affects only one of the two genes (Clark *et al.* 1994).

To determine whether the Muv phenotype of each of the *lin-15*AB mutants isolated in these screens is the result of the newly induced mutation alone or rather the result of an interaction between the newly induced mutation and the parental mutation, we used Southern hybridization to analyze the *lin-15* locus in these strains. Four of the 12 mutant strains showed polymorphisms, 3 of which were confined to only the A or only the B region. Specifically, the *lin-15(n2993 n433)* strain has a loss of an *Eco*RI site in the B region of *lin-15*, the *lin-15 (n744 n2733)* strain has a small deletion of 0.3 kb in the A region, and the *lin-15(n744 n2735)* strain has a larger deletion of several kilobases in the A region.

By contrast, the *lin-15(n744 n2726)* strain has a deletion of about 0.9 kb in an *Eco*RI-*Sac*I restriction fragment containing both A and B sequences. This region includes both the start of the class A mRNA and the end of the class B mRNA. The deletion probably eliminates the 5′ end of the class A mRNA and may eliminate some of the 3′ end of the class B mRNA. This deletion may be sufficient to cause a class B defect, and the Muv phenotype of this strain may result entirely from the new mutation, *n2726*.

Polymorphisms were not detected in the other *lin-15* strains. Since the parental mutation of these strains is either a *lin-15*A or a *lin-15*B point mutation, and EMS produces predominantly point mutations (Anderson 1995), it is likely that the Muv phenotype of most if not all of these *lin-15*AB strains is the result of a synthetic interaction between *lin-15* class A and class B point mutations.

Identification of *lin-13* mutations: Five newly identified putative class B mutations and *lin(n770)* failed to complement *lin-13(n387)* in a class A mutant background. *lin-13(n387)*, which causes a sterile Muv phenotype at 25°, had been shown to behave as a class B synMuv at 15° (Ferguson and Horvitz 1989). No class A mutations in *lin-13* were isolated.

Unlike *lin-15*, which encodes two nonoverlapping transcripts, *lin-13* encodes a single transcript encoding a nuclear protein predicted to contain 24 C2-H2 zinc fingers, 1 C4 zinc finger, and an LXCXE potential Rb-binding motif (Meléndez and Greenwald 2000). Sterile Muv mutations of *lin-13*, *n387*, and *n388* have been shown to be nonsense mutations at residues S524 and R857, respectively. The mutant gene products are predicted to be truncated proteins with either 2 or 5 complete zinc fingers (Meléndez and Greenwald 2000). To determine the molecular natures of the *lin-13* mutations we isolated, we used PCR to amplify DNA from the mutants and determined the sequence changes in these strains. Three mutants carry *lin-13* nonsense mutations: *lin-13(n770)*, *lin-13(n2238)*, and *lin-13(n2985)* (Table 2). These alleles are all predicted to encode truncated proteins, albeit ones longer than those generated by *lin-13(n387)* and *lin-13(n388)*. It is notable that the *lin-13(n2238)* predicted protein product of 995 amino acids is only slightly longer than the *lin-13(n388)* predicted protein product of 856 amino acids and has only 1 additional undisrupted zinc finger domain. Three other mutants carry *lin-13* missense mutations. *lin-13 (n2988)* and *lin-13(n2981)* each encode a cysteine-to-tryptophan change in the first cysteine of the first and fifth zinc fingers, respectively (Table 2). The first zinc finger may play a more important role than the fifth zinc finger, since *lin-13(n2988)* is notably stronger than *lin-13(n2981)* at low temperature (Table 1). The remaining missense mutation, *lin-13(n2984)*, encodes a glycine-to-glutamic acid change in the residue immediately preceding the first cysteine of the first zinc finger

TABLE 2

Sequences of *lin-13* class B mutations

Allele	Wild-type sequence	Mutant sequence	Substitution
n770	CAG	TAG	Q1988amber
n2238	CAA	TAA	Q996ochre
n2981	TGC	TAC	C814Y
n2984	GGA	GAA	G360E
n2985	CAA	TAA	Q1717ochre
n2988	TGT	TAT	C361Y

Wild-type and mutant codons are shown with the mutated nucleotide underlined. All mutations were GC-to-AT transitions as expected for EMS-induced mutations (Anderson 1995). Amino acid substitutions are shown as wild-type residue identity, residue number, and predicted mutant residue.

(Table 2). The bulky negatively charged mutant residue may partially interfere with the ability of the first cysteine to act in the coordination of zinc. Consistent with this hypothesis, the phenotype of *lin-13(n2984)* is weaker than that of *lin-13(n2988)* at lower temperatures (Table 1).

Phenotypes of newly isolated synMuv strains: Many of the newly isolated synMuv strains displayed a temperature-sensitive effect on vulval development such that the penetrance of the Muv defect increased at higher temperatures (Table 1). Similar observations were made by Ferguson and Horvitz (1989). The synMuv strains also often showed temperature-sensitive growth characteristics. Many strains showed a reduced growth rate or, in some cases, lethality at 25° (Table 1). Similar observations also were made by Ferguson and Horvitz (1989). Although there were some differences among strains in growth at 20°, the differences were much greater at 15° and 25°. Rare synMuv animals displayed a protruding excretory pore. Some animals ruptured as adults at the vulva or, rarely, at a pseudovulval protrusion. Strains isolated in the screen in which *lin-15(n744)* was used as the parental mutation had a fairly high percentage of rupture, often >50% of adults (data not shown).

Animals homozygous for the *lin-52(n3718)* mutation were sterile. The sterility of these animals is likely caused by a loss of *lin-52* gene function, as we always observed its cosegregation with the *lin-52* synMuv phenotype. Furthermore, animals heterozygous for *lin-52(n771) in trans* to *nDf40*, a deficiency that removes the *lin-52* locus, have diminished brood sizes and display maternal-effect lethality, indicating that a reduction of *lin-52* function leads to reduced fertility.

Strains carrying *lin-54* mutations differed from other synMuv strains in that a greater proportion of these animals had a ventral protrusion that was further posterior to the vulva than was the case for most synMuv mutants. Also, a number of these strains had two ventral protrusions posterior to the vulva, a rare occurrence for other synMuv strains. This phenomenon was observed with both alleles of *lin-54*. Among *lin-8(n111); lin-54 (n2231)* animals, 13% had a relatively far posterior ventral protrusion, and 11% had two posterior ventral protrusions (n = 126). Among *lin-54(n2231); lin-15(n767)* animals, 26% had a ventral protrusion further posterior than usual, and 19% had two ventral protrusions (n = 75). Among *lin-54(n2990); lin-15(n433)* animals, 13% had a ventral protrusion further posterior than usual, and 10% had two ventral protrusions (n = 112).

