Caenorhabditis elegans lin-13, a Member of the LIN-35 Rb Class of Genes Involved in Vulval Development, Encodes a Protein With Zinc Fingers and an LXCXE Motif

Alicia Meléndez and Iva Greenwald

Department of Biochemistry and Molecular Biophysics, Howard Hughes Medical Institute, Columbia University College of Physicians and Surgeons, New York, New York 10032

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ABSTRACT

The SynMuv genes appear to be involved in providing a signal that inhibits vulval precursor cells from adopting vulval fates in *Caenorhabditis elegans.* One group of SynMuv genes, termed class B, includes genes encoding proteins related to the tumor suppressor Rb and RbAp48, a protein that binds Rb. Here, we provide genetic evidence that *lin-13* behaves as a class B SynMuv gene. We show that null alleles of *lin-13* are temperature sensitive and maternally rescued, resulting in phenotypes ranging in severity from L2 arrest (when both maternal and zygotic activities are removed at 25°), to sterile Multivulva (when only zygotic activity is removed at 25°), to sterile non-Multivulva (when both maternal and zygotic activities are removed at 15°), to wild-type/class B SynMuv (when only zygotic activity is removed at 15°). We also show that LIN-13 is a nuclear protein that contains multiple zinc fingers and a motif, LXCXE, that has been implicated in Rb binding. These results together suggest a role for LIN-13 in Rb-mediated repression of vulval fates.

THE cell-cell interactions that specify cell fates during *Caenorhabditis elegans* vulval development have been intensively studied (reviewed in Greenwald 1997). The vulva is normally formed from the 22 descendants of three cells, P5.p, P6.p, and P7.p. However, each of six cells, numbered P3.p–P8.p, has the potential to generate vulval cells, and hence these six cells are named "vulval precursor cells" (VPCs). Each VPC adopts one of three fates, which can be recognized by the lineages they undergo. The "1°" and "2°" fates are termed "vulval" fates, because they lead to the production of vulval cells. The "3°" fate is termed the "nonvulval" or "hypodermal" fate, because it leads to the production of cells that join the hyp7 hypodermal syncytium. In wild-type hermaphrodites, P3.p–P8.p always adopt the same pattern of fates: 3°-3°-2°-1°-2°-3°.

Genetic screens have identified genes that are necessary for VPC patterning and vulval fates. These screens have relied on two vulval abnormalities. A "Vulvaless" phenotype results when P5.p, P6.p, and P7.p adopt nonvulval fates. The Vulvaless phenotype is readily visualized in the dissecting microscope when progeny hatch internally and form a "bag of worms." A "Multivulva" (Muv) phenotype results when P3.p, P4.p, and/or P8.p inappropriately adopt vulval (1° and 2°) fates. The Multivulva phenotype is readily visualized in the dissecting microscope by multiple pseudovulval protrusions, with each protrusion generally corresponding to progeny produced by a single VPC.

The analysis of Vulvaless and Multivulva mutants, in combination with cell ablation experiments, has revealed that three different signaling events specify the pattern of the VPC fates (reviewed in Greenwald 1997). One event is referred to as the "inductive" signal, which promotes the adoption of 1° fates. The inductive signal is produced by the anchor cell of the gonad and is transduced by the LET-23 receptor tyrosine kinase in the VPCs. The inductive signal may countermand another signal, referred to as the "inhibitory" signal, which prevents VPCs from adopting vulval fates. The inhibitory signal is believed to emanate from hyp7, the syncytial hypodermal cell that encompasses much of the animal. A third signal, referred to as the "lateral" signal, is believed to be produced by the VPC that is adopting the 1° fate to cause an adjacent cell to adopt the 2° fate.

The absence of inhibitory signaling results in a Multivulva phenotype because extra VPCs adopt vulval fates. Studies of a group of genes termed the "SynMuv" genes revealed the existence of inhibitory signaling (Herman and Hedgecock 1990). These genes are called "SynMuv" genes because a Multivulva phenotype is visible only if mutations in two different genes are combined (a *Syn*thetic *Mul*ti*v*ulva phenotype; Horvitz and Sulston 1980; Ferguson and Horvitz 1989). Ferguson and Horvitz (1989) identified two classes of SynMuv mutations, class A and class B; a Multivulva phenotype results only when a class A mutation is com-

Corresponding author: Iva Greenwald, HHMI/Biochemistry, 701 W. 168th St., Rm. 720, Columbia University College of Physicians and Surgeons, New York, NY 10032.
E-mail: greenwald@cuccfa.ccc.columbia.edu

bined with a class B mutation. These genetic properties led them to suggest that there are two functionally redundant pathways, one composed of class A genes and the other composed of class B genes (Ferguson and Horvitz 1989). Other genetic studies have led to the suggestion that SynMuv genes act upstream of or in parallel to the LET-23 receptor tyrosine kinase (Ferguson *et al.* 1987; Huang *et al.* 1994).

Several SynMuv genes have been characterized in molecular detail. The first three SynMuv genes that were cloned were found to encode novel proteins. The *lin-15* locus contains two genes, one class A and one class B, each encoding a novel protein product (Clark *et al.* 1994; Huang *et al.* 1994). The class B SynMuv gene *lin-36* encodes a novel protein that is expressed in many cell types (Clark *et al.* 1994; Huang *et al.* 1994; Thomas and Horvitz 1999).

Recently, Lu and Horvitz (1998) found that other SynMuv genes encode homologs of proteins known to regulate gene expression. They found that *lin-35*, a class B SynMuv gene, encodes a protein related to the tumor suppressor Rb, and *lin-53*, another class B SynMuv gene, encodes a protein related to RbAp48, a protein that binds Rb. Furthermore, they also obtained evidence that the *hda-1* histone deacetylase gene also acts as a class B SynMuv (Lu and Horvitz 1998), consistent with biochemical studies of the interactions of histone deacetylase and Rb in mammalian cells (Brehm *et al.* 1998; Luo *et al.* 1998; Magnaghi-Jaulin *et al.* 1998). These results suggest that other class B SynMuv genes may also be involved in Rb-mediated transcriptional regulation.

Here, extending an initial observation of Ferguson and Horvitz (1989), we show that the *lin-13* gene has genetic properties consistent with function as a SynMuv gene. We show that the null phenotype of *lin-13* is temperature sensitive and that a Multivulva phenotype results from the absence of zygotic *lin-13* activity at 25°, whereas the absence of zygotic activity at 15° causes a Multivulva phenotype only in the presence of a class A SynMuv mutation. In addition, we show that the predicted LIN-13 protein contains multiple zinc fingers and a motif (LXCXE) that has been implicated in Rb binding. Finally, we show that LIN-13 is a nuclear protein and is consistently expressed in many cell types, including hyp7, at the time of VPC specification. Our results are consistent with a role for LIN-13 in an Rb-mediated transcriptional control process that leads to repression of vulval fates.

