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The *C. elegans* gene *lin-36* acts cell autonomously in the *lin-35 Rb* pathway

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SUMMARY

The *Caenorhabditis elegans* gene *lin-36* acts to antagonize Ras-mediated vulval induction in a pathway that includes genes with products similar to the mammalian retinoblastoma (Rb) protein and the Rb-binding protein p48. We report that *lin-36* encodes a novel protein of 962 amino acids. We demonstrate that *lin-36* functions in and is expressed in the vulval precursor cells, establishing that the *lin-36* pathway is involved in intercellular signaling. We also report that the *lin-36* pathway and/or another pathway that is functionally redundant with the *lin-36* pathway antagonize a ligand-independent activity of the receptor tyrosine kinase/Ras vulval induction pathway.

Key words: *lin-36*, Signal transduction, Vulval development, Redundancy, *C. elegans*

INTRODUCTION

Receptor tyrosine kinase (RTK) and Ras-mediated signal transduction pathways have been found to function normally in the control of the development of numerous organisms (Pawson and Bernstein, 1990; Perrimon, 1994) and have been implicated in oncogenesis (Egan and Weinberg, 1993). The core signal transduction pathway is well conserved and well understood (Egan and Weinberg, 1993). However, little is known about how this pathway is regulated. To approach this problem, we are analyzing the regulation of the RTK- and Ras-mediated signal transduction pathway of vulval induction in *Caenorhabditis elegans* (for reviews, see Horvitz and Sternberg, 1991; Eisenmann and Kim, 1994; Sundaram and Han, 1996).

In vulval induction a signal from a single cell in the developing gonad, the anchor cell, activates an RTK- and Ras-mediated signal transduction pathway to induce three nearby hypodermal blast cells, P5.p, P6.p and P7.p, to adopt vulval fates. These three cells are members of the vulval equivalence group, a set of six cells collectively referred to as P(3-8).p, all of which have the potential to adopt either of two vulval fates (called 1° and 2°) or a non-vulval fate (called 3°). Mutations that decrease activity in this signaling pathway block vulval induction and result in a vulvaless (Vul) phenotype, whereas mutations that increase activity in this pathway can cause the P3.p, P4.p and P8.p cells to adopt vulval fates and result in a multivulva (Muv) phenotype.

A number of genes have been identified that appear to regulate the activity of the signal transduction pathway of vulval induction. Mutations in the genes *lin-2*, *lin-7* and *lin-10* produce a Vul phenotype similar to that produced by reduction-of-function mutations in components of the *let-60 ras* pathway. *lin-2*, *lin-7* and *lin-10* appear to act to localize the LET-23 receptor to the basolateral membrane of the P(3-8).p cells, thereby upregulating the activity of the receptor tyrosine kinase pathway (Ferguson et al., 1987; Hoskins et al., 1996; Simske et al., 1996).

Antagonists of the vulval inductive pathway have also been identified. A group of genes known as the synthetic Multivulva (synMuv) genes inhibit the adoption of vulval fates by the P(3-8).p cells. A synthetic interaction between two different synMuv mutations is required to produce a synMuv phenotype (Horvitz and Sulston, 1980; Ferguson and Horvitz, 1985, 1989). Specifically, the synMuv mutations define two classes of genes, A and B. Animals must carry both a class A and a class B mutation to have a Muv phenotype; animals that carry mutations in either class but not in both classes 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: if both pathways are blocked, P(3-8).p cells that would normally be prevented from adopting vulval fates instead adopt vulval fates. Four class A genes (*lin-8*, *lin-15*A, *lin-38* and *lin-56*) and ten class B genes (*lin-9*, *lin-15*B, *lin-35*, *lin-36*, *lin-37*, *lin-51*, *lin-52*, *lin-53*, *lin-54*, *lin-55*) have been identified (Horvitz and Sulston, 1980; Ferguson and Horvitz, 1989; J. H. Thomas and H. R. Horvitz, unpublished observations). *lin-15* is a complex locus with genetically separable class A and class B activities encoded by two nonoverlapping transcripts (Ferguson and Horvitz, 1989; Clark et al., 1994; Huang et al., 1994). Five synMuv genes have been cloned. Three, *lin-15*A, *lin-15*B and *lin-9*, encode novel proteins (Clark et al., 1994; Huang et al., 1994; Beitel, 1994). *lin-35*, a class B gene, encodes a protein similar to the retinoblastoma-susceptibility gene product Rb, and *lin-53*, another class B gene, encodes a protein similar to the RbAp48 protein, which interacts with Rb (Lu and Horvitz, 1998). Genetic mosaic analyses suggest that *lin-15*A, *lin-15*B and *lin-37* act cell non-autonomously and most likely in the

hypodermal syncytium, suggesting that the synMuv genes encode the components of two redundant systems by which the hypodermis inhibits vulval induction (Herman and Hedgecock, 1990; Hedgecock and Herman, 1995).

In this paper, we describe studies of the class B synMuv gene *lin-36*. We report that *lin-36* encodes a novel protein, is likely expressed in the nuclei of the P(3-8).p cells, and acts cell autonomously.

MATERIALS AND METHODS

General genetic methods and strains

C. elegans strains were maintained and genetically manipulated as described by Brenner (1974) and were grown at 20°C unless otherwise stated. N2 was the wild-type strain. Mutations used in this study are described by Hodgkin et al. (1988) or as indicated in the text.

Triple and double mutants for gene interaction studies were constructed essentially as described by Ferguson et al. (1987). For all genes tested, either null alleles or the strongest known mutations were used.

Non-complementation screens for *lin-36* alleles

L4 males of genotype *lin-8(n111)* were mutagenized with 4,5′,8-trimethylpsoralen (TMP) and ultraviolet radiation (Yandell et al., 1994) and mated with hermaphrodites of genotype *lin-8(n111); sma-3(e491) lin-36(n766) unc-32(e189)*. A total of 23,666 haploid genomes were screened, and one Muv non-Sma non-Unc F_1 animal was isolated. The new mutation was made homozygous and backcrossed three times.

In a second non-complementation screen, L4 males of genotype *lin-8(n111)* were mutagenized with ethyl methanesulfonate (EMS) (Brenner, 1974) and mated with hermaphrodites of genotype *lin-8(n111); lin-36(n766) unc-32(e189); lon-2(e678) xol-1(y70)*. A total of 21,003 haploid genomes were screened, and five Muv non-Unc non-Lon F_1 animals were isolated. The new mutations were made homozygous and backcrossed three or four times.

P(3-8).p cell lineage analysis

P(3-8).p cell lineages were observed using Nomarski optics as described by Sulston and Horvitz (1977).

General DNA manipulation

General DNA manipulations and analyses were performed essentially according to the protocols of Sambrook et al. (1989). The RNA filter used for northern blots was a gift from L. Bloom.

Subclones were constructed from cosmid E02E3. *Mlu*I and *Nco*I were used to delete cosmid DNA to construct pJHT1 and pJHT2, respectively. The following subclones contained the DNA between the listed restriction sites: pJHT9, *Mlu*I and *Kpn*I; pJHT12, *Spe*I and *Kpn*I; pJHT13, *Sal*I and *Kpn*I; pJHT15, *Sal*I and *Sac*I; pJHT16, *Sal*I and *Xba*I; pJHT18, *Cla*I and *Xba*I.

Germline transformation

Germline transformation by microinjection was done according to Mello and Fire (1995). DNA for injection was purified using Qiagen columns (Qiagen Inc., Chatsworth, CA). Cosmids and plasmids for rescue experiments were injected at concentrations of 20-80 μg/ml. The *lin-36::GFP* reporter was injected at 50 μg/ml. pRF4, a plasmid containing the *rol-6(su1006)* dominant allele, was used as a coinjection marker in all experiments at a concentration of 80 μg/ml (Mello and Fire, 1995).