To determine the origin of these unusually far posterior pseudovulvae and two posterior pseudovulvae in *lin-54* animals, we used Nomarski optics to observe the P(3–8).p cells and their descendants in *lin-8(n111); lin-54 (n2231)* animals. Our observations established that, as in the wild type, in these animals there are only six potential vulval precursor cells: P(3–8).p divided to give 1°, 2°, and 3° cell fates, as in the wild type, and other Pn.p cells, such as P9.p, which lies posterior to P8.p, were not transformed into potential vulva precursor cells (as seen in "superMuv" mutants, which have extra pseudovulval protrusions in a *lin-15* background; Clark 1992). Cell lineage analysis revealed that in *lin-8; lin-54* animals, the functional vulva sometimes formed from P(4–6).p rather than from P(5–7).p (data not shown). In these cases, the P5.p nucleus did not lie directly beneath the anchor cell nucleus; instead, the P5.p and P6.p nuclei were located to either side of and below the anchor cell nucleus (data not shown). This phenomenon has been observed in other synMuv strains, but is rare (Ferguson *et al.* 1987; Thomas and Horvitz 1999). In animals in which the functional vulva was formed from the descendants of P(4–6).p, P7.p and P8.p and their descendants were posterior to the developing vulva. These cells adopted vulval fates in the mutant animals and formed either two posterior pseudovulvae or one posterior pseudovulva at a greater relative distance from the misplaced vulva than that of a pseudovulva formed from only P8.p relative to a properly positioned functional vulva. In one case, the vulva was observed to derive from the descendants of P5.p, P6.p, and part of P7.p. The descendants of P7.p were spread over a greater distance than usual; some joined a number of P8.p descendants to form a pseudovulva, while other P8.p descendants formed a second posterior pseudovulva. Thus, the P(3–8).p cells and the anchor cell seem to be occasionally displaced relative to each other in *lin-54* animals. In some animals in which the anchor cell was located over P6.p, the descendants of P7.p and P8.p were spread over a wider distance than those in the wild type. These data suggest that the posterior members of the vulval equivalence group are displaced posteriorly in a significant number of *lin-54* animals.

Analysis of synMuv genes: We mapped three new synMuv genes, *lin-52*, *lin-54* (class B), and *lin-56* (class A), and three previously described synMuv genes, *lin-13*, *lin-53*, and *dpl-1* (all class B), using multifactor crosses and deficiencies (Tables 3 and 4; Figure 1).

dpl-1 mapped to the same linkage group II deficiency

TABLE 3

Three- and four-factor crosses

Gene	Genotype of heterozygote	Phenotype of selected recombinants	Genotype of selected recombinants (with respect to unselected markers)
lin-13	*lin-13 unc-32+ +/+ + unc-49 dpy-18; lin-15(n767)*	Unc-32	0/2 *unc-49 dpy-18/+ +*
		Dpy	7/7 *lin-13 unc-32/+ +*
		Unc-49	0/1 *lin-13 unc-32/+ +*
	+ + lin-13/unc-93 dpy-27 +; lin-15(n767)	Unc	30/30 *lin-13/+*
	+ + lin-13/unc-93 dpy-17 +; lin-15(n767)	Unc	8/8 *lin-13/+*
		Dpy	0/3 *lin-13/+*
	+ lin-13 +/dpy-17 + unc-32; lin-15(n767)	Dpy	1/8 *lin-13/+*
		Unc	5/10 *lin-13/+*
lin-52	*+ + unc-32 lin-52/lon-1 sma-3 + +; lin-15(n767)*	Lon	1/1 *lin-52 unc-32/+ +*
		Sma	0/4 *lin-52/+*
		Muv	7/7 *lon-1/+*
	+ + lin-52/sma-3 unc-32; lin-15(n767)	Sma	3/3 *lin-52/+*
		Unc	0/13 *lin-52/+*
	+ lin-52 +/dpy-19 + unc-69; lin-15(n767)	Dpy	17/31 *lin-52/+*
		Unc	2/11 *lin-52/+*
	+ lin-52 +/unc-16 + unc-49; lin-15(n767)	Unc-16	0/2 *lin-52/+*
		Unc-49	13/16 *lin-52/+*
	+ lin-52 +/unc-16 + unc-47; lin-15(n767)	Unc-47	7/9 *lin-52/+*
	lin-52 + unc-69/+ stP127 +; lin-15(n767)	Muv	3/12 *stP127/+*
	sma-3 + lin-52 +/+ sqv-3 + unc-69; lin-15(n767)	Sma	9/9 *sqv-3/+*
		Muv	1/27 *sqv-3/+*
		Unc	14/16 *lin-52/+*
lin-53	*dpy-5 lin-53 + +/+ + lin-11 unc-75; lin-15(n767)*	Dpy	7/7 *lin-11 unc-75/+ +*
		Muv	0/12 *lin-11 unc-75/+ +*
		Vul	0/10 *dpy-5 lin-53/+ +*
		Unc	8/8 *dpy-5 lin-53/+ +*
	+ lin-53 +/unc-29 + lin-11; lin-15(n767)	Unc	1/3 *lin-53/+*
		Vul	11/19 *lin-53/+*
lin-54	*lin-8; + + lin-54/unc-22 unc-30 +*	Unc-22	19/19 *lin-54/+*
		Unc-30	0/15 *lin-54/+*
	lin-8; + lin-54 +/unc-30 + dpy-4	Unc	2/11 *lin-54/+*
		Dpy	21/22 *lin-54/+*
	lin-54 + +/+ lev-1 unc-26; lin-15(n433)	Lev	0/25 *lin-54/+*
dpl-1	*+ dpl-1 +/dpy-10 + unc-53; lin-15(n433)*	Dpy	6/9 *dpl-1/+*
		Unc	6/11 *dpl-1/+*
	+ dpl-1 +/rol-6 + unc-4; lin-15(n433)	Rol	6/14 *dpl-1/+*
		Unc	9/14 *dpl-1/+*
	dpl-1 + +/+ let-240 unc-4; lin-15(n433)	Unc	25/25 *dpl-1/+*
lin-56	*+ + lin-56/unc-85 dpy-10 +; lin-15(n744)*	Unc	4/4 *lin-56/+*
		Dpy	0/6 *lin-56/+*
	+ lin-56 +/dpy-10 + unc-53; lin-15(n744)	Dpy	5/10 *lin-56/+*
		Unc	6/11 *lin-56/+*
	+ + lin-56/rol-6 unc-4 +; lin-15(n744)	Rol	16/16 *lin-56/+*
		Unc	0/9 *lin-56/+*

Three- and four-factor crosses were performed as described previously (Brenner 1974; Ferguson and Horvitz 1989).

interval as *let-23*, which encodes a receptor tyrosine kinase involved in inductive vulval signaling (Ferguson *et al.* 1987; Aroian *et al.* 1990). To determine if *dpl-1(n2994)* is allelic to *let-23*, we performed complementation tests against *let-23(mn23)*, *let-23(mn216)*, and *let-23(n1045)*. These *let-23* alleles were chosen because *mn23* and *mn216* represent putative null alleles, and *n1045* is a viable reduction-of-function allele that causes both a Vul and a hyperinduced phenotype [Hin; P(3–8).p cells immediately adjacent to the developing vulva often adopt vulval fates, producing an abnormal vulva with adjacent Muv-like protrusions; Ferguson and Horvitz 1985; Aroian and Sternberg 1991]. *dpl-1(n2994)* complemented *let-23* for all phenotypes: synMuv, Vul, Hin, and lethal (Let). The allele used in these tests, *dpl-1(n2994)*, is predicted to fail to complement a loss-of-function allele for the synMuv phenotype, since *dpl-1(n2994)/mnDf67; lin-15*A animals are Muv (Table 4).