MATERIALS AND METHODS

General methods and strains: General methods for the handling and maintenance of *C. elegans* are described by Brenner (1974). Experiments were usually performed at both 15° and 25°, as indicated in the tables and text. Mutations used were as follows:

LG I: *lin-35(n745)* (Ferguson and Horvitz 1989), *smg-1(r861)* and *unc-54(r293)* (Hodgkin *et al.* 1989).

LG II: *lin-8(n111)* (Ferguson and Horvitz 1985), *lin-38(n751)* (Ferguson and Horvitz 1989).

LG III: *unc-93(e1500)* (Greenwald and Horvitz 1980), *unc-36(e251)* (Brenner 1974), *lin-13(n387, n388)* (Ferguson and Horvitz 1985), *qC1* (Austin and Kimble 1987).

LG X: *lin-15(n767)*, *lin-15(n374)*, and *lin-15(n745)* (Ferguson and Horvitz 1989); *lin-15(sy197)* (cited in Huang *et al.* 1994).

The transgenes *arIs30*, used for *lin-13::lacZ* studies, and *arIs48* and *arIs49*, used for LIN-13::GFP (green fluorescent protein) studies, are described below.

Transformation rescue: Transgenic lines were generated by microinjecting *unc-36 lin-13(n387)/qC1* hermaphrodites with cosmid (10 μg/ml) or plasmid DNA (2 μg/ml) along with the dominant *rol-6* marker pRF4 at a concentration of 100 μg/ml (Mello *et al.* 1991). Stable Rol lines were reared at 25°, and individual Unc Rol hermaphrodites segregating from each line were analyzed for the Muv and sterility defects.

Plasmids: The following plasmids were generated during the course of this study and used for experiments described in the text.

pM16 (rescues *lin-13*): A 16-kb *Mlu*I fragment from cosmid C03B8 was cloned into the *Mlu*I site of pGem (Promega, Madison, WI).

pMB13: A 13-kb *Bst*EII to *Mlu*I fragment from cosmid C03B8 was cloned into the *Bst*EII and *Mlu*I sites of Litmus 38 (New England Biolabs, Beverly, MA).

pMS12 (rescues *lin-13*): A 12-kb *Mlu*I to *Sal*I site of cosmid C03B8 was cloned into the *Mlu*I and *Sal*I sites of Litmus 38 (New England Biolabs). This plasmid served as the basis for pMSN12, pMSΔ12, pNlin-13::GFP, and pClin-13::GFP.

pMSN12 (rescues *lin-13*): A *Not*I site was engineered by PCR at the predicted ATG of C03B8.4 in pMS12.

pMSNΔ12: The *Not*I site of pMSN12 was cut, treated with Klenow to fill in the overhangs, and religated to destroy the ATG site.

pNlin-13::lacZ (transcription reporter): pNlin-13::lacZ contains a modified *lacZ* gene (encoding β-galactosidase carrying a nuclear localization signal) derived from pPD95.11 (Fire *et al.* 1990). A 3.8-kb *Msc*I to *Stu*I fragment containing the *lacZ* gene (including a stop codon) was inserted in frame into the *Not*I site of plasmid pMSN12.

pNlin-13::GFP (N terminus tagged with GFP): A PCR product encoding GFP (variant S65T, I167T) without its own stop codon was cloned in frame at the predicted ATG where the *Not*I was inserted in pMS12. The resulting hybrid protein has GFP at the N terminus fused to the entire LIN-13 protein except for its initial methionine.

pClin-13::GFP (C terminus tagged with GFP): A PCR product encoding GFP (variant S65T, I167T) without its own stop codon was cloned in frame into the unique *Kpn*I site (codon 2063) of pMS12. The resulting hybrid protein has GFP inserted into LIN-13 near the C terminus, so that the LIN-13 C terminus is preserved.

Generation of lines expressing *lin-13::lacZ*: pN*lin-13::lacZ* was coinjected at a concentration of 50 μg/ml with pRF4 [*rol-6(su1006)*] (Mello *et al.* 1991) at a concentration of 100 μg/ml into *smg-1 unc-54* hermaphrodites. Four independent lines containing extrachromosomal arrays were established and

each was integrated. All of the analysis described in this article was performed using the integrated array *arIs30*, which displayed a consistent staining pattern.

Generation of lines expressing LIN-13::GFP: pNlin-13::GFP was coinjected at a concentration of 100 μg/ml with pRF4 [*rol-6(su1006)*] (Mello *et al.* 1991) at a concentration of 100 μg/ml into N2 hermaphrodites. Two lines carrying extrachromosomal arrays were examined using a Zeiss Axiophot microscope equipped with fluorescence optics and both showed GFP expression in embryos still in utero. One extrachromosomal array was integrated to generate *arIs48*.

pClin-13::GFP was coinjected at a concentration of 100 μg/ml with pNC4-21 [*unc-4*(+)] (Miller and Niemeyer 1995) at a concentration of 50 μg/ml into *unc-4(e120)* hermaphrodites. Four lines carrying extrachromosomal arrays were examined using a Zeiss Axiophot microscope equipped with fluorescence optics and all displayed the same expression pattern as the lines carrying extrachromosomal arrays of pNlin-13::GFP. One extrachromosomal array was integrated to generate *arIs49*.

Antibody staining: For indirect immunofluorescence of fixed larval populations, hermaphrodites were synchronized by treating a mixed population with hypochlorite solution to isolate embryos. Staged preparations were fixed as described in Bettinger *et al.* (1996). Fixed worms were incubated with a polyclonal anti-β-galactosidase antibody (Capell) or a polyclonal anti-GFP antibody (Clontech, Palo Alto, CA), diluted 1:200. In some cases, monoclonal antibody MH27 (Priess and Hirsh 1986) diluted 1:1000 in PTB (1× PBS, 1% BSA, 0.5% Triton X-100) was also added. The primary incubation proceeded overnight at 4°. Worms were washed with 1× PBS, 0.1% BSA, 0.5% Triton-X 100 with several changes of buffer for 4 hr at room temperature. Cy3-conjugated goat anti-rabbit secondary antibody and FITC-conjugated goat anti-mouse secondary antibody (both from Jackson Immunoresearch, West Grove, PA) were diluted 1:300 in PTB and incubated with the fixed worms overnight at 4°. Worms were washed as before, for 4 hr at room temperature. 4′,6-diamidino-2-phenylindole (DAPI) was added to the last wash at a final concentration of 1 μg/ml. Worms were mounted on a 2% agarose pad with 3 μl of 10% N-propyl gallate and viewed with a Zeiss LSM 410 laser scanning confocal attachment on a Zeiss Axiovert 100 microscope.

