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lin-8, Which Antagonizes *Caenorhabditis elegans* Ras-Mediated Vulval Induction, Encodes a Novel Nuclear Protein That Interacts With the LIN-35 Rb Protein

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ABSTRACT

Ras-mediated vulval development in *C. elegans* is inhibited by the functionally redundant sets of class A, B, and C synthetic Multivulva (synMuv) genes. Three of the class B synMuv genes encode an Rb/DP/E2F complex that, by analogy with its mammalian and Drosophila counterparts, has been proposed to silence genes required for vulval specification through chromatin modification and remodeling. Two class A synMuv genes, *lin-15A* and *lin-56*, encode novel nuclear proteins that appear to function as a complex. We show that a third class A synMuv gene, *lin-8*, is the defining member of a novel *C. elegans* gene family. The LIN-8 protein is nuclear and can interact physically with the product of the class B synMuv gene *lin-35*, the *C. elegans* homolog of mammalian Rb. LIN-8 likely acts with the synMuv A proteins LIN-15A and LIN-56 in the nucleus, possibly in a protein complex with the synMuv B protein LIN-35 Rb. Other LIN-8 family members may function in similar complexes in different cells or at different stages. The nuclear localization of LIN-15A, LIN-56, and LIN-8, as well as our observation of a direct physical interaction between class A and class B synMuv proteins, supports the hypothesis that the class A synMuv genes control vulval induction through the transcriptional regulation of gene expression.

THE retinoblastoma (Rb) gene was the first tumor-suppressor gene to be cloned, and the Rb pathway has been found to be a frequent target of inactivation in many human cancers (Nevins 2001). The nematode *Caenorhabditis elegans* possesses a single homolog of Rb, *lin-35*, which functions in the inhibition of both cellular proliferation and differentiation (Lu and Horvitz 1998; Boxem and van den Heuvel 2001). The class A synthetic Multivulva (synMuv) genes function redundantly with the *lin-35* Rb gene to inhibit Ras-mediated vulval induction. The analysis of the class A synMuv genes may further our understanding of activities that interact with the Rb pathway in the regulation of cell-fate determination and in the prevention of oncogenic transformation.

The vulva of the *C. elegans* hermaphrodite is the conduit through which embryos are expelled and is also the point of entry for sperm after mating with a male. The vulva is formed by the descendants of three of six equipotent cells, P(3–8).p. These six cells are all able to express any one of three fates: the 1° vulval fate, the 2° vulval fate, and the 3° nonvulval fate. Vulval development is induced by activation of a receptor tyrosine kinase (RTK)/Ras pathway (Kornfeld 1997; Sternberg and Han 1998). During wild-type larval development, signaling from the anchor cell of the somatic gonad activates an RTK/Ras pathway, causing P6.p to adopt the 1° vulval fate and directly or indirectly causing P5.p and P7.p to adopt the 2° vulval fate. P(5–7).p then divide to produce 22 cells that migrate and fuse to form the toroidal vulva. P3.p, P4.p, and P8.p normally express the 3° nonvulval fate, dividing once and fusing with the hypodermis. Loss of RTK/Ras pathway signaling results in the expression of the nonvulval 3° fate by P(5–7).p and thus in a Vulvaless (Vul) phenotype. Vul animals lack a functional vulva and are consumed by their internally hatched progeny. Ectopic activation of RTK/Ras pathway signaling results in the expression of 1° or 2° vulval fates by P3.p, P4.p, and P8.p, causing a Multivulva (Muv) phenotype. The extra vulval tissue produced in Muv animals forms ectopic ventral protrusions.

Class A, B, and C synMuv genes act to antagonize RTK/Ras function in potential vulval cells (Ferguson and Horvitz 1989; Ceol and Horvitz 2004). As a result of a functional redundancy among these three classes of synMuv genes, only hermaphrodites mutant in two sets of genes exhibit the synMuv phenotype. Genetic screens and targeted studies have identified at least four class A synMuv genes: *lin-8*, *lin-15A*, *lin-38*, and *lin-56*; at least 17 class B synMuv genes: *lin-9*, *lin-13*, *lin-15B*, *lin-35*,