TABLE 4

Deficiency heterozygotes

Gene	Genotype of heterozygote	Phenotype of heterozygote
dpl-1	*dpy-10 dpl-1(n2994) +/+ mnDf46 unc-4; lin-15(n433)*	Wild type
	dpy-10 dpl-1(n2994) +/+ mnDf85 unc-4; lin-15(n433)	Wild type
	dpy-10 dpl-1(n2994) +/+ mnDf67 unc-4; lin-15(n433)	Muv
lin-52	*unc-36 lin-52 +/+ nDf40 dpy-18; lin-15(n767)*	Muv

Deficiency heterozygotes were constructed, and the vulval phenotype was scored. The presence of the deficiency was confirmed in each animal on the basis of the segregation of one-quarter dead eggs or larvae.

The class B synMuv gene *dpl-1* was represented by only one mutant allele in these screens. To test whether the phenotype produced by this allele is weaker than that expected from a null phenotype, this mutation was tested *in trans* to a deficiency (Table 4). Animals of genotype *dpl-1(n2994)/mnDf67; lin-15(n433)* had a Muv phenotype and an incidence of sterility similar to those of animals of genotype *dpl-1(n2994); lin-15(n433)* when both were progeny of a mother of genotype *dpl-1(n2994)/mnDf67; lin-15(n433)*. However, the fertile animals of genotype *dpl-1(n2994)/mnDf67; lin-15(n433)* had a much greater incidence of maternal-effect lethality than did animals of genotype *dpl-1(n2994); lin-15(n433)* when both were progeny of a mother of genotype *dpl-1(n2994)/mnDf67; lin-15(n433)*. Animals of both of these genotypes had a stronger Muv phenotype and were less fertile than animals of genotype *dpl-1(n2994); lin-15(n433)* when descended from animals of genotype *dpl-1(n2994); lin-15 (n433)*. These results suggest that *dpl-1(n2994)* is a weak allele of a locus that has a stronger, possibly sterile and maternal-effect lethal, null phenotype. Ceol and Horvitz (2001) have subsequently isolated a putative null allele of *dpl-1*, which causes sterility.

To demonstrate formally that *lin-52*, *lin-53*, *lin-54*, *dpl-1*, and *lin-56* are indeed synMuv genes, we conducted tests similar to those used by Ferguson and Horvitz (1989). First, we separated an allele of each gene from its parental mutation and showed that strains that carried only the isolated allele in homozygous condition displayed wild-type vulval development at the level of resolution of the dissecting microscope (Table 5). Double mutants were then constructed between alleles of the new synMuv genes and alleles of previously defined synMuv genes. Double mutants carrying class A and class B mutations were Muv; double mutants carrying two class A mutations or two class B mutations were wild type for vulval development (Table 5). *lin-13(n770)* has also been shown to behave as a class B synMuv mutation by these criteria (Table 5 and data not shown).

Maternal rescue of the synMuv phenotype depends on both class A and class B genes: Many of the new synMuv strains displayed maternal rescue of the Muv phenotype,

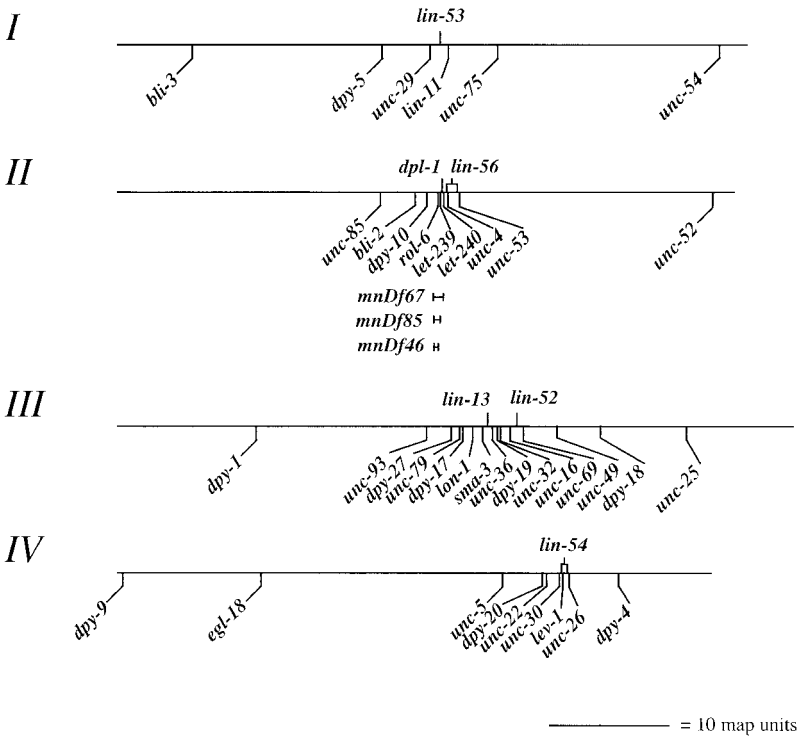

Figure 1.—Partial genetic map of *C. elegans* showing the locations of newly identified or newly characterized synMuv genes and the markers that were used to position these genes on the map. New synMuv genes are shown above the line representing the linkage group; marker genes are shown below the line. Deficiencies are drawn below the line and indicate which genes are deleted.

TABLE 5

Phenotypes of single and double mutants

	New mutation	Single mutant	Double mutant with class A		Double mutant with class B	
			lin-8(n111)	*lin-15(n767)*	*lin-36(n766)*	*lin-15(n744)*
Class A	*lin-56(n2728)*	WT	WT	WT	Muv	Muv
Class B	*lin-13(n770)*	WT	Muv	Muv	WT	WT
	lin-52(n771)	WT	Muv	Muv	WT	WT
	lin-53(n833)	WT	Muv	Muv	WT	WT
	lin-54(n2231)	WT	Muv	Muv	WT	WT
	dpl-1(n2994)	WT	Muv	Muv	WT	WT

Mutations in new genes were separated from the original mutation present in the synMuv strain as described in MATERIALS AND METHODS. Double mutants carrying mutations in these new genes and mutations in previously known class A or class B genes were constructed as described in MATERIALS AND METHODS. WT, animals had wild-type vulval morphology as observed using a dissecting microscope, *i.e.*, 0% Muv ($n > 500$). Muv, animals had a Muv phenotype of >50% penetrance ($n \geq 100$). *lin-13*, *lin-52*, and *lin-54* animals displayed a weaker Muv phenotype in double mutants with *lin-8(n111)* than in those with *lin-15(n767)*. *dpl-1* animals displayed a weaker Muv phenotype in a *lin-15(n433)* background than in a *lin-15(n767)* background.