Sequence analysis: Standard molecular biology protocols were performed as described in Sambrook *et al.* (1989). The DNA sequence of C03B8 was obtained from the *C. elegans* genome sequencing project (Waterston *et al.* 1997). The exons of *lin-13* were predicted by GENEFINDER (Edgley *et al.* 1997). To examine the accuracy of this prediction, we analyzed cDNAs isolated by screening a mixed-stage cDNA library (Stratagene, La Jolla, CA) with the ^{32}P-labeled 12-kb *Mlu*I-*Sal*I fragment. Four cDNAs were identified among 120,000 PFUs screened. These cDNAs were sequenced on one strand in their entirety and spanned the 3′ end and somewhat more than half of the predicted coding region. To determine the structure of the 5′ end and the remaining coding region, we generated PCR products using DNA prepared by Q. Dong from a mixed-stage cDNA library (Barstead and Waterston 1989). The 5′ end was determined from a PCR product generated using the SL1 *trans*-spliced leader sequence (Krause and Hirsh 1987) and an internal *lin-13* primer (data not shown).

The lesions associated with *lin-13* mutations were found by sequencing the *lin-13* coding region of the mutants. We amplified the *lin-13* genomic region by PCR reactions from individual *lin-13* homozygous mutant hermaphrodites segregating from *unc-36 lin-13/qC1* parents. The PCR products were directly sequenced using established methods.

RNA-mediated interference: Double-stranded RNA was prepared using the RNA transcription kit (Stratagene) and injected without dilution according to Fire *et al.* (1998). Injected hermaphrodites were transferred every day for 3 days. A total of 82 injected hermaphrodites were incubated at 25° and their progeny were examined daily for up to 9 days. A total of 24 injected hermaphrodites were incubated at 15° and their progeny were examined daily until they reached adulthood.

RESULTS

***lin-13* genetic interactions with SynMuv genes:** *lin-13(n387)* and *lin-13(n388)* were identified in screens for vulval mutants and genetically characterized (Ferguson and Horvitz 1985). Both mutations, which cause similar phenotypes, are heat sensitive and display maternal effects: at 25°, *lin-13* homozygous hermaphrodites segregating from a *lin-13*/+ mother are sterile and display a Muv phenotype, but at 15° they are fertile, non-Muv, and grandchildless (*i.e.*, their progeny are sterile; Ferguson and Horvitz 1985). The focus of the work described in this article is the role of *lin-13* in VPC specification.

Ferguson and Horvitz (1989) identified two classes of mutations, class A and class B, that behave as SynMuv mutations: mutations in class A are non-Muv, and mutations in class B are non-Muv, but double mutants carrying a mutation in class A along with a mutation in class B are Muv. Ferguson and Horvitz (1989) also reported that *lin-13(n387); lin-15A(n767)* hermaphrodites are Muv at 15°, suggesting that *lin-13* may be a class B SynMuv gene. However, they did not report any combinations of *lin-13* alleles with any other class A alleles or any class B alleles, raising the possibility that the interaction with *lin-15A(n767)* is specific for the *lin-15A* gene or for the *n767* allele.

We therefore examined double mutants between *lin-13(n387)* and several different class A and class B SynMuv mutations at 15° (Table 1). Double mutants containing *lin-13(n387)* and the class A mutations *lin-8(n111)*, *lin-15(sy197)*, *lin-15(n767)*, or *lin-38(n751)* are Muv at 15°. Double mutants containing *lin-13(n387)* and the class B mutations *lin-15(n374)* or *lin-35(n745)* are non-Muv at 15°. The double mutant analysis therefore indicates that *lin-13(n387)* consistently behaves as if it has reduced class B SynMuv gene activity at 15°.

In addition to these genetic interactions at 15°, we note also that for the class A mutation *lin-38(n751)*, a strong semidominant interaction was seen at 25°: 22% (111/507) of hermaphrodites of genotype *lin-38(n751); lin-13(n387) unc-36(e251)/dpy-17(e164)* were Muv at 25°. The interaction with *lin-13(n387)*/+ was weak or not detectable for other class A mutations and not detectable for class B mutations (data not shown).

Molecular cloning and sequence analysis of *lin-13*: *lin-13* was known to map very near and to the left of the cloned gene *mab-5* (Costa *et al.* 1988 and personal communication). Cosmids for the implicated genomic re-

TABLE 1

***lin-13(n387)* acts as a class B SynMuv at 15°**

SynMuv allele combined with *lin-13(n387)*	Class	% Muv at 15°
+	–	(0/38)
lin-8(n111)	A	56 (32/57)
lin-15(sy97)	A	20 (17/87)
lin-15(n767)	A	54 (109/203)
lin-38(n751)	A	65 (39/60)
lin-15(n374)	B	0 (0/178)
lin-35(n745)	B	0 (0/157)

Note that all SynMuv alleles are wild type in a *lin-13(+)* background (Ferguson and Horvitz 1989). The *lin-13(n387)* chromosome was marked with either *unc-93(e1500)* or *unc-36(e251)*. Hermaphrodites of genotype *lin-x; unc lin-13(n387)/qC1* were allowed to self-fertilize at 15°; 50–200 Unc hermaphrodite progeny were scored for the presence of multiple pseudovulvae in the dissecting microscope. % Muv, number of Unc Multivulva/total Unc hermaphrodite progeny.

gion were tested in germline transformation experiments (G. Kao, A. Meléndez and I. Greenwald, unpublished observations), and cosmid C03B8 (*C. elegans* Sequencing Consortium 1998) was found to rescue both the sterility and Muv phenotypes of *lin-13(n387)* (Figure 1). We analyzed subclones of C03B8 in rescue assays and identified a 16-kb *Mlu*I fragment (pM16) and a smaller 12-kb *Mlu*I-*Sal*I fragment (pMS12) that have rescuing activity (Figure 1). These fragments include a predicted gene, C03B8.4, which encodes a predicted zinc-finger protein; the only predicted gene present on the 12-kb *Mlu*I-*Sal*I fragment, and the only complete gene present on the 16-kb *Mlu*I fragment, is C03B8.4. The introduction of a frameshift mutation at the predicted start ATG of C03B8.4 on the 12-kb *Mlu*I fragment (pMS–DN12) abolished rescuing activity (Figure 1). These results suggested that C03B8.4 corresponds to *lin-13*, and this inference was confirmed by finding sequence changes in both *lin-13* alleles (see below).

We isolated and characterized cDNAs corresponding to the zinc-finger protein and verified the exon/intron junctions as predicted by GENEFINDER (Edgley *et al.* 1997). Our results were in agreement with the predicted sequence except for the following: the GENEFINDER prediction contained two putative small exons that were not identified by the cDNA analysis, one exon that was instead found to be longer by the cDNA analysis, and a predicted intron that was instead found to be in cDNA. The sequence we deduced from our cDNA analysis has been submitted to GenBank (accession no. AF245435). Northern and 5′ rapid amplification of cDNA ends analyses indicate that the *lin-13* transcript is ~7 kb in length and appears to be SL1 *trans*-spliced at the 5′ end (data not shown).

The predicted LIN-13 protein contains 2248 amino acids and includes recognizable amino acid sequence motifs (Figure 2). Using the sequence analysis program SMART (Schultz *et al.* 1998) and inspection, we identified 24 apparent zinc fingers of the C2-H2 class [C-X(2-4)-C-X(3)-F-X(5)-L-X(2)-H-X(3)-H] and 1 apparent zinc finger of the C4 class (Klug and Schwabe 1995). All of these apparent zinc fingers appear to be conserved in *C. briggsae lin-13* (based on a sequence available via the Washington University genome sequencing consortium; data not shown). Zinc-finger motifs have been implicated in DNA binding and in protein-protein interactions (Berg 1990; Klug and Schwabe 1995).