Isolation of cDNAs and sequence determination

We screened a mixed-stage cDNA library (Barstead and Waterston, 1989) with the ^{32}P-labeled 5.0 kb insert in pJHT16 and isolated one cDNA. After limited *Exo*III digestion, M13 phage was used to prepare single-stranded DNA (Sambrook et al., 1989), and DNA sequences were determined using the method of dideoxy chain termination and the Sequenase enzyme (US Biochemical, Cleveland, OH). One region in the 3′ untranslated region required the use of *Taq* polymerase at 95°C for sequence determination (Promega, Madison, WI).

Molecular analysis of *lin-36* lesions

Genomic DNA was purified from N2 and strains carrying all known *lin-36* mutations. *lin-36(n3090)* DNA was digested with *Sal*I and *Xba*I, *Nco*I, *Nco*I and *Xba*I, *Eco*RV and *Hinf*I, and analyzed by Southern blot analysis (Sambrook et al., 1989) using the ^{32}P-labeled insert of pJHT16 as a probe. The *lin-36* coding region and the regions of introns near the splice sites were amplified using the polymerase chain reaction (PCR), and the sequences of these PCR products were determined using an automated ABI 373A cycle sequencer (Applied Biosystems, Foster City, CA).

Genetic mosaic analysis

Genetic mosaic analysis of *lin-36* was conducted using the method of free duplication loss described by Herman (1984). Two strategies were adopted to find mosaic animals using a strain of genotype *lin-8(n111); dpy-17(e164) ncl-1(e1865) lin-36(n766) unc-36(e251); sDp3[dpy-17(+) ncl-1(+) lin-36(+) unc-36(+)]*. First, mosaic L3 and L4 larvae were isolated on the basis of gross morphology and then observed using Nomarski optics to define the cell division at which duplication loss occurred by observing the enlarged nucleoli seen in cells mutant for *ncl-1* (Hedgecock and Herman, 1995). Specifically, Unc non-Dpy animals result from duplication loss in AB or ABp; Dpy non-Unc animals result from loss in P_1, P_2 or C (Yuan and Horvitz, 1990). In these animals, a P_2 loss can be distinguished from a C loss by the segregation of all Dpy Ncl Unc progeny. Semi-Unc non-Dpy animals are the result of a loss in either ABpl or ABpr. The second strategy was to examine 1,515 seemingly wild-type L3 and L4 larvae for the Ncl phenotype using Nomarski optics. This approach enabled us to identify ABa losses and losses in the ABp lineages later than ABpl/r. The Ncl phenotype was scored primarily in neurons; it cannot be scored reliably in the Pn.p cells themselves. From the P_1 lineage, the pharyngeal neurons I4, I6, M1, M4 and M5, the tail neurons PVR and DVC, and occasionally hyp11 were scored. From ABp, the HSNs and most of the ABp-derived neurons of the head and tail were scored. The subset of ABp-derived neurons with a Ncl phenotype and their lineage relationship (Sulston and Horvitz, 1977; Sulston et al., 1983; White et al., 1986) allowed us to determine the cell division at which duplication loss occurred. From ABa, the BDUs, the ALMs, the pharyngeal neurons I5, MI, the M2s and the M3s, and most of the ABa-derived neurons of the head were scored.

Construction of a *lin-36::GFP* reporter

A *lin-36::GFP* reporter was constructed by ligating a 4.4 kb *Sal*I *Sac*I fragment from pJHT16 to a linker fragment and *Sal*I *Xma*I double-digested pPD95.79, a vector containing a green fluorescent protein (GFP) coding region with a *C. elegans unc-54* 3′ end and a fluorescence enhancing S65C mutation (Chalfie et al., 1994; Heim et al., 1994, 1995; A. Fire, personal communication). The linker fragment was constructed by using the primers pG5F (5′ AGAGCAATGGAAGACGC) and pG3W (5′ TCCCCCCGGGTTGTTGAGAATCCGATG) to amplify DNA from pJHT16 and digesting with *Xma*I and *Sac*I. pG3W anneals to the last codons of *lin-36*, and adds an *Xma*I site followed by four additional nucleotides.

Five rescued lines were isolated after germline transformation of *lin-36(n766); lin-15(n767)* animals. To induce chromosomal integration, transformants were exposed to 4,100 rads of γ-irradiation from a ^{60}Co source (Mello and Fire, 1995). Two lines that retained *lin-36* rescue ability and GFP staining were isolated.

Laser microsurgery

Laser microsurgery was conducted essentially as described previously (Sulston and White, 1980; Avery and Horvitz, 1987; Thomas et al.,

1990). 60 mM sodium azide was used as an anesthetic. Mock-ablated animals were used as an operation control.

RESULTS

Identification of new *lin-36* alleles

Seven *lin-36* mutations had been identified previously in screens for class B synMuv mutations (Ferguson and Horvitz, 1989; J. H. Thomas and H. R. Horvitz, unpublished observations). To isolate additional *lin-36* mutations, we conducted two non-complementation screens using a *lin-8(n111)* class A background. In one screen, 4,5′,8-trimethylpsoralen was used as a mutagen. One *lin-36* mutation, *n3090*, was isolated and shown by Southern hybridization to be associated with an *Eco*RV restriction fragment length polymorphism (RFLP). This RFLP was produced by a point mutation rather than a deletion (see below). In the other screen, EMS was used as a mutagen, and five *lin-36* mutations (*n3093*, *n3094*, *n3095*, *n3096*, *n3097*) were isolated. This frequency of EMS-induced *lin-36* alleles, 2.4×10^{-4} per haploid genome, is close to that expected for the average EMS-induced loss-of-function mutation in *C. elegans*, 5×10^{-4} (Brenner, 1974; Meneely and Herman, 1979; Greenwald and Horvitz, 1980).

The phenotypes caused by the strongest *lin-36* mutant alleles (*n747*, *n766*, *n2243*, *n3093*, *n3095*, *n3096*) were essentially identical (Table 1), suggesting that they may be complete loss-of-function alleles. The frequency of isolation in our non-complementation screen is consistent with this hypothesis. However, animals heterozygous for a strong *lin-36* allele and a deficiency of the *lin-36* region displayed a phenotype weaker than that of the corresponding homozygote. For example, the penetrance of the Muv phenotype of *lin-8; lin-36(n766)/nDf20* animals was 87% (*n*=259), whereas that of *lin-8; lin-36(n766)* homozygotes carrying the same heterozygous marker, *unc-32(e189)*, was 95% (*n*=214). Similar results were observed for *lin-36(n3096)*. In addition, animals heterozygous for a weak *lin-36* allele and a strong *lin-36* allele displayed a stronger phenotype than animals heterozygous for a weak allele and a deficiency. Specifically, the penetrance of the Muv phenotype of *lin-8; lin-36(n2235)/nDf20* animals was 74% (*n*=194), whereas that of *lin-8; lin-36(n2235)/lin-36(n766)* animals was 87% (*n*=214) and that of *lin-8; lin-36(n2235)/lin-36(n747)* animals was 91% (*n*=188); all animals scored were heterozygous for *unc-32(e189)*. These observations suggest that strong alleles of *lin-36* reduce *lin-36* function more than does a deficiency. Using the polymerase chain reaction (PCR), we showed that all of the *lin-36* coding sequence was deleted from the *nDf20* chromosome (data not shown). It is possible that a linked, haplo-insufficient suppressor of the synMuv phenotype is deleted by this deficiency. Alternatively, the strong *lin-36* alleles may interfere with the activities of other class B synMuv genes in addition to reducing *lin-36* activity. Although our data are consistent with the hypothesis that we have isolated complete loss-of-function alleles, it remains possible that the strongest *lin-36* alleles are not nulls but rather partially reduce *lin-36* activity and also act to antagonize the activity of other members of the *lin-35 Rb* pathway.