such that animals of genotype *a/a; b/b* descended from animals of genotype *a/+; b/+* had lower penetrance and reduced expressivity compared to animals of genotype *a/a; b/b* descended from animals of *a/a; b/b* genotype (Table 6). Similar results have been shown for other synMuv strains (FERGUSON and HORVITZ 1989). To determine whether this maternal rescue was conferred by only one of the two classes, we compared the Muv phenotypes of animals of genotype *a/a; b/b* descended from animals of genotypes *a/+; b/+ vs. a/a; b/+ vs. a/+; b/b*. Several different combinations of synMuv mutations were tested. In many cases, neither class alone produced significant maternal rescue compared to that produced by both classes together. We concluded that maternal rescue displayed in synMuv strains is the result of a synergistic interaction between genes of the two classes rather than the result of the maternal contribution of genes of just one class (Table 6).

Molecular identification of *lin-52*: We further characterized the class B synMuv gene *lin-52*. Using standard three- and four-factor mapping techniques, we localized *lin-52* to a small genetic interval between *sqv-3* and the Tc1 transposon polymorphism *stP127* (Figure 2). We generated transgenic animals using DNA clones from this interval and found that the overlapping cosmids ZK630 and C26C12 and subclones of DNA common to both of these cosmids rescued the Muv phenotype of *lin-52(n771); lin-15(n767)* mutants. Typically >70% of transgenic animals in the first generation of a stable transgenic line (*i.e.*, in the transgenic F_2 progeny of an injected animal) were rescued. However, transgenic lines containing these cosmids or their subclones displayed a progressive reduction in the penetrance of rescue in each subsequent generation. The reason for this trend is unknown; we speculate that it may have resulted from transgene silencing. Such generation-dependent transgene silencing occurs in the *C. elegans* germline and is thought to be caused by the preferential recruitment of silencing factors to repetitive stretches of DNA (KELLY *et al.* 1997).

Because two complete predicted genes, ZK632.9 and ZK632.13, were present on the minimal rescuing fragment, we performed further experiments to define *lin-52* (Figure 2). Into the minimal rescuing fragment we cloned a small double-stranded oligonucleotide that is predicted to introduce an in-frame stop codon into the ZK632.13 gene. This altered subclone was unable to rescue the Muv phenotype of *lin-52(n771); lin-15(n767)* mutants, whereas a subclone in which the oligonucleotide was removed, thereby restoring the ZK632.13 open reading frame, rescued like the clones described above. In addition, we found that RNA-mediated interference of ZK632.13 in a *lin-15(n767)* background resulted in a highly penetrant Muv phenotype. Finally, we determined the sequence of ZK632.13 in *lin-52(n771)* and *lin-52(n3718)* mutants. *lin-52(n771)* mutants contain a missense mutation that is predicted to substitute a positively charged lysine in place of a negatively charged glutamate, and *lin-52(n3718)* mutants contain a nonsense mutation that is predicted to truncate the ZK632.13 protein after 30 amino acids (Figure 3). These results identify ZK632.13 as *lin-52*.

We assembled a cDNA clone of *lin-52* (Figure 3A; see MATERIALS AND METHODS). This clone contains a 5′ SL2 splice leader sequence and a poly(A) tail, indicating that it is full length. An SL2 leader sequence is often *trans*-spliced upstream of genes that are initially transcribed as downstream genes of an operon (ZORIO *et al.* 1994). The SL2 leader sequence and the proximity of *lin-52* to the gene immediately upstream of it suggest that *lin-52* is transcribed as part of a polycistronic operon. The large open reading frame of this cDNA is predicted to encode a 161-amino-acid protein that is similar to uncharacterized proteins predicted by human, mouse, and Drosophila cDNA and genomic sequences (Figure 3B; C. J. CEOL and H. R. HORVITZ,

TABLE 6

Maternal rescue of synMuv phenotype

Class A mutation	Class B mutation	Penetrance of the Muv phenotype in animals of *a/a; b/b* genotype descended from animals of maternal genotype (%)			
		a/+; b/+	*a/+; b/b*	*a/a; b/+*	*a/a; b/b*[b]
lin-8(n111)	*lin-9(n112)*	72 (n = 57)[a]	83 (n = 141)[a]	100 (n = 183)	100 (n = 165)
lin-8(n111)	*lin-35(n745)*	45 (n = 65)[a]	ND	97 (n = 149)	100 (n = 209)
lin-8(n111)	*lin-36(n766)*	14 (n = 93)[a]	23 (n = 216)[a]	84 (n = 300)	98 (n = 207)
lin-8(n111)	*lin-37(n758)*	31 (n = 69)[a]	98 (n = 133)[a]	85 (n = 188)	100 (n = 161)
lin-15(n433)	*lin-54(n2231)*	9 (n = 89)[a]	ND	21 (n = 211)	99 (n = 164)
lin-15(n767)	*lin-13(n770)*	58 (n = 64)[a]	97 (n = 238)[a]	90 (n = 220)	95 (n = 230)
lin-15(n767)	*lin-52(n771)*	83 (n = 98)[a]	103 (n = 225)[a]	90 (n = 294)	100 (n = 211)
lin-15(n767)	*lin-53(n833)*	103 (n = 76)[a]	ND	100 (n = 64)	100 (n = 116)
lin-15(n433)	*dpl-1(n2994)*	11 (n = 95)[a]	75 (n = 125)[a]	50 (n = 298)	78 (n = 234)
lin-38(n751)	*lin-9(n112)*	81 (n = 65)[a]	100 (n = 146)	99 (n = 189)[a]	100 (n = 165)
lin-56(n2728)	*lin-15(n744)*	93 (n = 92)[a]	99 (n = 200)	ND	100 (n = 163)

The contribution of class A and class B genes to the maternal rescue of the synMuv phenotype of doubly homozygous animals descended from either singly or doubly heterozygous mothers was determined by counting the number of Muv animals of phenotype R, where R is the phenotype produced by the *cis* marker *r*. Doubly heterozygous mothers were obtained by mating N2 males with marked doubly homozygous hermaphrodites. Singly heterozygous mothers were obtained by mating males homozygous for one of the synMuv mutations with marked doubly homozygous hermaphrodites. A strain homozygous for *lin-35* was obtained by the general procedure used for the isolation of strains homozygous for single synMuv mutations. *dpy-14* was used to balance *lin-35*. The *lin-8; lin-9* strain was marked with *lon-1*. The *lin-35; lin-8* strain was marked with *unc-13*. The *lin-8; lin-36* strain was marked with *unc-32*. The *lin-8; lin-37* strain was marked with *lon-1*. The *lin-38; lin-9* strain was marked with *unc-52*. The *lin-13; lin-15(n767)* strain was marked with *unc-93* or *unc-32*. The *lin-52; lin-15(n767)* strain was marked with *sma-3*. The *lin-53; lin-15(n767)* strain was marked with *dpy-5* or *lin-11*. The *lin-54; lin-15(n433)* strain was marked with *dpy-20*. The *dpl-1; lin-15(n433)* strain was marked with *rol-6*. The *lin-56; lin-15(n744)* strain was marked with *rol-6*. ND, not determined.