By inspection of the LIN-13 sequence, we also identified an LXCXE motif. The LXCXE motif was identified in several Rb-binding proteins and has been shown to mediate the binding of such proteins to Rb (Fattaey *et al.* 1993; Lee *et al.* 1998; Magnaghi-Jaulin *et al.* 1998). The sequence data available for the apparent *C. briggsae lin-13* indicate that this LXCXE motif is conserved (Figure 2).

***lin-13* mutations:** We expected that the two existing *lin-13* alleles would be missense mutations, as both alleles are temperature sensitive, a property that is not usually associated with null alleles. Furthermore, Ferguson and Horvitz (1989) reported that *lin-13(n387)/nDf16* larvae produced when *lin-13(n387)* heterozygous males were mated with *nDf16* heterozygous hermaphro-

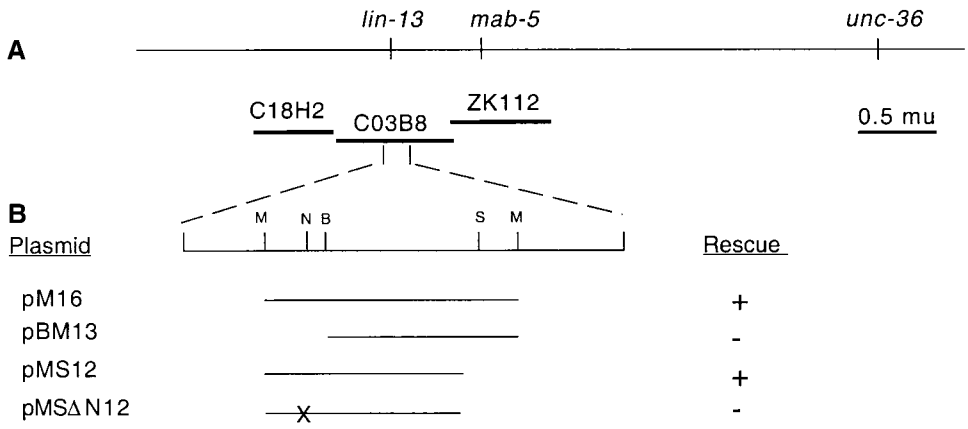


Figure 1.—Molecular cloning of *lin-13*. Rescue of *lin-13* mutant phenotypes was assessed as described in materials and methods. Relevant restriction sites mentioned in the text are indicated: M, *Mlu*I; N, *Not*I; B, *Bst*EII; S, *Sal*I. (A) Simplified genetic map. (B) Schematic physical map.

dites at 25° arrested at what appeared by size to be the L2 stage. This result suggested that the null phenotype of *lin-13* is likely to be zygotic lethal.

However, when we identified the molecular lesions associated with *lin-13(n387)* and *lin-13(n388)*, we found that both are early nonsense mutations. *lin-13(n387)* is a T-to-A (S524stop) change that disturbs the third zinc finger and would encode a predicted protein of 523 amino acids (see Figure 3). *lin-13(n388)* is a C-to-T (R857stop) change immediately after the fifth zinc finger and would encode a predicted protein of 856 amino acids (see Figure 3). Thus, both nonsense mutations are predicted to result in greatly truncated LIN-13 protein products that are likely to eliminate or strongly reduce *lin-13* activity. Furthermore, *lin-13* mRNAs containing such early premature stop codons are likely to be unstable in *C. elegans* (Pulak and Anderson 1993). In the next section, we describe results that are consistent with these *lin-13* mutations being null alleles.

The *lin-13* null phenotype: As noted above, one salient feature of *lin-13* alleles is that they are temperature sensitive. Although temperature sensitivity is not usually associated with null alleles, temperature-sensitive null alleles have been identified for other genes (*e.g.*, *daf-4*; Estevez *et al.* 1993) and have been interpreted as an indication that a process rather than a mutant gene product is temperature sensitive. To investigate whether the process that requires *lin-13* is temperature sensitive, as opposed to whether truncated gene products encoded by *lin-13(n387)* and *lin-13(n388)* are temperature sensitive, we used RNA-mediated interference (RNAi; Fire *et al.* 1998) to inactivate endogenous *lin-13* activity.

N2 hermaphrodites were injected with *lin-13* double-stranded RNA (dsRNA) and individual hermaphrodites were incubated on separate plates at 25° or at 15°. We consistently observed stronger phenotypes at 25° than at 15°. After injection of dsRNA into 82 N2 hermaphrodites at 25°, we observed four different kinds of broods: 13 broods were composed entirely of progeny that appeared to be arrested at the L2 stage (see below), 18 broods were composed entirely of sterile Muv hermaphrodites, 31 broods were composed of a mixture of sterile Muv, arrested larvae, and wild-type hermaphrodites, and 9 broods were composed of entirely wild-type hermaphrodites. After injection of dsRNA into 24 N2 hermaphrodites at 15°, we observed two different kinds of broods: 18 broods were composed entirely of progeny that were sterile and generally had a protruding vulva, and 6 broods were composed of a mixture of wild-type hermaphrodites and hermaphrodites with a protruding vulva.

With respect to VPC specification, the observation that when the progeny were raised at 25°, some were Muv, whereas when progeny were raised at 15°, none were Muv suggests that the process that requires *lin-13* activity is temperature sensitive and is consistent with the interpretation that existing *lin-13* alleles strongly reduce or eliminate *lin-13* activity. Thus, with respect to VPC specification, *lin-13* activity appears to be necessary only at higher temperatures. We do not know the precise nature of the cellular defects underlying the protruding vulva phenotype observed at 15°. As the hermaphrodites that have a protruding vulva are generally sterile, their phenotype is reminiscent of the phenotype caused by somatic gonad defects that cause abnormal vulval eversion (Seydoux *et al.* 1993). Preliminary observations suggest that sterile *lin-13* hermaphrodites do have somatic gonadal abnormalities (A. Meléndez, unpublished observations; J. Hubbard, personal communication).

As mentioned above, substantial larval arrest was observed among the progeny of dsRNA-injected hermaphrodites raised at 25°. This phenotype was unexpected, as larval arrest is not observed among the progeny of *lin-13*/+ parents. Larval arrest appears to occur in the L2 stage on the basis of body size and gonadal morphology. The apparent L2 arrest occurred either transiently (for several days) or "permanently" (for at least 9 days at 25°). The arrested L2 larvae show normal motility and are not rod-like and so do not resemble null mutations in Ras pathway components (*e.g.*, Beitel *et al.* 1990).