Table 1. Penetrance of the Muv phenotype in strains carrying *lin-36* mutations

	lin-8(n111)		*lin-15(n767)*	
lin-36 allele	% Muv	*n*	% Muv	*n*
n747[a]	98	177	ND	ND
n750[a]	75	357	ND	ND
n766[a]	98	207	100	226
n772[a]	ND	ND	99	369
n2235[b]	79	313	ND	ND
n2240[b]	79	373	ND	ND
n2243[b]	94	593	ND	ND
n3090	81	265	ND	ND
n3093	96	320	ND	ND
n3094	53	278	ND	ND
n3095	94	420	ND	ND
n3096	97	456	99.7	317
n3097	57	334	99	309

The percentage of Muv animals of each genotype was determined at 20°C. Complete genotypes are shown. % Muv, percentage of animals having ventral protrusions. n, number of animals scored. ND, not determined.

[a]Data from Ferguson and Horvitz (1989).

[b]J. H. Thomas and H. R. Horvitz, unpublished observations.

Vulval lineage defects of *lin-36*

We analyzed the vulval defects of strains carrying *lin-36* mutations at the cell lineage level by directly observing the lineages of the P(3-8).p descendants. Animals carrying only a strong *lin-36* mutation, *n766*, displayed wild-type P(3-8).p cell lineages, as did animals carrying only the strong class A mutation, *lin-15(n767)* (Table 2). The cells of the vulval equivalence group that are not induced in the wild type, P(3,4,8).p, were induced in animals of genotype *lin-36(n766); lin-15(n767)*. In the 10 animals examined, 29/30 of these cells were completely induced and the remaining 1/30 (a P8.p cell that divided to give an OOS lineage; see Sternberg and Horvitz, 1986, 1989, for nomenclature) showed partial induction.

Cloning of *lin-36*

Previous data placed *lin-36* between *lin-13* and *unc-36* on linkage group III (Ferguson and Horvitz, 1989). We mapped *lin-36* under *nDf20* and between *egl-5* and *unc-36* (Fig. 1A), both of which had been cloned and positioned on the physical map (Wilson et al., 1994). The genetic data suggested that *lin-36* was located about midway between *egl-5* and *unc-36*, and we injected cosmid pools from this region into animals bearing a class A synMuv mutation and a *lin-36* mutation in germline transformation experiments. Cosmid E02E3 rescued the Muv phenotype of both *lin-8(n111); lin-36(n747)* and *lin-36(n766); lin-15(n767)* animals and was used to construct subclones. These experiments defined a 5.0 kb minimal rescuing fragment, the *Xba*I *Sal*I insert of pJHT16 (Fig. 1B).

This 5.0 kb fragment was used to probe a northern blot containing poly(A)+ RNA from mixed-stage animals and recognized a single band of approximately 3.5 kb. The same fragment was used as a probe to screen a cDNA library and isolate a single clone. The 3,400 nucleotide cDNA included a poly(A) tail and nine nucleotides of the SL1 *trans*-spliced leader (Krause and Hirsh, 1987; Huang and Hirsh, 1989), indicating that it was full-length. The sequence of the cDNA was compared to the genomic sequence from the region generated by the *C. elegans* genome sequencing project (Wilson et al., 1994) to define the exon and intron structure of the gene (Fig. 1C).

lin-36 encodes a novel protein

The coding sequence of *lin-36* was determined from the cDNA clone (Fig. 2). Conceptual translation yielded a novel gene

Table 2. P(3-8).p cell lineages in *lin-36* mutants

Genotype	*n*	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	Gross phenotype
N2[a]	Many	S	SS	(**LL**TN)	[TTTT]	(NT**LL**)	SS	WT
	Many	SS	SS	(**LL**TN)	[TTTT]	(NT**LL**)	SS	WT
lin-15(n767)	5	S	SS	(**LL**TN)	[TTTT]	(NT**LL**)	SS	WT
	4	SS	SS	(**LL**TN)	[TTTT]	(NT**LL**)	SS	WT
	1	SS	SS	(**LL**TN)	[TTTT]	(NO**LL**)	SS	WT
lin-36(n766)	5	S	SS	(**LL**TN)	[TTTT]	(NT**LL**)	SS	WT
	5	SS	SS	(**LL**TN)	[TTTT]	(NT**LL**)	SS	WT
lin-36(n766); lin-15(n767)	1	(LO**LL**)	[LLTT]	(**LL**ON)	[TTTL]	(NO**LL**)	[OTOL]	2a1p
	1	OT**L**O	(**LL**OO)	(**LL**TN)	[TTTT]	(NT**LL**)	[LLTO]	2a1p
	1	LSOO	(**LL**TL)	(**LL**ON)	[TTTT]	(NT**LL**)	[LTOT]	3a1p
	1	[TOOT]	(**LL**ON)	(**LL**ON)	[TTTT]	(NT**LL**)	[LTTT]	2a1p
	1	[LLTT]	(**LL**TN)	(**LL**ON)	[TTTT]	(NT**LL**)	[OOLO]	2a1p
	1	[OOLO]	(**LL**TT)	(**LL**ON)	[TTTT]	(NL**LL**)	OOS	2a1p
	1	TTL**L**	(**LO**TO)	(**LL**ON)	[TTTT]	(NO**LL**)	[OOOO]	2a1p
	1	(**LL**ON)	[TTTL]	(**LL**LN)	[TTTT]	(NT**LL**)	[LTOO]	1a1p
	1	(**LL**TN)	[TTOO]	(**LL**TN)	[TTTT]	(NL**LL**)	[OTTO]	1a1p
	1	(**LL**TO)	[TOOO]	(**LL**TN)	[TTTT]	(NL**LL**)	[TOTO]	1a1p
lin-7(e1413); lin-36	1	LLTN	??OO	(**LL**ON)	[LOTO]	(NO**LL**)	SOS	2a1p
(n766); lin-15(n767)	1	S	(**LL**ON)	(**LL**TT)	[TTOO]	(LO**OL**)	[LLTL]	1a1p
	1	SOT	(**LL**LN)	[LTTT]	(NT**LL**)	SS	SS	1a
	1	**LLL**L	[OOOT]	(**LL**TN)	[TTTT]	(NT**LL**)	SSS	1a
	1	LTTN	(**OO**ON)	SOT	**L**ONO	SS	SS	3a, Vul

P(3-8).p cell lineages are reported using the nomenclature of Sternberg and Horvitz (1986, 1989) to describe nuclear division and morphology. S, the nucleus adopted a morphology characteristic of fusion with the hypodermis; T, the nucleus divided transversely (left-right); L, the nucleus divided longitudinally (anterior-posterior); O, the nucleus divided obliquely; N, the nucleus did not divide and adopted a distinct morphology; ?, the nucleus had not divided 4 hours after the last divisions but did not adopt the distinct morphology of an N fate.

Underlining and boldface type indicate that the daughter nuclei adhered to the cuticle.

1° cell lineages are indicated by brackets, [].

2° cell lineages are indicated by parentheses, ().

Gross phenotype is described in terms of pseudovulval invaginations in larvae and subsequent pseudovulval protrusions in adults.