[a] These data were obtained by estimating the penetrance and by assuming that the number of doubly homozygous animals were one-quarter of the R progeny.

[b] These data are from Table 1 of Ferguson and Horvitz (1989).

unpublished observations). LIN-52 is most similar to these proteins in a short carboxy-terminal domain. Over a stretch of 28 amino acids, LIN-52 is 50% identical and 75% similar to the human predicted protein LOC91750. This region of similarity may represent a functional domain within the LIN-52 protein.

DISCUSSION

In this article we describe the isolation and characterization of 50 new synMuv mutants. We define and describe two new genes, *lin-54* and *lin-56*; describe two other newly named genes, *lin-52* and *lin-53*, of which one allele each had been previously isolated; describe the initial identification and characterization of a previously described gene, *dpl-1*; and identify class B synMuv alleles of *lin-13*. In sum, at least 4 class A genes (*lin-8*, *lin-15*A, *lin-38*, and *lin-56*) and at least 14 class B genes (*lin-9*, *lin-13*, *lin-15*B, *lin-35 Rb*, *lin-36*, *lin-37*, *lin-52*, *lin-53*, *lin-54*, *dpl-1*, *tam-1*, *let-418*, *efl-1*, *hda-1*, and *mep-1*) are now known (Table 7). We showed that the maternal rescue of the synMuv phenotype is dependent on a synergistic interaction between the wild-type alleles of both classes. We also cloned the *lin-52* gene and found that it encodes a small protein that may be evolutionarily conserved.

Null phenotypes of synMuv genes: The null phenotypes of most synMuv genes have not been rigorously established. Most likely, not all synMuv genes have the same null phenotypes (Table 7). Several synMuv genes are likely to have a synMuv null phenotype. The class A mutation *lin-15(n767)* is a likely null allele by molecular criteria: it is a deletion in the middle of the coding sequence with a small insertion producing a frameshift in the class A transcript (Huang *et al.* 1994). *lin-15(n767)* mutants display a class A synMuv phenotype and are viable and fertile. The *lin-15* class B gene is also likely to have a synMuv null phenotype. The *lin-15*AB mutations *lin-15(n309)* and *lin-15(e1763)* are deletions of most of the DNA of the locus and are therefore molecular nulls for both A and B *lin-15* transcripts (Clark *et al.* 1994; Huang *et al.* 1994). These mutants are Muv, viable, and fertile, indicating that the *lin-15* class B gene also has a synMuv null phenotype. *lin-36* is also likely to have a synMuv null phenotype: nonsense mutations isolated in a noncomplementation screen are synMuv, viable, and fertile (Thomas and Horvitz 1999).

Several other synMuv genes have a null phenotype

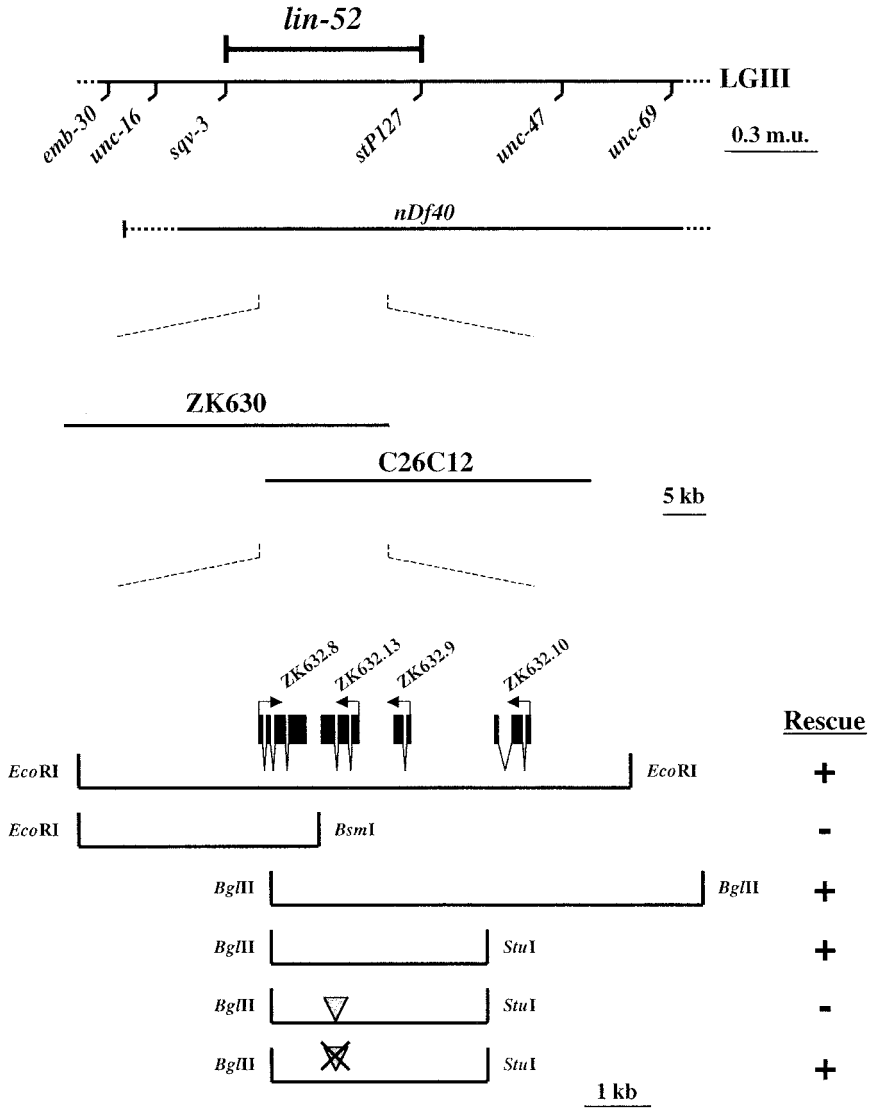

FIGURE 2.—Molecular cloning of *lin-52*. (Top) The genetic map location of *lin-52* on linkage group III. The dashed portion of *nDf40* indicates that the left endpoint of this deficiency is not precisely known but maps between *emb-30* and *sqv-3*. (Middle and bottom) The rescuing cosmids ZK630 and C26C12 and subclones assayed for *lin-52* rescue. The restriction sites used to generate subclones are indicated. "+," a majority of transgenic lines were rescued in the first generation of establishing the line. When rescue was observed, typically >70% of transgenic animals in a line were rescued in this generation. Arrows indicate the direction of transcription. Coding sequences of predicted genes are shaded. An oligonucleotide encoding an in-frame stop codon was inserted into (arrowhead) and subsequently removed from (arrowhead with X) the ZK632.13 predicted gene.