As RNAi reduces both maternal and zygotic gene activity (Fire *et al.* 1998), we hypothesized that this L2 arrest phenotype reflected the concomitant depletion of both maternal and zygotic *lin-13* activity. To test this hypothesis, we allowed *lin-13(n387)* homozygous hermaphrodites to segregate from *lin-13*/+ mothers at 15°. These hermaphrodites are fertile and non-Muv. If *lin-13(n387)* is a null allele, then these hermaphrodites should be unable to provide maternal *lin-13(+)* activity to their progeny. Indeed, we have found that when their progeny are raised at 25°, they arrest as L2 larvae. This observation, in conjunction with the sequence analysis and RNAi data, suggests that the null phenotype of *lin-13* is temperature sensitive and that the phenotype resulting from the absence of both maternal and zygotic *lin-13* activity is L2 arrest.

As described above, Ferguson and Horvitz (1989) observed that *lin-13(n387)*/*nDf16* arrest as larvae at 25°. It is not clear how their observation relates to the null phenotype we have now described. The *lin-13(n387)*/*nDf16* larvae were obtained from *nDf16*/+ mothers, so perhaps maternal *lin-13* activity was somewhat depleted. In this context, it is perhaps worth noting that at 15°, *lin-13(n387)*/*nDf16* hermaphrodites have a single vulval protrusion (Ferguson and Horvitz 1989), as observed in our RNAi experiments. As *lin-13(n387)* hermaphrodites segregating from heterozygous parents at 15° do not have a protrusion, the difference may be attributable to reduced maternal *lin-13* activity in *nDf16*/+ parents. Alternatively, the *lin-13(n387)*/*nDf16* larvae may arrest for a different reason: as *nDf16* is relatively large, it is

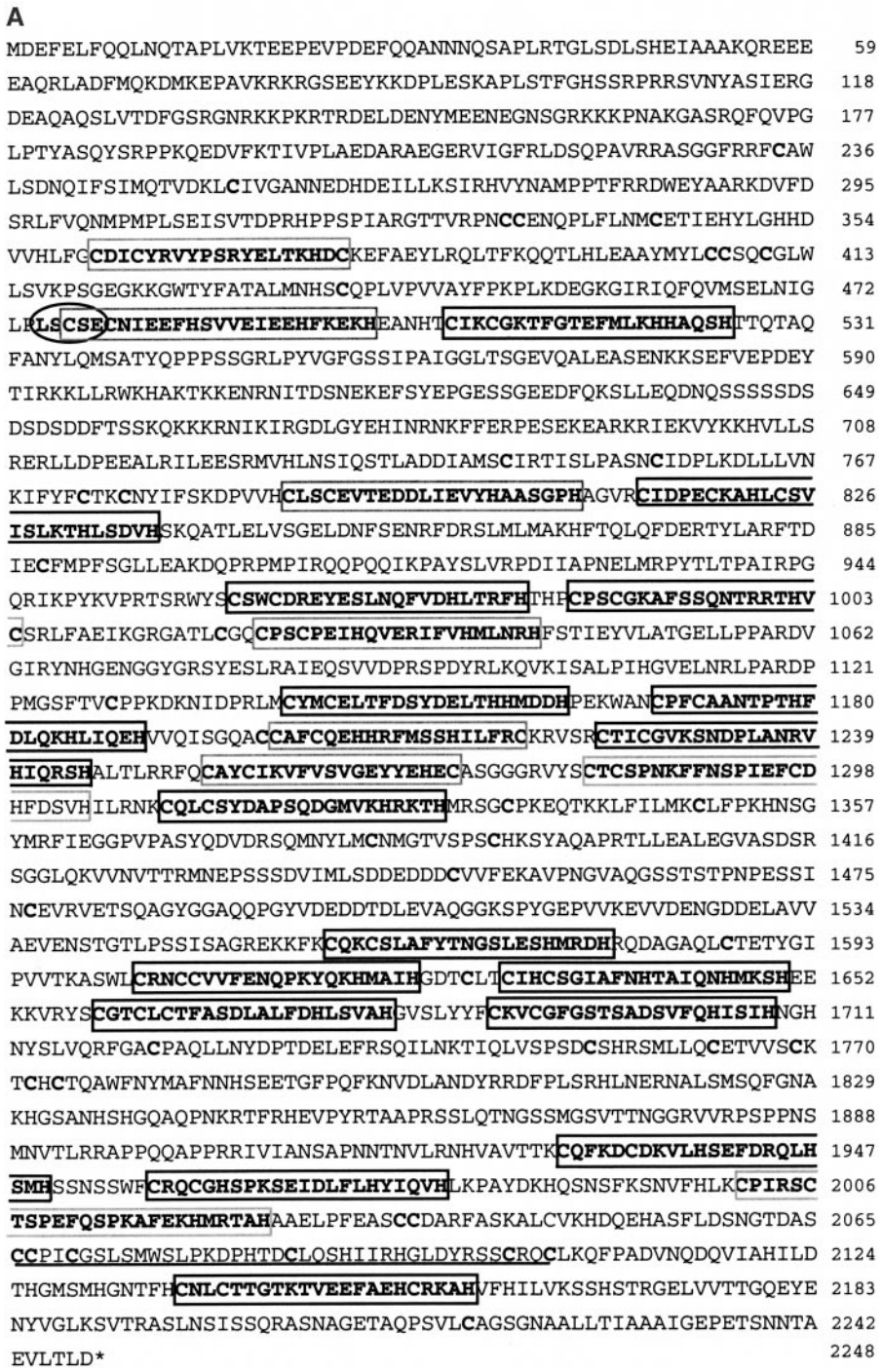


Figure 2.—Features of the LIN-13 predicted protein sequence. (A) LIN-13 predicted protein. All cysteines are indicated in bold. The 24 predicted zinc fingers of the C2-H2 class are boxed. Predicted zinc fingers boxed in solid lines are closer to the consensus sequence (Klug and Rhodes 1987; Berg 1990); the remainder are boxed in shaded lines (see Figure 2B). The single predicted zinc finger of the C4 class is underlined. The LXCXE motif is outlined by an oval. See text for further information. (B) *C. elegans* LIN-13 zinc fingers. The 24 predicted zinc fingers of the C2-H2 class are aligned, along with a consensus sequence. Amino acids that are diagnostic of the zinc-finger motif (Klug and Rhodes 1987; Berg 1990) are in boldface. An asterisk indicates the zinc fingers boxed in solid lines in Figure 2A. (C) LXCXE motif alignments. The LXCXE motifs from *C. elegans* LIN-13 (this study), *C. briggsae* LIN-13 (CbLIN-13; Washington University genome sequence consortium) and several mammalian and viral proteins that are known to interact with Rb via the LXCXE motif are shown (see Jones *et al.* 1990; Fattaey *et al.* 1993; Buyse *et al.* 1995; Taunton *et al.* 1996; Tevosian *et al.* 1997). (D) Molecular alterations identified in *lin-13* alleles. The LIN-13 protein is depicted schematically. Solid bars represent the C2-H2 zinc fingers and a shaded bar represents the C4 zinc finger. The position of the LXCXE motif is indicated by a triangle. *lin-13(n387)* is a C to G alteration (S524stop), and *lin-13(n388)* is a C to T alteration (R857stop).

possible that the deficiency removes other genes whose dosage becomes critical in the absence of *lin-13* activity.