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lin-36, *lin-37*, *lin-52*, *lin-53*, *lin-54*, *lin-61*, *dpl-1*, *efl-1*, *hda-1*, *hpl-2*, *let-418*, *mep-1*, and *tam-1*; and four class C synMuv genes: *trr-1*, *mys-1*, *epc-1*, and *ssl-1* (Horvitz and Sulston 1980; Ferguson and Horvitz 1985, 1989; Lu and Horvitz 1998; Hsieh *et al.* 1999; Solari and Ahringer 2000; von Zelewsky *et al.* 2000; Ceol and Horvitz 2001, 2004; Couteau *et al.* 2002; Unhavaithaya *et al.* 2002; Thomas *et al.* 2003; X. Lu, M. M. Harrison, P. W. Sternberg and H. R. Horvitz, unpublished results). A subset of the class B synMuv genes encode proteins that mediate histone modification, chromatin remodeling, and transcriptional repression. In particular, *efl-1*, *dpl-1*, *lin-35*, *lin-53*, *hda-1*, *let-418*, and *hpl-2* encode *C. elegans* homologs of E2F, DP, Rb, the Rb-associated protein RbAp48, a class I histone deacetylase (HDAC), the Mi-2 chromatin-remodeling ATPase, and heterochromatin protein 1 (HP1), respectively (Lu and Horvitz 1998; Solari and Ahringer 2000; von Zelewsky *et al.* 2000; Ceol and Horvitz 2001; Couteau *et al.* 2002). The mammalian homologs of LIN-53 RbAp48, HDA-1 HDAC, and LET-418 Mi-2 are components of the histone deacetylase and chromatin-remodeling NuRD complex, while HP1 has been shown to function as a histone H3 methyl-lysine-9-binding protein (Knoepfler and Eisenman 1999; Richards and Elgin 2002). Because of their molecular identities, the synMuv B genes are thought to antagonize RTK/Ras function in the vulva by silencing transcription of vulval specification genes through chromatin modification and remodeling. LIN-35 Rb is likely to play a pivotal role in this process, as evidence suggests that mammalian pRb mediates the association of the sequence-specific heterodimeric transcription factor DP/E2F with the NuRD complex components HDAC1 and RbAp48 as well as with a histone H3 K9 methyltransferase (Nicolas *et al.* 2000; Nielsen *et al.* 2001; Zhang and Dean 2001). The resultant likely recruitment of Mi-2 and HP1 may induce a facultative heterochromatic state around the targeted genes, preventing transcription. The class C synMuv genes encode a putative *C. elegans* Tip60/NuA4-like histone acetyltransferase complex; it has not yet been determined if this putative complex acts in transcriptional activation or repression (Ceol and Horvitz 2004).

The class A synMuv genes may inhibit vulval development through the regulation of transcription. Of the four known class A synMuv genes, two—*lin-56* and *lin-15A*—have been cloned and encode novel nuclear proteins that likely associate in a functional complex *in vivo* (Clark *et al.* 1994; Huang *et al.* 1994; E. M. Davison, A. M. Saffer, L. S. Huang, J. DeModena, P. W. Sternberg and H. R. Horvitz, unpublished results). Furthermore, LIN-56 and LIN-15A share a novel C2CH motif related to the THAP domain, shown in the human protein THAP1 to possess zinc-dependent sequence-specific DNA-binding activity *in vitro* (Clouaire *et al.* 2005; E. M. Davison, A. M. Saffer, L. S. Huang, J. DeModena, P. W. Sternberg and H. R. Horvitz, unpublished results). Here we report our characterization of a third class A synMuv gene, *lin-8*.

MATERIALS AND METHODS

Strains and general techniques: *C. elegans* strains were cultivated on NGM agar seeded with *Escherichia coli* strain OP50 as described by Brenner (1974) and were grown at 20° unless otherwise indicated. Bristol strain N2 was used as the wild-type strain. The mutant alleles used in this study are listed below, and a description of each can be found in Riddle *et al.* (1997) unless noted otherwise:

LG I: *lin-35(n745).*
LG II: *lin-8(n111, n2376, n2378, n2403, n2731, n2738, n2739, n2741)* (Thomas *et al.* 2003), *dpy-10(e128).*
LG III: *lin-52(n771)* (Ferguson and Horvitz 1989; Thomas *et al.* 2003).
LG X: *lin-15B(n744, n2245).*

pPK5363 is a Tc1-transposon insertion polymorphism on LG II found in the NL7000 but not in the N2 strain (Korswagen *et al.* 1996). *nIs128* contains a GFP transgene integrated into LG II (H. T. Schwartz and H. R. Horvitz, unpublished results). In addition, the following deficiencies were used: *ccDf1*, *ccDf2*, and *ccDf11* (Chen *et al.* 1992).

Deletion and polymorphism mapping: To test complementation of *lin-8* with the deficiencies *ccDf1*, *ccDf2*, and *ccDf11*, *lin-8(n111) dpy-10(e128); lin-15B(n744)* hermaphrodites were mated with *ccDf/+* males. The Muv phenotype of the non-Dpy male progeny, half of which should possess *lin-8* in *trans* to the relevant deficiency, was scored. In the *ccDf1* and *ccDf2* crosses, 0/88 and 0/84 male offspring, respectively, exhibited ventral protrusions, whereas 20/63 male offspring of the *ccDf11* cross exhibited ventral protrusions.