that is either lethal or sterile. *lin-13* Muv mutants carry nonsense mutations and have a zygotic sterile and maternal effect larval arrest phenotype (MELÉNDEZ and GREENWALD 2000). It is likely that the null phenotype of *lin-9* is sterile. A noncomplementation screen for *lin-9* mutants led to the isolation of *lin-9* alleles that were sterile and behaved as synMuv mutations (FERGUSON and HORVITZ 1989). These sterile *lin-9* mutations were shown to be nonsense mutations (BEITEL *et al.* 2000). Similarly, we found that the *lin-52(n3718)* mutation causes a sterile and synMuv phenotype and is a nonsense mutation that is predicted to severely truncate the LIN-52 protein. Therefore, the *lin-52* null phentoype is likely sterile. The class B mutation *dpl-1(n2994)* was tested *in trans* to a deficiency that spanned the locus. *dpl-1(n2994)* mutants have a stronger Muv phenotype, are less fertile, and have a greater incidence of maternal-effect lethality when they and their mothers are *trans*-heterozygotes for a deficiency of this locus. This observation suggests that *dpl-1(n2994)* is probably a reduction-of-function mutation rather than a complete loss-of-function mutation. A null mutation of *dpl-1* isolated by CEOL and HORVITZ (2001) causes the sterile phenotype predicted by the analysis described here.

The screens described in this article were not designed to isolate synMuv mutations that caused lethality or sterility [*lin-52(n3718)* was isolated in a separate screen that allowed the identification of lethal or sterile mutations. However, the other mutants isolated in this screen are not described in this article, and for this reason this screen is not considered in the following discussion.] Thus, complete loss-of-function alleles of loci with such null phenotypes were not isolated. However, viable and fertile reduction-of-function mutations in such loci could have been isolated. Mutations in complementation groups with few alleles are candidates for being such reduction-of-function mutations. Loci that are not readily mutated to a viable synMuv phenotype may not have been identified.

From our general screens, we isolated mutations in

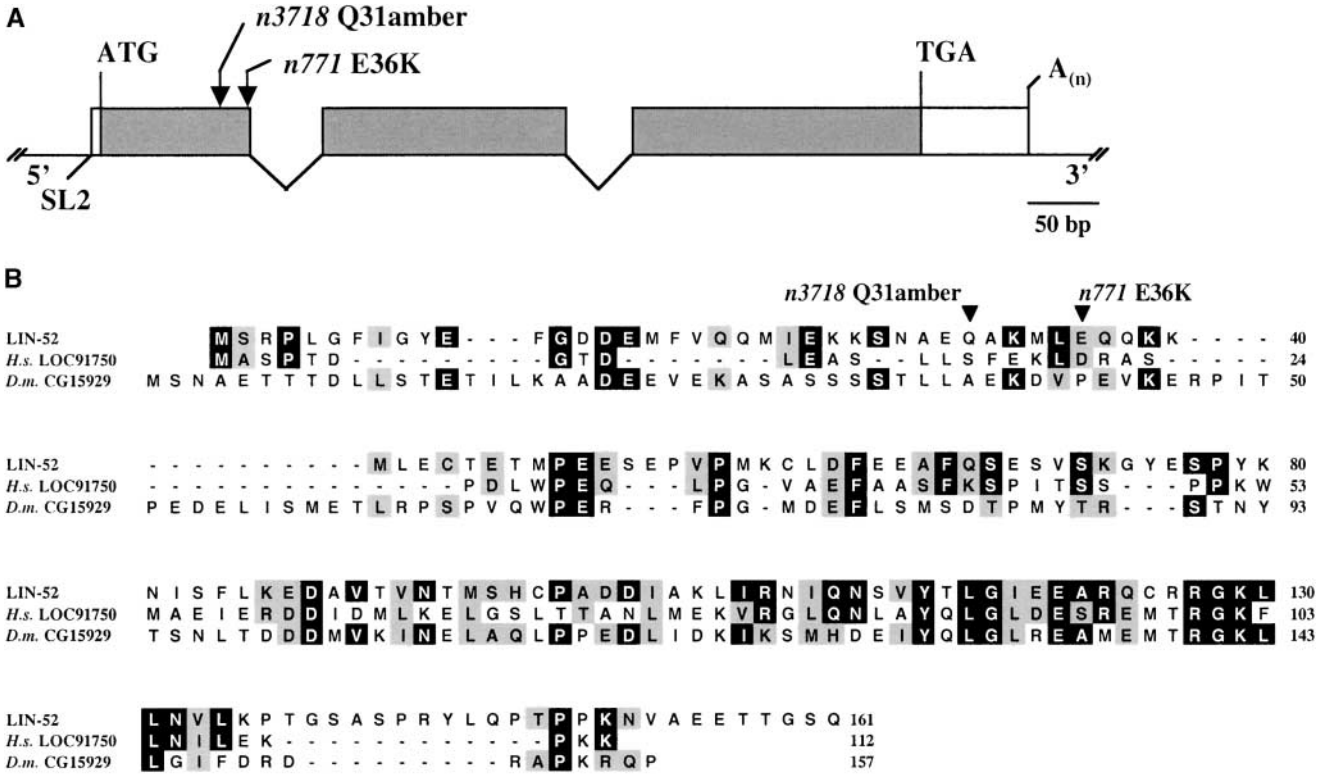

FIGURE 3.—*lin-52* gene structure and predicted protein sequence. (A) *lin-52* gene structure as derived from cDNA and genomic sequences. Exons (shaded boxes), 5′ and 3′ untranslated regions (open boxes), predicted translational start and stop codons, SL2 splice leader sequence, and poly(A) tail are indicated. Arrows indicate the locations of the *n771* and *n3718* mutations. (B) Alignment of the predicted LIN-52, human LOC91750, and Drosophila CG15929 proteins. Solid boxes indicate identities and shaded boxes indicate similarities. Arrowheads indicate the positions of *lin-52* mutations.

different complementation groups at different frequencies. Class A mutations fell into either a frequently isolated group, *lin-8* and *lin-15*A (8 alleles each), or an infrequently isolated group, *lin-38* and *lin-56* (1 or 2 alleles each). Class B mutations included 10 alleles of *lin-15*B; 6 of *lin-35*; 5 of *lin-13*; 3 of *lin-36*; 2 of *lin-54*; 1 each of *lin-37*, *lin-53*, and *dpl-1*; and no alleles of *lin-9*, *lin-52*, *tam-1*, *let-418*, *efl-1*, *hda-1*, or *mep-1*. Given the number of haploid genomes screened and the expected frequency of mutation of the average *C. elegans* gene by EMS, 5×10^{-4}, we expected to isolate ~9 alleles of each gene with a synMuv null phenotype (BRENNER 1974; GREENWALD and HORVITZ 1980), suggesting that *lin-8*, *lin-15*A, *lin-15*B, and *lin-35* are such genes. In contrast to these results, class B alleles of *lin-13*, which are not null alleles, were isolated at a relatively high frequency. *lin-13*, which encodes a protein of 2248 amino acids (MELÉNDEZ and GREENWALD 2000), may provide a large mutagenic target.