The behavior of *lin-13* null alleles is summarized in Table 2. The phenotypes observed range in severity from L2 arrest (when both maternal and zygotic activities are removed at 25°), to sterile Multivulva (when only zygotic activity is removed at 25°), to sterile non-Multivulva (when both maternal and zygotic activities are removed at 15°), to wild type (when only zygotic activity is removed at 15°).

***lin-13::lacZ* expression:** The inductive and lateral signals specify VPCs to adopt vulval fates during the L3 stage. To examine the cell types in which *lin-13* is expressed at that time, we constructed a *lin-13::lacZ* transcriptional reporter gene in which *lacZ* was inserted in frame at a site engineered at the ATG codon. This reporter gene contains all *lin-13* genomic rescuing sequences and was designed to be analogous to the *smg-1*-dependent *lin-12::lacZ* reporter described in Wilkinson and Greenwald (1995; see materials and methods for details). L3 hermaphrodites showed consistent expression of LacZ in hyp7 nuclei (Figure 3). Consistent

B

	CX_{1-5} C X_3 F X_5 LX_2 HX_{2-6} H/C
1	VHLFG**C**DI..**C**YRVYPSRYE**L**TK**H**D...**C**
2	GLPLS**C**SE..**C**NIEEFHSVVEIEE**H**FKEK**H**
*3	EANHT**C**IK..**C**GKT**F**GTEFM**L**KH**H**AQS.**H**
4	DPVVH**C**LS..**C**EVTEDDLIEVY**H**AASGP**H**
*5	HAGVR**C**IDPE**C**KAHLCSVIS**L**KT**H**LSDV**H**
*6	SRWYS**C**SW..**C**DREYESLNQFVD**H**LTRF**H**
*7	FHTHP**C**PS..**C**GKA**F**SSQNTRRT**H**V...**C**
8	TLCGQ**C**PS..**C**PEIHQVERIFV**H**MLNR**H**
*9	DPRLM**C**YM..**C**ELT**F**DSYDE**L**TH**H**MDD.**H**
*10	EKWAN**C**PF..**C**AANTPTHFD**L**QK**H**LIQE**H**
11	SGQAC**C**AF..**C**QEHHRFMSS...**H**ILFR**C**
*12	KRVSR**C**TI..**C**GVKSNDPLANRV**H**IQRS**H**
13	LRRFQ**C**AY..**C**IKV**F**VSVGEYYE**H**E...**C**
14	GRVYS**C**T...**C**SPNKFFNSPIEFCD**H**FDSV**H**
*15	ILRNK**C**QL..**C**SYDAPSQDGMVK**H**RKT.**H**
*16	EKKFK**C**QK..**C**SLA**F**YTNGS**L**ES**H**MRD.**H**
*17	KASWL**C**RN..**C**VV**F**ENQPKYQK**H**MAI.**H**
*18	DTCLT**C**IH..**C**SGIAFNHTAIQN**H**MKS.**H**
*19	KVRYS**C**GT..**C**LCT**F**ASDLA**L**FD**H**LSVA**H**
*20	SLYYF**C**KV..**C**GFGSTSADSVFQ**H**ISI.**H**
*21	AVTTK**C**QFKD**C**DKVLHSEFDRQL**H**SM..**H**
*22	NSSWF**C**RQ..**C**GHSPKSEID**L**FL**H**YIQV**H**
23	VFHLK**C**PIRS**C**TSPEFQSPKAFEK**H**MRTA**H**
*24	GNTFH**C**NL..**C**TTGTKTVEEFAE**H**CRKA**H**

C

CeLIN-13	N	I	G	L	P	**L**	S	**C**	S	**E**	C	N	I	E	E	F	H	S
CbLIN-13	D	I	G	F	P	**L**	S	**C**	Q	**E**	C	E	T	E	E	F	K	S
HBP1 1	D	S	L	E	L	**L**	Q	**C**	N	**E**	N	V	P	S	S	P	G	Y
HBP1 2	V	V	Q	H	G	I	P	**C**	C	**E**	I	H	I	G	D	V	C	L
HBRM	A	E	V	E	R	**L**	T	**C**	E	**E**	E	E	E	K	I	F	G	R
RBP1	I	G	P	E	T	**L**	V	**C**	H	**E**	V	D	L	D	D	L	D	E
HRIZ	V	K	E	P	E	I	R	**C**	D	**E**	K	P	E	D	L	L	E	E
HDAC1	S	D	-	K	R	I	A	**C**	E	**E**	E	-	F	S	D	S	E	E
SV40	N	E	-	E	N	**L**	F	**C**	S	**E**	E	-	M	P	S	S	D	D
E1A Ade5	P	E	V	I	D	**L**	T	**C**	H	**E**	A	G	F	P	P	S	D	D
E7 HPV	P	E	T	T	D	**L**	Y	**C**	Y	**E**	Q	L	N	D	S	S	E	E

D

Figure 2.—*Continued.*

expression was also seen in body wall muscles, intestinal nuclei, distal tip cells, and many neurons (data not shown). In contrast, only occasional expression was observed in the VPCs. We do not know whether this occasional staining reflects artifactual expression of LacZ from the multicopy transgene or a very low level of normal expression in the VPCs.

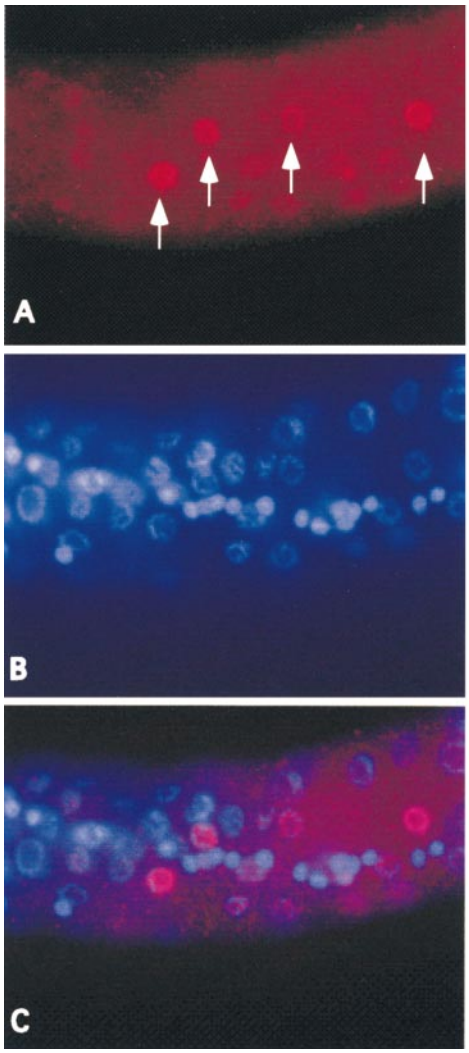
LIN-13 subcellular localization: We constructed LIN-13::GFP hybrid proteins in which GFP (Chalfie *et al.* 1994) is fused in frame at either the predicted amino terminus or carboxy terminus (materials and methods). Transgenes expressing either of these LIN-13::GFP hybrid proteins fully rescue the Muv and sterility defects of *lin-13(n387)* and appear to have identical expression patterns (data not shown). The LIN-13::GFP hybrid proteins localize to the nucleus (Figure 3, D and E), consistent with a role in transcriptional regulation. In the nucleus, there are also discrete bodies or speckles of fluorescence; other nuclear proteins have been found to localize in such patterns, but in most cases the functional significance of these structures is not clear (Lamond and Earnshaw 1998). Recently, Fukushige *et al.* (1999) have visualized binding of the GFP-tagged transcription factor ELT-2::GFP to an array carrying known ELT-2 binding sequences in living embryos as discrete foci of fluorescence that resemble the speckles we have observed, raising the intriguing possibility that they reflect binding of LIN-13::GFP to specific binding sites for LIN-13.