The number of pseudovulval invaginations anterior to the vulva is indicated by the number preceding the letter a; the number of pseudovulval invaginations posterior to the vulva is indicated by the number preceding the letter p. WT (wild type), no pseudovulval invaginations formed. Vul, no functional vulva was formed; in the case described here, the vulval tissue protruded. P(3-8).p cell lineages of *lin-7(e1413)* mutants show no or partial vulval induction (Sternberg and Horvitz, 1989).

[a]The N2 lineage data are from Sulston and Horvitz (1977), Sulston and White (1980) and Sternberg and Horvitz (1986), as well as from controls for this manuscript.

product of 962 amino acids with a predicted molecular mass of 108 kDa. The predicted protein product is highly hydrophilic and has no hydrophobic stretches of significant length. There are few obvious protein motifs. There is a potential nuclear localization sequence (AKKRK) at amino acids 528-532 (for review, see Goldfarb, 1989) and a poly(glutamine) stretch at the C terminus. The region between residues 101 and 376 is cysteine- and histidine-rich; it contains 15 histidines and 14 cysteines. One region containing two cysteines (residues 268 and 271), two histidines (residues 284 and 288) and a leucine (residue 281) fits zinc-finger spacing requirements; however, other amino acids are not conserved (Rosenberg et al., 1986; Tautz et al., 1987), and we suspect that this region does not form a zinc finger.

Molecular determination of *lin-36* lesions

We used PCR to amplify *lin-36* DNA from mutants to determine the sequence changes in these strains. Six mutations are missense mutations, five are nonsense mutations, one is a splice-site mutation, and one contains a missense mutation as well as a silent mutation (Table 3; Fig. 1C). Three of the ochre mutations, *lin-36(n766)*, *lin-36(n3095)*, *lin-36(n3096)* and the amber mutation *lin-36(n3093)* eliminate 167, 394, 521 and 384 amino acids of the 962 amino acid coding sequence, respectively. All produced a strong phenotype (Table 1). The fourth ochre mutation, *lin-36(n2235)*, eliminates only 65 amino acids of the coding sequence and produced a weaker phenotype.

lin-36 acts cell autonomously

To determine the site of action of *lin-36*, we conducted a genetic mosaic analysis. A strain of genotype *lin-8(n111); dpy-17(e164) ncl-1(e1865) lin-36(n766) unc-36(e251); sDp3(f)* was used to generate genetic mosaics as described in Materials and methods. Mosaics were identified and characterized as L3 and L4 larvae and subsequently scored for the Muv phenotype upon reaching adulthood. Strains mutant for *lin-36* exhibit maternal rescue of the Muv phenotype (Ferguson and Horvitz, 1989; J. H. Thomas and H. R. Horvitz, unpublished observations). Presumably, maternally deposited wild-type *lin-36* RNA or protein persists through several cell divisions and is present in sufficient quantities in the relevant cells to affect the penetrance of the Muv phenotype. This maternal effect affects the mosaic analysis, so we determined that the penetrance of the Muv phenotype of Dpy Ncl Unc animals homozygous for *lin-36* descended from duplication-bearing mothers was 69% (n=240). This number sets an upper limit on the fraction of Muv animals we expected from a mitotic loss of the duplication in the lineage from which the cellular focus of *lin-36* is derived.

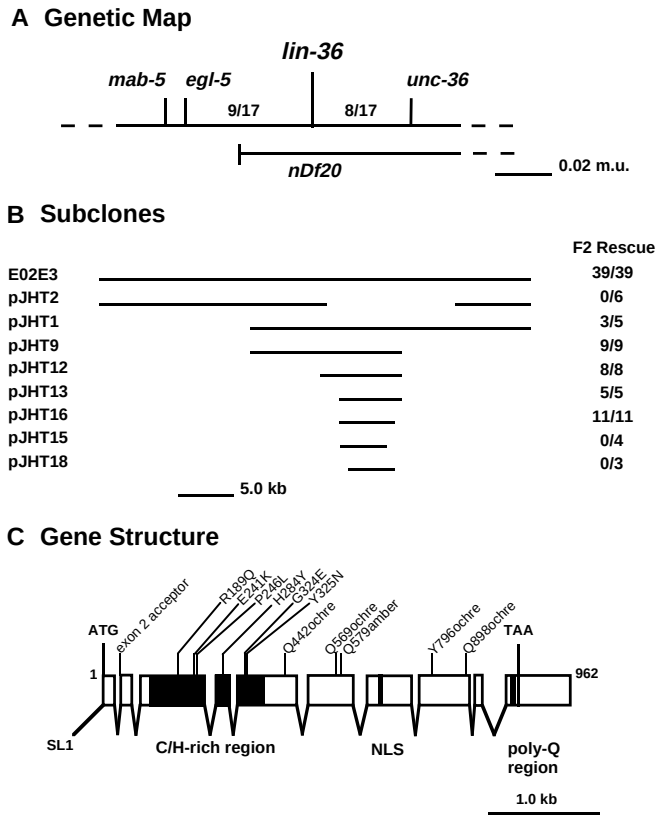

Fig. 1. Cloning of *lin-36*. (A) Genetic map of the *lin-36* region of linkage group III. The deficiency *nDf20* failed to complement *lin-36*, and of 17 recombination events scored between *egl-5* and *unc-36*, nine were between *egl-5* and *lin-36* and eight were between *lin-36* and *unc-36*. Three cosmids from the corresponding region of the physical map (Wilson et al., 1994), C04H11, F44B9 and E02E3, rescued the Muv phenotype of *lin-8(n111); lin-36(n747)* animals. (B) Subclones constructed from cosmid E02E3. The E02E3 F_2 rescue data shown combines the results of injection experiments using both *lin-8(n111); lin-36(n747)* animals and *lin-36(n766); lin-15(n767)* animals; the subclones were tested only in the latter strain. The subclone insert is depicted by the line shown adjacent to each subclone plasmid name (see Materials and methods). (C) Gene structure of *lin-36*. Exons are shown as blocks. Start and stop sequences, poly(glutamine) (poly-Q) and cysteine- and histidine-rich regions and mutational changes are indicated. SL1, *trans*-spliced leader sequence. NLS, nuclear localization sequence.

Duplication loss in AB, ABp, ABpl or ABpr often produced Muv animals (Fig. 3). Loss in ABa or P_1, P_2 or C failed to result in Muv animals. These data are consistent with an anatomical focus in both the ABpl and ABpr lineages. The P(3-8).p cells derive from both of these lineages. The somatic gonad is derived from EMS, a daughter of P_1. By contrast, the major hypodermal syncytium, hyp7, derives from ABa, ABp and C; C, a descendant of P_1, makes the largest hyp7 contribution during embryogenesis. ABa makes a larger contribution to hyp7 than either ABpl or ABpr, yet losses in ABpl or ABpr often resulted in a Muv phenotype, whereas losses in ABa or P_1 did not (Fig. 3). Since vulval development is thought to involve the P(3-8).p cells, hyp7 and the gonadal anchor cell, these observations indicate that the P(3-8).p cells, which are derived from ABpl and ABpr, are the best candidates for the focus of *lin-36* activity. Thus, *lin-36* probably acts cell autonomously. As discussed below, synMuv genes with a postulated focus in hyp7 show very different clonal behavior. We observed three double losses (Fig. 3 legend). One occurred in AB and MS/EMS and resulted in a Muv phenotype, presumably because of the AB loss. Another occurred in ABpl and C and resulted in a Muv phenotype, presumably because of the ABpl loss. A third occurred in ABprapp and ABa and resulted in a Muv phenotype, presumably because of the ABprapp loss. Thus, we can account for the phenotypes of mosaic animals with double losses on the basis of one of the two losses.