There are several reasons why mutations may have been isolated at a frequency $<5 \times 10^{-4}$. Some of the genes may have a sterile or lethal loss-of-function phenotype, so that only rare reduction-of-function mutations were isolated. We probably failed to isolate any *lin-9* alleles for this reason. Mutations in such genes should be easily obtained in screens that allow the isolation of sterile and lethal mutants. Other genes may provide a small mutagenic target. Only one allele of *lin-37* was isolated in the screens described in this article. This gene is physically small, and the allele we isolated is consistent by molecular criteria with its being a loss-of-function allele (X. LU, personal communication). Mutations affecting *lin-52* were likewise difficult to isolate, probably because of the likely sterile loss-of-function phenotype and the small physical size of *lin-52*. In addition, there may have been a bias in our experiments as a consequence of the parental mutations we used in our screens. The class A mutations *lin-8(n111)* and *lin-15 (n433)* do not produce highly penetrant Muv phenotypes in conjunction with some class B mutations, which may have resulted in a lower frequency of isolation of alleles of certain genes.

Many class A and class B synMuv genes probably act in distinct pathways: Most genes isolated in the screens described here or by FERGUSON and HORVITZ (1989) seem to be distinctly members of either the class A or the class B pathway. With the exception of *lin-15* alleles, no class A and class B mutations mapped to the same site. However, the *lin-15* locus consists of two adjacent genes, a class A gene and a class B gene (CLARK *et al.* 1994; HUANG *et al.* 1994). These results suggest that most synMuv genes act in only one of the two pathways.

TABLE 7

SynMuv genes and alleles

Gene	No. of alleles	Mutant alleles	Probable null phenotype (evidence)
Class A			
lin-8	9	*n111*, *n2376*, *n2378*, *n2403*, *n2724*, *n2731*, *n2738*, *n2739*, *n2741*	SynMuv[a] (no. alleles)
*lin-15*A	13	*n433*, *n749*, *n767*, *n2375*, *n2725*, *n2726*, *n2733*, *n2734*, *n2735*, *n2737*, *n2742*, *sy211*, *sy212*	SynMuv (molecular data[b], no. alleles)
lin-38	4	*n751*, *n761*, *n2402*, *n2727*	Unknown
lin-56	1	*n2728*	Unknown
Class B			
lin-9	3	*n112*, *n942*, *n943*	SynMuv, sterile (molecular data[c], non-complementation screen[d])
lin-13	8	*n770*, *n2238*, *n2981*, *n2984*, *n2985*, *n2988* (*n387* and possibly *n388* at 15°[d])	SynMuv, sterile, maternal-effect lethal[a] (molecular data[e])
*lin-15*B	14	*n374*, *n743*, *n744*, *n2230*, *n2233*, *n2241*, *n2244*, *n2245*, *n2980*, *n2983*, *n2987*, *n2989*, *n2991*, *n2993*	SynMuv (molecular data[b], no. alleles)
lin-35	8	*n373*, *n745*, *n2232*, *n2236*, *n2239*, *n2242*, *n2977*, *n2996*	SynMuv (molecular data[f], no. alleles)
lin-36	13	*n747*, *n750*, *n766*, *n772*, *n2235*, *n2240*, *n2243*, *n3090*, *n3093*, *n3094*, *n3095*, *n3096*, *n3097*	SynMuv (molecular data[g], noncomplementation screen[g])
lin-37	2	*n758*, *n2234*	SynMuv (molecular data[h])
lin-52	2	*n771*, *n3718*	SynMuv, sterile (molecular data[i])
lin-53	3	*n833*, *n2978*, *n3368*[j]	SynMuv, sterile, protruding vulva (molecular data[j], *n833* and *n2978* are dominant negative[k])
lin-54	2	*n2231*, *n2990*	Unknown
dpl-1	4	*n2994*, *n3316*, *n3643*, *zu355*	SynMuv, sterile, maternal-effect lethal (phenotype enhanced by Df, molecular data[l], *zu355* isolated in maternal-effect lethal screen[m])
tam-1[n]	18	*cc566*, *cc567*, *cc587*, *sy272*, and 14 others	SynMuv (molecular data[o], no. alleles[o], phenotype not enhanced by Df[o])
let-418	3	*ar113*, *ar114*, *s1617*	SynMuv, sterile, maternal-effect lethal, everted vulva (molecular data[p], phenotype not enhanced by Df[p])
efl-1	3	*n3318*, *n3639*, *se1*	SynMuv, sterile, maternal-effect lethal[q] (molecular data[l], *se1* isolated in maternal-effect lethal screen[m])
hda-1	1	*e1795*[r]	SynMuv, protruding vulva, gonad development abnormal and sterile (molecular data[s])
mep-1	1	*q660*	SynMuv[t,u], sterile[t,v], protruding vulva[t,v], larval lethal[v] (molecular data[t,v])

Mutant alleles not described in the text are described by Horvitz and Sulston (1980), Ferguson and Horvitz (1985, 1989), Desai *et al.* (1988), Huang *et al.* (1994), Thomas and Horvitz (1999), Hsieh *et al.* (1999), Von Zelewsky *et al.* (2000), Ceol and Horvitz (2001), and Page *et al.* (2001). Probable null phenotype is given at 20°; see Table 1. Under evidence, no. alleles indicates that a high number of mutations were isolated in the screens described in the text, consistent with the hypothesis that some of these alleles are null alleles.

[a] See Ferguson and Horvitz (1985) for possibly contradictory deficiency data.
[b] Clark *et al.* (1994); Huang *et al.* (1994).
[c] Beitel *et al.* (2000).
[d] Ferguson and Horvitz (1989).
[e] Meléndez and Greenwald (2000).
[f] Lu and Horvitz (1998).
[g] Thomas and Horvitz (1999).
[h] Lu (1999).
[i] See text.
[j] X. Lu and H. R. Horvitz (personal communication).
[k] Text and Lu and Horvitz (1998).
[l] Ceol and Horvitz (2001).
[m] Page *et al.* (2001).
[n] This gene does not act as a class B synMuv in double mutants with *lin-8*, but does with *lin-15*A and *lin-38* (Hsieh *et al.* 1999). Such an interaction had not been previously observed for the synMuv genes, but *lin-8(n111)* synMuv double mutants have occasionally been observed to be weaker than corresponding synMuv double mutants carrying other class A mutations (Thomas and Horvitz 1999).
[o] Hsieh *et al.* (1999).
[p] Von Zelewsky *et al.* (2000).
[q] Page *et al.* (2001); C. J. Ceol and H. R. Horvitz (unpublished results).
[r] *hda-1* is also known as *gon-10* (Dufourcq *et al.* 2002). The *hda-1(e1795)* mutation alone causes a weakly penetrant Muv phenotype (Dufourcq *et al.* 2002), but in combination with the class A mutation *lin-15(n767)* this phenotype is fully penetrant and displays stronger expressivity (C. J. Ceol, E. C. Andersen and H. R. Horvitz, unpublished results).
[s] Dufourcq *et al.* (2002).
[t] (C. J. Ceol, F. Stegmeier, M. M. Harrison and H. R. Horvitz, unpublished results).
[u] Unhavaithaya *et al.* (2002).
[v] Belfiore *et al.* (2002).