In living animals, we see significant GFP fluorescence in embryos (Figure 3D); we have not determined the cell types expressing GFP in embryos. In living larvae, however, expression is very low, and in particular we could not detect expression during the L3 stage. To improve the sensitivity of LIN-13::GFP detection, we fixed and stained hermaphrodites with antibodies to GFP. In such preparations, we see consistent LIN-13::GFP accumulation in the nuclei of hyp7 and in seam cells throughout development, including the L3 stage. We also see nuclear accumulation in other cell types; we did not characterize these in detail, but the expression pattern of LIN-13::GFP seems roughly equivalent to that of *lin-13::lacZ*. As with *lin-13::lacZ*, we do not see a reliable expression of LIN-13::GFP in the VPCs, although we occasionally detect GFP in one or two VPCs in fixed and antibody-stained hermaphrodites (data not shown).

DISCUSSION

Genetic properties of *lin-13*: We have obtained evidence that the two existing temperature-sensitive *lin-13* mutations are null alleles. Both *lin-13* alleles contain premature stop codons that are predicted to result in severely truncated LIN-13 proteins. RNA-mediated interference causes a temperature-sensitive phenotype similar to that caused by mutations in *lin-13*. The phenotypes observed are variable and are influenced by maternal rescue. These phenotypes range in severity from L2 arrest (when both maternal and zygotic activities are removed at 25°), to sterile Multivulva (when only zygotic activity is removed at 25°), to sterile non-Multivulva (when both maternal and zygotic activities are removed at 15°), to wild-type/class B SynMuv (when only zygotic activity is removed at 15°). The apparent temperature-sensitive null phenotype suggests that the process or complex involving LIN-13 is temperature sensitive.

Extending an earlier observation of Ferguson and Horvitz (1989), we have found that *lin-13* has genetic properties consistent with function as a class B SynMuv. At 15°, *lin-13* alleles consistently behave as class B SynMuv alleles; *i.e.*, when segregating from a *lin-13*/+



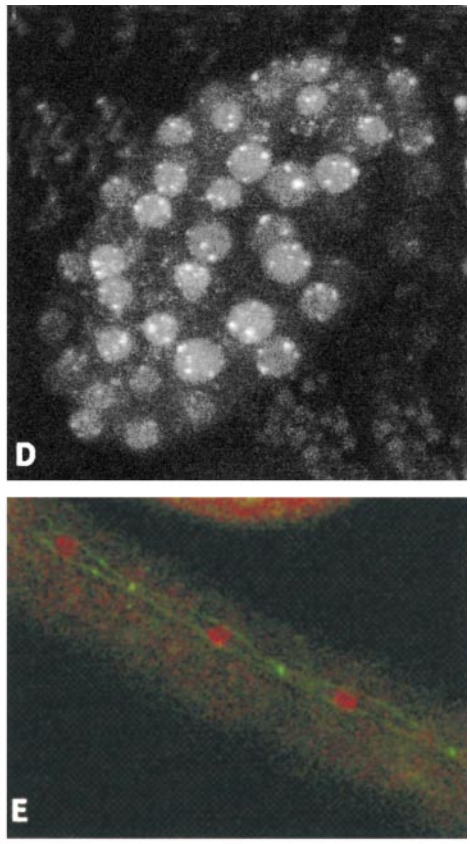


Figure 3.—Analysis of *lin-13::lacZ* and LIN-13::GFP. (A–C) *lin-13::lacZ* expression in hyp7 in L3 larvae. Larvae were fixed and stained with an anti-LacZ antibody (red) as well as DAPI to visualize nuclei. (A) Anti-LacZ; (B) DAPI; (C) merged anti-LacZ antibody (red) and DAPI. (D and E) Nuclear localization of LIN-13::GFP. (D) Living embryo. Fluorescence is nuclear. Note also the discrete fluorescent foci within the nuclei. (E) Lateral hypodermal seam cells in an L3 larva. Larvae were fixed and stained with an anti-GFP antibody to visualize LIN-13::GFP (red). Larvae were also stained with MH27, a monoclonal antibody that detects adherens junctions (green; Priess and Hirsh 1986).

mother, they display a Muv phenotype in combination with all class A mutations tested, but they did not display a Muv phenotype in combination with any class B mutation tested. The consistent behavior of *lin-13* as a class B SynMuv gene implicates *lin-13* in inhibitory signaling.

lin-13 appears to be absolutely required for inhibitory signaling. At 25° *lin-13* homozygous hermaphrodites segregating from a *lin-13*/+ mother are Muv even in the presence of class A gene activity (Ferguson and Horvitz 1985). One plausible explanation for this behavior is that *lin-13* might also possess class A activity. If so, then *lin-13* might provide a physical or functional link between the class A and class B genes.

While we were revising this article, Solari and Ahringer (2000) published another linkage between class A and class B SynMuv genes. Using RNA-mediated interference, they obtained evidence that five components of the chromatin remodeling complex NuRD (Tong *et al.* 1998; Xue *et al.* 1998) have both class A and class B SynMuv gene activity. Interestingly, these components include HDA-1 (histone deacetylase) and LIN-53 (RbAp48), which have been physically as well as functionally associated with Rb (Brehm *et al.* 1998; Luo *et al.* 1998; Magnaghi-Jaulin *et al.* 1998; see also below).

The nature of the *lin-13* mutations and the *lin-13* RNAi phenotype suggests that the putative class A activity, or other activity, of *lin-13* is dispensable at 15°. In this discussion, we focus on the class B SynMuv activity of *lin-13*, as it is this activity that is apparent at all temperatures.

LIN-13 and Rb: We have shown that LIN-13 is a nuclear protein that is predicted to contain 24 zinc fingers. These features are consistent with a role for LIN-13 in transcriptional regulation of target genes. The zinc fingers of LIN-13 might bind DNA directly or function in protein-protein interactions (Berg 1990; Klug and Schwabe 1995).