The simplest interpretation of these results is that *lin-36* acts cell autonomously in the P(3-8).p cells, although it is conceivable that *lin-36* is instead required in close relatives of the P(3-8).p cells, possibly hyp7 cells derived from V3 or V5 in both the ABpl and ABpr lineages. It is also conceivable that *lin-36* acts in both the P(3-8).p cells and hyp7 but plays a more critical role in the P(3-8).p cells. In such a model, loss of *lin-36* activity in both the P(3-8).p cells and some hyp7 nuclei results in a Muv phenotype, although loss in only hyp7 does not. Although the penetrance of the Muv phenotype in animals that have lost *lin-36* activity in AB or ABp (64%, *n*=44) is essentially the same as that in animals that have lost *lin-36* activity meiotically (69%, *n*=240), the penetrance of the Muv phenotype in animals that have lost *lin-36* activity in ABpl or ABpr is substantially lower (2/10, excluding double losses). These data are consistent with a *lin-36* contribution from hyp7 in addition to that from P(3-8).p cells. However, these data are also consistent with the cell autonomous model. Specifically, the control animals that lost *lin-36* activity meiotically displayed not only incomplete penetrance but also variable expressivity of the Muv phenotype, in that not all P(3-8).p cells adopted vulval fates. Since ABpl and ABpr mosaic animals have only one or two P(3-8).p cells that can adopt ectopic cell fates, the cell autonomous model predicts a reduced frequency

Table 3. Sequences of *lin-36* mutations

Allele	Wild-type sequence	Mutant sequence	Substitution or splice-site change
n747	GAA	AAA	E241K
n750	CCT	CTT	P246L
	GGC	GGT	G103G (silent)
n766	TAT	TAA	Y796ochre
n772	CGC	CAC	R189Q
n2235	CAA	TAA	Q898ochre
n2240	CCT	CTT	P246L
n2243	GGA	GAA	G324E
n3090	TAT	AAT	Y325N
n3093	CAG	TAG	Q579amber
n3094	atttttag/GAT	atttttaa/GAT	Exon 2 acceptor
n3095	CAA	TAA	Q569ochre
n3096	CAA	TAA	Q442ochre
n3097	CAT	TAT	H284Y

Exon sequences are shown in uppercase letters and intron sequences are shown in lowercase letters. The *C. elegans* consensus splice acceptor site is wwtttcag/NNN, where W is A or T, and N is any nucleotide (Fields, 1990). Amino acid substitutions are shown as wild-type residue identity, residue number, and predicted mutant residue. *n750* had two changes, one of which was identical to the *n2240* lesion; the other was a silent glycine-to-glycine mutation. All mutations except *n3090* were EMS-induced and therefore expected to be GC-to-AT transitions (Anderson, 1995), as was the case for all of them except *n766*, which was a TA-to-AT transversion.

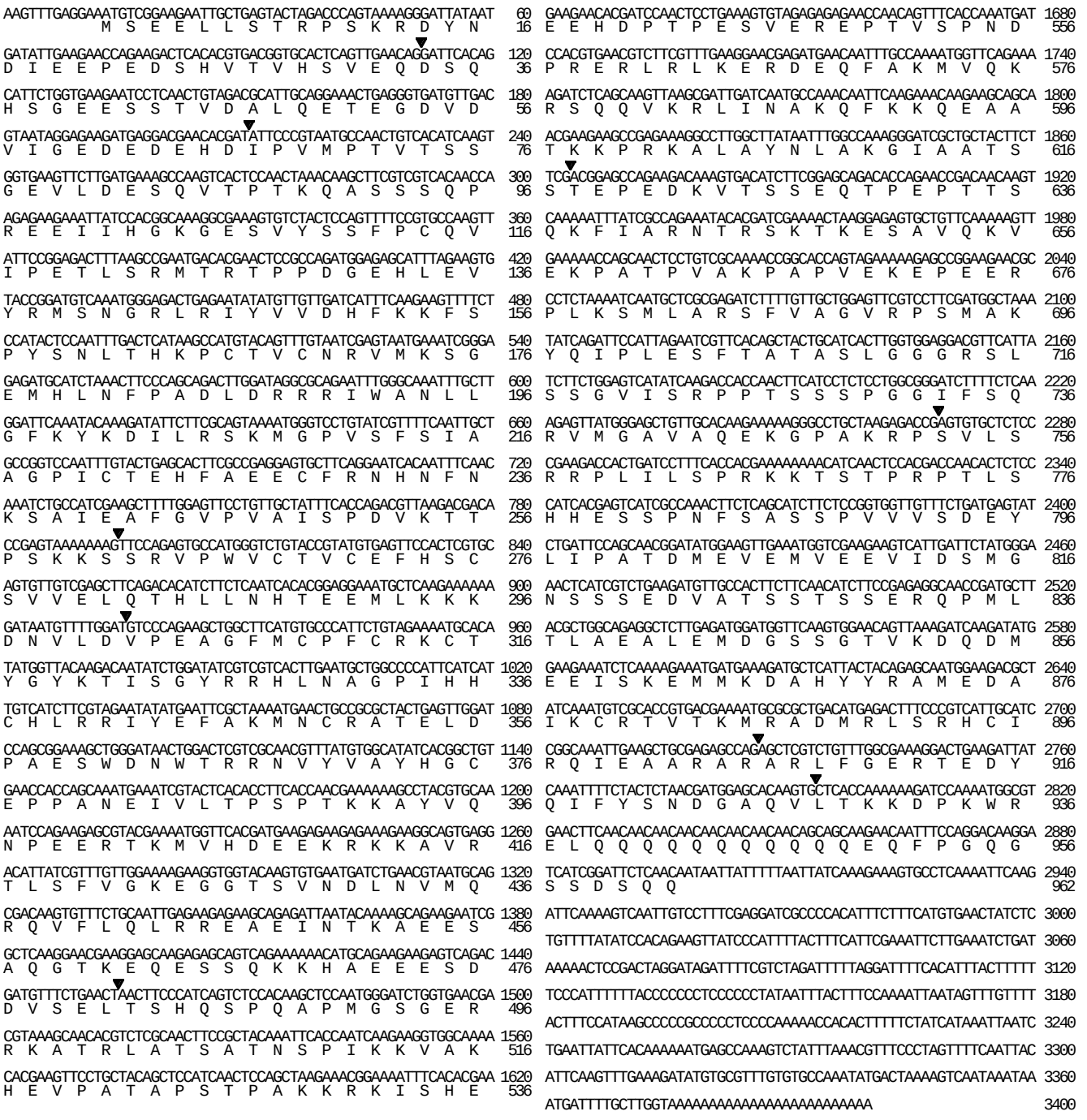

Fig. 2. *lin-36* cDNA and LIN-36 predicted amino acid sequences. Nucleotides (upper lines) are numbered beginning at the first nucleotide after the *Eco*RI linker. The first nine nucleotides correspond to the 3′ end of the SL1 *trans*-spliced leader. Amino acids (lower lines) are numbered beginning at the presumptive ATG start codon and ending at the residue just before the presumptive TAA stop codon. Arrowheads indicate the locations of intervening sequences. The nine introns are 56, 75, 101, 75, 101, 123, 66, 48 and 215 nucleotides long, respectively. The GenBank accession number for the *lin-36* cDNA is AF104917.

of Muv animals from these mosaic classes compared to the AB and ABp mosaic classes.