Genetic evidence suggests that *lin-13* may act in both pathways. It was previously shown that at 25° the *lin-13 (n387)* mutation produces a Muv phenotype similar to that of the synMuv double mutants but acts like a class B synMuv at 15° (Ferguson and Horvitz 1989; Meléndez and Greenwald 2000). We have identified a class B allele of *lin-13*, *n770*. Thus, all described mutations in *lin-13* cause either a Muv phenotype or a class B synMuv phenotype. The Muv phenotype of certain *lin-13* alleles may be caused by either the elimination of both class A and class B activities of *lin-13* or the elimination of another activity of *lin-13* that is independent of the synMuv pathways. If *lin-13* has both class A and class B activities, why might it be difficult to isolate class A alleles of *lin-13*? Particular regions of *lin-13* may be required for class A and class B activities, and class B regions may provide a larger or easier target for mutagenesis. Alternatively, some regions, possibly certain zinc fingers, are required for only class B activity while others are required for both, but none is required solely for class A activity. It is possible that *lin-13* mutations that cause a class A phenotype are not fertile or viable; however, no other class A mutations exhibit these phenotypes (Table 7). It is also possible that weaker *lin-13* alleles show only a class B synMuv phenotype. Either the class B pathway may be more sensitive to disruption or *lin-13* may play a more vital role in the class B pathway; if so, it would be impossible to get a mutation strong enough to exhibit class A activity without concomitantly exhibiting class B activity.

Synthetic phenotypes: Synthetic phenotypes are produced by combinations of mutations in different genes. Many synthetic lethal phenotypes have been studied in yeast, affecting such processes as cytoskeletal organization and secretion (Kaiser and Schekman 1990; Hufaker *et al.* 1987). Often, a synthetic phenotype is indicative of functional redundancy. Several genes are functionally redundant for various developmental processes in metazoa. In Drosophila, *achaete* and *scute* are functionally redundant for the specification of larval sense organs; however, individually each of these genes is required for the specification of a specific group of adult bristles (reviewed by Ghysen and Dambly-Chaudiere 1988). In *C. elegans*, *lin-12* and *glp-1* are molecularly similar and functionally redundant for some aspects of development, since double mutants exhibit defects not found in either single mutant (Lambie and Kimble 1991). Partial functional redundancy also is seen among *C. elegans* genes that function in the engulfment of cell corpses during programmed cell death; there are two classes of genes, such that animals carrying mutations in both classes have a more severe defect in engulfment than do animals carrying mutations in only one class (Ellis *et al.* 1991). The three *C. elegans* genes that encode Rac-like proteins, *ced-10*, *mig-2*, and *rac-2/3*, function redundantly in axon guidance and subsets of these genes function redundantly in certain cell migrations (Lundquist *et al.* 2001). Whereas many synMuv genes are individually necessary for fertility or viability, others are not known to be individually required in any process other than vulval development. In contrast to lone mutations in many partially functionally redundant genes that have slight defects in a particular process, lone mutations in the synMuv genes do not have any discernable defects in vulval development (Ferguson and Horvitz 1989; Thomas and Horvitz 1999).

Functional redundancy at the genetic level suggests that two sets of genes implement the same biological effect, *e.g.*, the negative regulation of vulval induction. The precise molecular mechanisms by which these genes act can be completely distinct, and the two classes of synMuv genes need not act at the same point in the pathway for vulval development. At what point(s) in the vulval pathway might the synMuv genes act? Mutations in the LET-23 receptor tyrosine kinase produce a Vul phenotype that is epistatic to the Muv phenotype caused by synMuv mutations, indicating that for the synMuv phenotype to be expressed *let-23* gene function is needed. If the Muv phenotype were caused by mutation in a single gene, this gene could act either parallel to or upstream of *let-23*. However, the synMuv phenotype is instead caused by mutations in two genes. Thus, if the effects of mutation in either of these two genes are blocked by a *let-23* mutation, the synMuv phenotype would be suppressed. These considerations indicate that at least one of the two classes of synMuv genes (A or B) must act parallel to or upstream of *let-23*, but the other class of synMuv gene could act parallel to, upstream of, or downstream of *let-23*. Specific models of how the class B synMuv genes may act in parallel to the *let-23* signal transduction pathway have been discussed by Lu and Horvitz (1998) and Thomas and Horvitz (1999).

Class B synMuv genes including *lin-52* define an Rb-mediated pathway: *lin-35*, a member of the class B synMuv pathway, encodes a protein similar to the mammalian tumor suppressor pRb (Lu and Horvitz 1998). Other genes with class B synMuv activity encode DP (*dpl-1*), E2F (*efl-1*), RbAp48 (*lin-53*), histone deacetylase (*hda-1*), and HP1 family proteins (*hpl-2*; Lu and Horvitz 1998; Ceol and Horvitz 2001; Couteau *et al.* 2002). In addition to their role in vulval development, many class B genes have been shown to regulate G1-to-S phase progression in the cell cycle. These genes include *dpl-1*, *efl-1*, *lin-9*, *lin-15*B, *lin-35*, and *lin-36*; other class B genes, *hda-1*, *let-418*, *lin-37*, *lin-53*, and *tam-1*, do not appear to be involved in cell cycle control (Boxem and Van Den Heuvel 2001, 2002). Even among the subgroup of class B genes that are involved in cell cycle control, *lin-35* and *lin-15*B have been shown to have partially nonoverlapping functions (Boxem and Van Den Heuvel 2002). These results suggest that either the class B synMuv genes act differently in vulval development and cell cycle control or we have not yet distin-

guished more subtle differences in their roles in vulval development.

Mammalian homologs of some of these class B synMuv proteins are known to functionally, and in some cases physically, interact with pRb. These and other parallels indicate that the class B synMuv pathway is an analog of Rb pathways in other organisms, particularly those pathways in which Rb is involved in chromatin remodeling. Consequently, additional class B synMuv genes may have homologs with analogous functions in other organisms. One such gene is *lin-52*. *lin-52* encodes a small protein, portions of which are conserved in similarly small proteins predicted by the human, mouse, and Drosophila genome sequences. The further analysis of *lin-52* and other synMuv genes should help elucidate the mechanisms of action of Rb-like proteins and their regulators and effectors. The determination of how the class B synMuv genes negatively regulate the vulval induction process should provide insight concerning the antagonistic actions of Rb-mediated and Ras-mediated pathways.

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