LIN-13 also contains an LXCXE motif. The LXCXE motif has been identified in several different Rb-binding proteins and furthermore has in numerous cases been shown to be necessary and sufficient for binding to the "pocket" of Rb and Rb-related proteins (Fattaey *et al.* 1993; Lee *et al.* 1998; Magnaghi-Jaulin *et al.* 1998). For example, Rb has been found to interact via the

TABLE 2

The *lin-13* null phenotype

Genotype of progeny	Genotype of mother	Temperature	Muv	L2 arrest	Sterile
N2	N2	25°	No	No	No
lin-13(n387)[a]	*lin-13*/+	25°	Yes	No	Yes
lin-13(RNAi)[b]	N2[c]	25°	Yes	Yes	Yes
N2	N2	15°	No	No	No
lin-13(n387)	*lin-13*/+	15°	No	No	No
lin-13(n387)	*lin-13(n387)*	15°	No	No	Yes
lin-13(RNAi)[d]	N2[c]	15°	No	No	Yes
N2	N2	15° → 25°	No	No	No
lin-13(n387)	*lin-13(n387)*	15° → 25°[e]	Yes	Yes	Yes

lin-13(n387) is marked with *unc-36(e251)*. The data in this table are based on Ferguson and Horvitz (1985, 1989) and this study.

[a] Hermaphrodites also grow slowly.

[b] Data are described in the text.

[c] The maternal contribution of *lin-13* may be depleted in the progeny of these injected hermaphrodites.

[d] Hermaphrodites have a protruding vulva but are not Multivulva.

[e] Gravid *lin-13(n387)* hermaphrodites segregated from *lin-13*/+ parents at 15° were shifted to 25°, and the embryos were hatched and maintained at 25°. Most of these embryos developed until the L2 stage and arrested, but some became sterile Multivulva adults.

pocket domain with the LXCXE motif of HDAC1 (Magnaghi-Jaulin *et al.* 1998), leading to repression of target genes. Rb has also been found to interact via the pocket domain with the LXCXE motif of various viral oncoproteins (see Figure 2C), leading to activation of target genes (Fattaey *et al.* 1993; Lee *et al.* 1998). The presence of this motif in LIN-13, in the context of the class B SynMuv genetic activity of *lin-13*, suggests that LIN-13 may directly interact with LIN-35 Rb, the product of another class B SynMuv gene (Lu and Horvitz 1998).

Loss of the activity of any one class B SynMuv gene can result in a Multivulva phenotype if class A activity is also removed. These results suggest that class B SynMuv genes function within a single pathway, process, or complex. Available biochemical data for the class B SynMuv gene products LIN-35 Rb, LIN-53 Rbp48, and HDA-1 histone deacetylase support this interpretation, as they all appear to be constituents of a single complex (Lu and Horvitz 1998).

The presence of zinc fingers and its nuclear localization suggest that LIN-13 plays a role in gene regulation. In *C. elegans* both LIN-53 RbAp48 and HDA-1 HDAC lack LXCXE motifs. Although these proteins are able to interact with LIN-35 Rb *in vitro* (Lu and Horvitz 1998), it is possible that *in vivo* LIN-13 serves as a bridge among these proteins. Another possible function for LIN-13 is to serve as a transcription factor that, like E2F, is regulated by Rb binding. Biochemical characterization of LIN-13, including an examination of potential physical interactions with various components of the LIN-35 Rb complex, will be necessary to distinguish among these possibilities.

Recently, Hsieh *et al.* (1999) showed that several class B SynMuv genes, including *lin-35* Rb, are required for the silencing of genes present in tandemly repeated transgenic arrays. Hsieh *et al.* (1999) showed that the expression of several repetitive transgenes is reduced in class B SynMuv mutant backgrounds. Although we have not yet tested the effect of eliminating *lin-13* activity on the expression of the same arrays examined by Hsieh *et al.* (1999), we note that we have never observed reduced expression of any of the various repetitive transgenes we have placed in a *lin-13* background in the course of other studies (A. Meléndez and I. Greenwald, unpublished observations). If further analysis demonstrates that *lin-13* indeed behaves differently from other class B SynMuvs with respect to context-dependent gene silencing, we might speculate that LIN-13 serves to target the LIN-35 Rb/HDAC complex to specific target genes involved in inhibition of vulval fates.

The role of SynMuv genes in VPC specification: A Multivulva phenotype can in principle result from activation of a pathway or process that promotes vulval fates or from the inhibition of a pathway or process that represses vulval fates. Genes that influence this process can in principle act in an external signaling cell or in the VPCs. There have been two views to date. One view is that SynMuv genes act in an external signaling cell, hyp7, to control expression of a factor that antagonizes the effects of the inductive signal or otherwise to promote nonvulval fates (Herman and Hedgecock 1990). The other view is that SynMuv genes act in the VPCs to repress transcription of genes that are involved in vulval differentation (Lu and Horvitz 1998).

The proposal that SynMuv genes act in hyp7 was based

on genetic mosaic studies of a *lin-15* mutation that concomitantly removes both class A and class B activities (Herman and Hedgecock 1990) and was supported by mosaic analysis of the class B SynMuv gene *lin-37* (Hedgecock and Herman 1995). Our findings that *lin-13::lacZ* and LIN-13::GFP are consistently expressed in hyp7 and are generally undetectable in VPCs at the time of VPC specification are consistent with this view. If SynMuv genes act in hyp7 and not in the VPCs, they may promote the expression in hyp7 of an inhibitory factor that represses vulval fates. This hypothetical factor may activate an as-yet-unknown but independent signal transduction pathway in the VPCs, so that whether a VPC adopts a vulval fate or not depends on the relative strengths of the inductive and inhibitory signaling inputs. Alternatively, the inhibitory factor may be a direct antagonist of the LET-23 epidermal growth factor (EGF) receptor. Such antagonists have been identified in Drosophila: the secreted protein Argos and the transmembrane protein Kekkon 1 appear to inhibit EGF receptor signaling via direct binding mechanisms (Freeman *et al.* 1992; Ghiglione *et al.* 1999).

Alternatively, Lu and Horvitz (1998) have proposed that *lin-35* Rb and *lin-53* Rbp48 function in the VPCs in response to inhibitory signaling from hyp7 to antagonize transcription factors that are activated by Ras. They favored a model in which these genes function in the VPCs because an anti-LIN-35 antibody appeared to stain VPC nuclei but not hyp7 nuclei and because genetic mosaic analysis of the class B SynMuv gene *lin-36* had been interpreted as indicating a focus in ABp, the ancestor of the VPCs (Thomas and Horvitz 1999). However, LIN-53::GFP is detected in hyp7 as well as in the VPCs (Lu and Horvitz 1998), and about half of the mosaic individuals lacking *lin-36* activity in ABp were non-Muv (Thomas and Horvitz 1999), consistent with the possibility that *lin-36* also has a complex focus for VPC specification. As it remains possible that some SynMuv genes act in hyp7 while others act in the VPCs, and as expression data can be misleading as to cellular focus, systematic genetic mosaic analyses of all the SynMuv genes, including *lin-35* and *lin-13*, will be necessary before this issue can be resolved.

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