A *lin-36::GFP* reporter is expressed in the nuclei of Pn.p cells

We determined the expression pattern of a *lin-36::GFP* reporter construct containing 1 kb of sequence 5′ to the ATG start site, the entire *lin-36* open reading frame, all *lin-36* introns and the GFP gene fused in-frame to the last codon of *lin-36*. This *lin-36::GFP* reporter transgene rescued the Muv phenotype of a *lin-36; lin-15(n767)* strain, indicating that it was expressed in cells needed for *lin-36* function. *lin-36::GFP* was expressed in the nuclei of the P(3-8).p cells and their descendants during vulval cell determination, division and

invagination (Fig. 4). Similar observations have been made with an independently constructed *lin-36::GFP* reporter (A. Hajnal and S. Kim, personal communication). The expression pattern of *lin-36* in the P(3-8).p cells and their descendants is consistent with our conclusion from the genetic mosaic analysis that *lin-36* acts cell autonomously. The P(3-8).p cell expression of *lin-36::GFP* was not altered by mutations in other class B genes, specifically *lin-9, lin-15, lin-35, lin-51, lin-52, lin-53* and *lin-55* (Ferguson and Horvitz, 1989; J. H. Thomas and H. R. Horvitz, unpublished observations) (data not shown).

Besides the P(3-8).p cells, many other cells expressed GFP in strains bearing our reporter construct. Most notably, neurons of the head, tail and ventral cord expressed *lin-36::GFP* throughout development, and sporadic fluorescence was infrequently observed in the germline (data not shown). Very weak staining was observed in hypodermal and intestinal nuclei.

lin-36::GFP expression in all cases was localized to nuclei. The GFP reporter gene used in the construction of this reporter construct did not contain a nuclear localization sequence, but as mentioned above, the *lin-36* coding region contains a potential nuclear localization sequence. These data suggest that *lin-36* may act in the nuclei of the P(3-8).p cells to mediate its effect on vulval development.

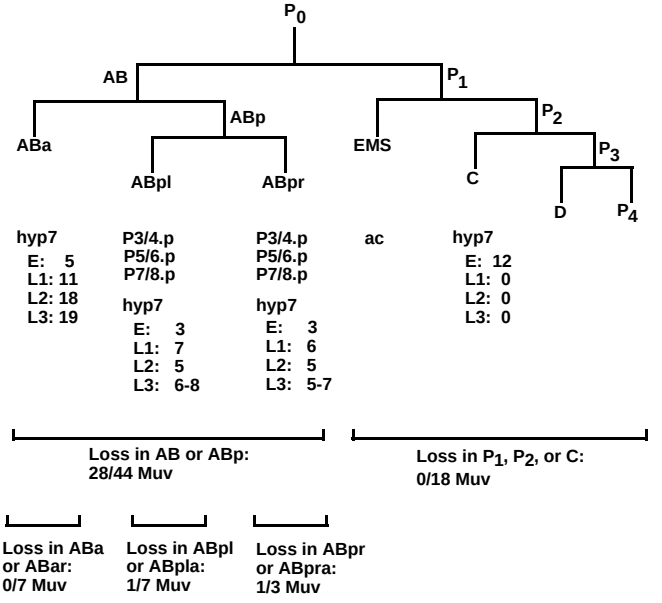

Fig. 3. Mosaic analysis of *lin-36*. A partial embryonic cell lineage (Sulston et al., 1983) showing the origins of cells involved in vulva development. The gonadal anchor cell (ac) is derived from EMS; P(3-8).p are derived from ABplapp and ABprapp; hyp7, the main body hypodermal syncytium, is derived from ABa, ABp and C. The number of hyp7 nuclei that each of these lineages contributes at each developmental stage (E, embryonic; L1, L2, L3, first, second and third larval stages, respectively) is noted under the lineage (Sulston and Horvitz, 1977; Sulston et al., 1983). Contributions from the P3.p, P4.p and P8.p 3° cell lineages are noted in the L3 totals even though it is not likely that these hyp7 nuclei play a role in vulval cell determination; these totals include two nuclei each from ABpl and ABpr and two additional nuclei from P8.p, which descends either from ABpl or ABpr, accounting for the variable contribution to hyp7 of the L3 ABpl and ABpr lineages. In Muv animals, P3.p, P4.p and P8.p descendants often adopt vulval cell fates rather than hyp7 cell fates. Brackets below the lineage diagram show the lineages in which duplication loss occurred and the fraction of mosaic animals in this category with a Muv phenotype. The summary of losses and phenotypes in the figure is derived from the following data where the cell in which the loss of *sDp3* occurred is reported followed by the fraction of such mosaic animals that were Muv: AB, 11/13; ABp, 9/22; AB or ABp, 8/9; ABpl, 1/6; ABpla, 0/1; ABpr, 1/2; ABpra, 0/1; ABa, 0/6; ABar, 0/1; P_1, 0/1; P_2, 0/3; C, 0/4; P_1 or P_2 or C, 0/5; P_1 or P_2, 0/1; P_2 or C, 0/4; AB and EMS or MS, 1/1; ABpl and C, 1/1; ABprapp and ABa, 1/1. Animals in which double losses occurred are excluded.

The synMuv genes inhibit a ligand-independent activity of the *let-23* RTK

The Vul phenotype of mutations in the *let-23* receptor tyrosine kinase gene is epistatic to the Muv phenotype of the *lin-8; lin-9* and *lin-15*AB synMuv mutations (Ferguson et al., 1987;

Table 4. The synMuv phenotype is coexpressed with the *lin-2,7,10* Vul phenotype

SynMuv genotype	Vul genotype	Muv (%)	Vul (%)	Muv Vul (%)	WT (%)	*n*
WT	*let-23*[e]	0	100	0	0	100+
	lin-2[c]	0	99	0	1	280
	lin-7[d]	0	98	0	2	341
	lin-10[d]	0	97	0	3	211
*lin-36; lin-15*A	WT[a]	100	0	0	0	226
	let-23	0	100	0	0	203
	lin-2	39	14	37	9	180
	lin-7	43	9	47	2	202
	lin-10	29	25	42	4	224
*lin-15*AB	WT	97	0	3[b]	0	119
	let-23[f]	0	100	0	0	40
	lin-2	52	0	48	0	106
	lin-7	63	1	35	1	140
	lin-10	56	2	42	0	113

The vulval phenotypes of strains of various genotypes are described. Complete genotypes are shown (no markers were used).

SynMuv mutations used: *lin-36(n766)*; *lin-15*A, *lin-15(n767)*; *lin-15*AB, *lin-15(e1763)* (Ferguson and Horvitz, 1985, 1989).

Vul mutations used: *lin-2(n397)*, *lin-7(e1413)*, *lin-10(n299)*, *let-23(sy97)* (Ferguson and Horviz, 1985; Aroian and Sternberg, 1991; Hoskins et al., 1996). Phenotypic categories were assigned as follows: Muv, animal had a Multivulva phenotype characterized by ectopic ventral protrusions; Vul, animal had a Vulvaless phenotype such that it was bloated with eggs or contained many internally hatched larvae (bag of worms); Muv Vul, animal coexpressed both a Multivulva and a Vulvaless phenotype, that is it had ectopic protrusions characteristic of a Muv phenotype and was bloated with eggs or larvae; WT, wild type, i.e. animal had a functional vulva and was non-Muv (phenotype), or animal was wild type for the particular genotype (genotype).

n, number of animals scored.

Several animals displayed a Hyperinduced (Hin) phenotype (P(3-8).p cells immediately adjacent to the developing vulva adopt vulval fates such that an abnormal vulva with adjacent vulval protrusions is formed) in addition to the phenotype described. These animals were assigned a phenotype based upon whether they had Multivulva-like ectopic vulval tissue (Muv), a nonfunctional vulva (Vul), both (Muv Vul), or no Multivulva-like ventral protrusions and a functional vulva (WT).

[a]Taken from Ferguson and Horvitz (1989).

[b]These animals were scored as Muv Vul, since they were somewhat bloated with eggs.

[c]Taken from Hoskins et al. (1996).

[d]Taken from Ferguson and Horvitz (1985).

[e]Taken from Arioan and Sternberg (1991).

[f]Taken from Huang et al. (1994).

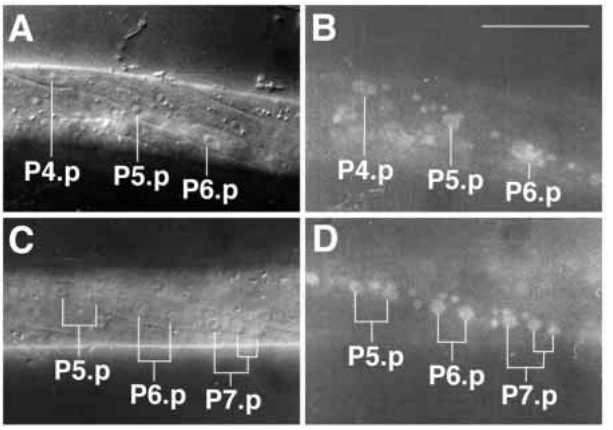

Fig. 4. *lin-36::GFP* reporter expression. Anterior is to the left; dorsal is up. (A,C) Photomicrographs taken using Nomarski optics. (B,D) Fluorescent images of the animals seen in A and C observed using an LP-FITC filter. Animals are from the rescued Roller line 3L26a, which carries an integrated reporter transgene and a co-integrated marker transgene. (A,B) L3 hermaphrodite with reporter expression in the nuclei of the P(3-8).p cells. The nuclei of ventral cord neurons adjacent to the P(3-8).p cells also expressed the reporter construct. (C,D) L3 hermaphrodites showing the nuclear expression of the reporter in the descendants of the P(3-8).p cells during vulval cell divisions. The lineage relationships of these cells are shown by lineage diagrams superimposed on the images. Bar, 20 μm.

Huang et al., 1994). To determine if *lin-36* acts similarly in the genetic pathway controlling vulval induction, we constructed triple mutants using *let-23* mutations and a *lin-36* mutation (Table 4). The Vul phenotype of *let-23* mutations is epistatic to the *lin-36; lin-15*A synMuv phenotype. Thus, *let-23* activity is required for the adoption of vulval fates by P(3-8).p cells in synMuv mutants. The *let-23* receptor tyrosine kinase acts genetically downstream of, or in parallel to, *lin-36* as well as the other synMuv genes.

lin-2, *lin-7* and *lin-10* positively regulate the *let-23*-mediated signal transduction cascade, apparently by localizing the LET-23 receptor to the basolateral membrane of the P(3-8).p cells (Simske et al., 1995; Hoskins et al., 1996). A mixture of Muv and Vul phenotypes, i.e. animals both with ectopic ventral protrusions and bloated with eggs (or bags of worms), has been reported for animals triply mutant for *lin-8; lin-9* and either *lin-2*, *lin-7* or *lin-10*; however, double mutants between the synMuv mutation *lin-15(n309)* and a *lin-2*, *lin-7* and *lin-10* mutation have been reported to be Muv (Ferguson et al., 1987). Since the *lin-9* mutation used in these experiments was not a null allele (Ferguson and Horvitz, 1989), we repeated these experiments using the strong *lin-36(n766)* allele. Some animals were Muv, some were Vul, some had a wild-type phenotype, and some expressed both a Vul and a Muv phenotype (Table 4). Thus, in these triple mutants the Muv and Vul phenotypes were coexpressed. Since this result is similar to that observed by Ferguson et al. (1987) with *lin-8; lin-9* and *lin-2*, *lin-7* or *lin-10* triple mutants, we repeated the *lin-15*AB experiment, but used another allele, *lin-15(e1763)*, which is a slightly larger deletion of *lin-15* than is *lin-15(n309)*. Both alleles eliminate both the *lin-15*A and *lin-15*B gene products and their activities (Clark et al., 1994). The *lin-15*AB double mutants with *lin-2*, *lin-7* and *lin-10* mutations showed coexpression of the Muv and Vul phenotypes (Table 4). These results are consistent with our observations of the *lin-36; lin-15*A mutants; however, unlike the *lin-36* triple mutants, most animals of these genotypes were either Muv or Muv Vul; few were wild type or Vul. Since almost all animals expressed the Muv phenotype and some expressed the Vul phenotype in addition to the Muv phenotype, it is likely that in the previous studies by Ferguson et al. (1987) the Vul phenotype was not noticed. We conclude that in mutants carrying class A and class B synMuv mutations and a *lin-2*, *lin-7* or *lin-10* mutation there is a coexpression of the Muv and Vul phenotypes.

The anchor cell is necessary for the adoption of vulval cell fates by the P(3-8).p cell in wild-type animals, as demonstrated by experiments in which the anchor cell was ablated by laser microsurgery (Kimble, 1981). *lin-8; lin-9* and *lin-15*AB synMuv mutants in which the anchor cell was ablated using laser microsurgery are Muv, suggesting that in the absence of negative regulation by the synMuv genes, P(3-8).p cells do not require the anchor cell signal to adopt vulval cell fates (Ferguson et al., 1987). We tested whether anchor cell-deficient synMuv mutants carrying *lin-36* mutations were similarly Muv. We eliminated the entire somatic gonad, which includes the anchor cell progenitor, using laser microsurgery to ablate Z1 and Z4, the precursors of the somatic gonad (Kimble and Hirsh, 1979). These operations were performed at the early L1 larval stage. The ablation of Z1 and Z4 prevents germline proliferation and produces a sterile animal with no gonad (Kimble and White, 1981). This phenotype served as an internal control to confirm the ablation of the somatic

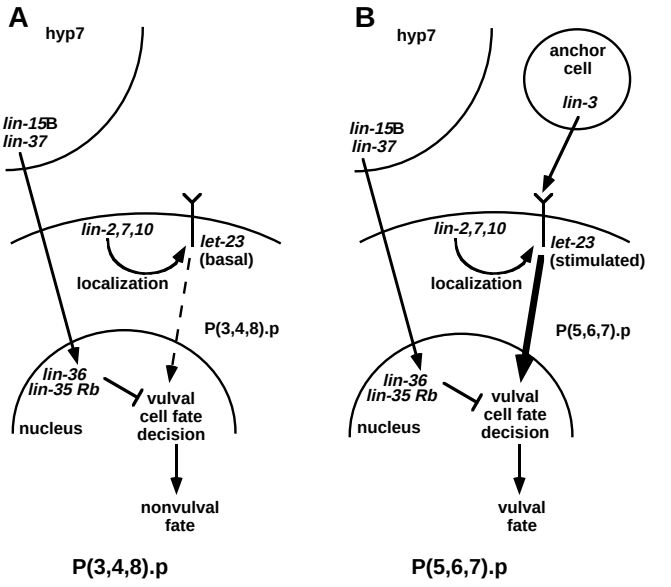

Fig. 5. Model for the control of the P(3-8).p cell-fate decision in wild-type animals. See text for details. (A) Cells far from the anchor cell, P(3,4,8).p, adopt nonvulval cell fates. SynMuv gene activity is sufficient to inhibit the ligand-independent (basal) activity of the *let-23* signal transduction pathway. (B) Cells closer to the anchor cell, P(5,6,7).p, adopt vulval cell fates. The *lin-3* ligand stimulates the *let-23* receptor. The synMuv gene-mediated antagonism of the *let-23* signal transduction activity is insufficient to block the signal from the activated induction pathway.

gonad. We ablated Z1 and Z4 in *lin-36; lin-15*A animals and found that 11/11 of these animals produced ectopic vulval tissue.

We tested whether the anchor cell signal is responsible for the adoption of vulval fates by P(3-8).p cells in which receptor localization is disrupted and the negative regulatory pathways are eliminated by triple mutants carrying synMuv mutations and mutations in *lin-2*, *lin-7* or *lin-10*. We ablated Z1 and Z4 in *lin-7; lin-36; lin-15*A animals and observed that 14/14 operated animals had ectopic vulval tissue. Thus, in animals in which the gonadal signal, the receptor localization system and the synMuv-mediated inhibitory pathways have been disrupted, the P(3-8).p cells can adopt vulval fates. These results, together with the triple mutant analyses, are consistent with the hypothesis that a ligand-independent and receptor localization-independent activity of the *let-23* signal transduction pathway can lead to the adoption of vulval cell fates. Presumably, it is this ligand-independent and receptor localization-independent activity that the synMuv genes normally inhibit.

DISCUSSION

lin-36 encodes a novel protein and acts cell autonomously

Our results suggest that *lin-36* encodes a novel protein that is required within the P(3-8).p cells to negatively regulate vulval development. Our *lin-36* genetic mosaic data are in marked contrast to the *lin-15* and *lin-37* genetic mosaic data that led to the conclusion that these synMuv loci act cell non-autonomously (Herman and Hedgecock, 1990; Hedgecock and Herman, 1995). Specifically, Herman and Hedgecock (1990) found that loss of *lin-15* function in P_1, AB or ABp, ABpl or ABpr can result in a Muv phenotype. hyp7, which derives from these lineages, was postulated to be the anatomical focus of both *lin-15*A and *lin-15*B activity, since both activities must be lost to produce a Muv phenotype. Hedgecock and Herman (1995) found that loss of *lin-37* function in P_1, ABp, ABpl or ABpr did not cause a Muv phenotype and that loss in AB infrequently resulted in a Muv phenotype. Loss in AB results in many hyp7 nuclei lacking *lin-37* function. Hedgecock and Herman (1995) postulated that *lin-37* is also required in hyp7, but that a relatively low dosage is sufficient for wild-type function. Our results, which show a requirement for *lin-36* function in only those lineages that generate the P(3-8).p cells (e.g. ABp but not P_1), suggest that *lin-36* acts in the P(3-8).p cells and not in hyp7. This hypothesis is consistent with our *lin-36* expression data.

Taken together, our findings and those of Herman and Hedgecock (1990) and Hedgecock and Herman (1995) suggest that some class B synMuv genes function in the P(3-8).p cells and others function in hyp7. Thus, the class B synMuv genes appear to encode components of an intercellular signaling system between hyp7 and P(3-8).p. Since *lin-36* is expressed in the nucleus, it is likely that the inhibitory signal from the class B pathway is interpreted within the nucleus of the P(3-8).p cell.

Antagonism of the inductive signaling pathway by the Rb-mediated class B synMuv pathway

The class B synMuv genes *lin-35* and *lin-53* encode proteins similar to the products of the mammalian retinoblastoma (Rb) tumor suppressor gene and the Rb-binding protein RbAp48, respectively (Lu and Horvitz, 1998). Both Rb and RbAp48 are believed to be involved in transcriptional regulation (Qian et al., 1993; Taya, 1997). *lin-35 Rb* appears to be expressed in the nuclei of the P(3-8).p cells (Lu and Horvitz, 1998). Since *lin-36* is expressed in the nuclei of the P(3-8).p cells and seems to act in these cells, it is possible that *lin-36* acts as a regulator or effector of *lin-35 Rb* activity.

How do *lin-36* and the other class B synMuv genes antagonize the *let-23* signal transduction cascade? Our observations suggest that the class B synMuv genes may inhibit the adoption of vulval cell fates by acting in the nucleus, possibly at the level of transcription. If so, the class B genes would provide an input into the cell-fate decision independently of the *let-23* signal transduction pathway. The nuclear role of Rb-like proteins is consistent with this hypothesis.

SynMuv mutants are Vul in a strain in which *let-23* receptor gene activity is reduced but are Muv in gonad-ablated animals. These observations indicate that whereas activation of the inductive signal transduction cascade by the gonadal ligand is not necessary for the adoption of vulval fates in the absence of negative regulation by *lin-36* and the other synMuv genes, the presence of the intracellular signal transduction cascade nonetheless is necessary. These results are consistent with a model in which the two classes of synMuv genes antagonize either the gonadal ligand-independent (basal) activity of the *let-23*-mediated inductive pathway or a weaker activity stimulated by a second, nongonadal signal of the *let-23*-mediated pathway. In either case, a gonadal ligand-independent activity is necessary for the adoption of vulval fates by the P(3-8).p cells in a synMuv mutant. The inhibition of this activity by the synMuv genes can be overcome by the activation of the inductive pathway by the binding of the *lin-3* ligand to receptor in the subset of P(3-8).p cells nearest the anchor cell.

lin-2, *lin-7* and *lin-10* positively regulate the inductive signal transduction cascade by localizing the LET-23 receptor to the P(3-8).p cell junctions (Hoskins et al., 1996; Simske et al., 1996). Our gene interaction studies showed that the synMuv phenotype is coexpressed with the Vul phenotype of *lin-2*, *lin-7* and *lin-10* in mutants with *lin-36* and *lin-15*A or with *lin-15*AB. This coexpression suggests that the synMuv genes act to antagonize the *let-23* pathway independently of the *lin-2*, *lin-7*, *lin-10* regulatory system, since neither set of genes requires the presence of the other to exert its effect on vulval development. In the absence of the activity of the synMuv genes, the proper subcellular localization of LET-23 receptor is sufficient to allow all P(3-8).p cells to adopt vulval fates. However, in the absence of both synMuv gene-mediated negative regulation and proper subcellular localization of LET-23 receptor, the P(3-8).p cells variably adopt either vulval or nonvulval fates, despite the presence of the inductive signal.

Our laser ablation experiments showed that the P(3-8).p cell fates seen in animals lacking the stimulatory anchor cell signal, positive regulation by *lin-7* and negative regulation by *lin-36* and *lin-15*A, are similar to the fates seen in animals lacking just the positive regulatory pathway mediated by *lin-2*, *lin-7*, *lin-10* and the negative regulatory pathway mediated by the

synMuv genes. This observation suggests that the P(3-8).p cells that adopt vulval cell fates in animals lacking both synMuv activity and receptor localization do not adopt these fates in response to the anchor cell signal. We hypothesize that LET-23 receptor clustering can amplify the signal independently of ligand binding. Not all P(3-8).p cells adopt vulval cell fates, however. Unstimulated and unlocalized *let-23* activity is sufficient only for some cells to adopt vulval fates in the absence of synMuv gene-mediated inhibition.

The data described above suggest the following model. The six Pn.p cells are predisposed to adopt vulval fates by an unstimulated basal activity of the *let-23* receptor tyrosine kinase (Fig. 5A). In the absence of this activity, the P(3-8).p cells cannot adopt vulval fates. However, this predisposition to adopt vulval fates is inhibited by the action of the two functionally redundant signaling pathways encoded by the synMuv genes, which antagonize the basal activity of the *let-23* signal transduction pathway. The inductive signal from the anchor cell overcomes this inhibition and causes nearby P(3-8).p cells to adopt vulval cell fates, either by overriding or by inhibiting the synMuv genes (Fig. 5B).

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