The left endpoint of *ccDf1* was defined relative to the physical map by determining if cosmid sequences from the interval between *sup-9* and *lin-31* could be amplified by polymerase chain reaction (PCR) from *ccDf1* homozygous inviable embryos. A drop of chitinase solution (20 mg/ml chitinase, 50 mm NaCl, 70 mm KCl, 2.5 mm $MgCl_2$, 2.5 mm $CaCl_2$) was placed over single inviable embryos, which were promptly transferred to 10 μl of lysis buffer (50 mm KCl, 10 mm Tris, pH 8.2, 2.5 mm $MgCl_2$, 0.45% NP-40, 0.45% Tween-20, 0.01% gelatin, 60 μg/ml proteinase K) and subsequently frozen at −80°. Embryos were thawed and lysed by incubation at 60° for 1 hr. Proteinase K was inactivated by incubation at 95° for 15 min. The lysate from each inviable embryo was used for three PCR reactions: the test amplification; amplification of a sequence from cosmid F34D6, which served as a positive control for successful lysis; and amplification of *lin-31*, which served as a negative control to confirm identification of each egg as a *ccDf1* homozygote. Sequences from cosmids B0454, ZC239, F39E9, and W10G11 but not from cosmids M151 or F19B10 were successfully amplified from *ccDf1* homozygous inviable embryos, placing the left endpoint of *ccDf1* between W10G11 and M151. The primers used were as follows: F34D6.7, 5′-CACCT GAAGATTCAAGTTTAG-3′; F34D6.11, 5′-GTGTGAGCTCAG CAGCTTC-3′; B0454 Fwd, 5′-GGTTCTCGTTAGCTGAGT GG-3′; B0454 Rev, 5′-GTACGGAGCCAAGATCATACG-3′; ZC239 Fwd, 5′-GCAGAGACGTTGGATCCTAGC; ZC239 Rev, 5′-CTTCAGGAGTCGGTGAACTCG-3′; F39E9 Fwd, 5′-CAGT CTCAGGCTAGACTTGG-3′; F39E9 Rev, 5′-GCTGAGCAGAT CTCGAATGG-3′; W10G11 Fwd1, 5′-GCTTCCACATTCAGTG AAGG-3′; W10G11 Rev1, 5′-CAAGCCAGAAGAGCAAGTCG-3′; W10G11 N1, 5′-CGAGATGTAAGCTCAGTATGG-3′; M151 Fwd, 5′-CATCGGTCTCCCATAGTTACC-3′; M151 Rev, 5′-GCT CTGGCTGCTCGAGTTCC-3′; F19B10 Fwd, 5′-CTGAAGCATT

GGCTCAGAGG-3′; F19B10 Rev, 5′-CGTCATTGATGGACCA TGTGC-3′; *lin-31* Fwd1, 5′-GCTATTCAGGACTCTGACG-3′; *lin-31* Rev1, 5′-CCTTCCCAGGACGATCG-3′.

The *pPK5363* polymorphism is an insertion of the Tc1 transposon into cosmid C17F4 present in the NL7000 but not in the N2 strain (Korswagen *et al.* 1996). To map *lin-8* against *pPK5363*, *lin-8(n111) dpy-10(e128)/NL7000; lin-15B(n744)* males were crossed with *lin-8(n111) dpy-10(e128); lin-15B(n744)* hermaphrodites, and resulting Muv non-Dpy and Dpy non-Muv hermaphrodite progeny were picked and used to establish homozygous recombinant lines. PCR employing Tc1-specific (5′-GCTGATCGACTCGATGCCACGTCG-3′) and C17F4-specific (5′-CCATCAACGAGTACGATACG-3′) primers was used to determine if polymorphism *pPK5363* was present. Of the Muv non-Dpy chromosomes, 4/13 carried *pPK5363*, and of the Dpy non-Muv chromosomes, 2/14 carried *pPK5363*. These results placed *lin-8* to the left of *pPK5363*, which is itself to the left of *dpy-10*.

Transgenic animals: Germline transformation by microinjection was performed as described by Mello *et al.* (1991). The coinjection marker pRF4 was injected at a concentration of 80 ng/μl. Transgenic animals were identified using the Roller phenotype generated by expression of the *rol-6(su1006)* dominant allele from pRF4. Experimental constructs were injected at a concentration of 20 or 50 ng/μl.

Antibody preparation, immunoblotting, and immunocytochemistry: Anti-LIN-8 antibodies were generated using purified maltose-binding protein (MBP)-LIN-8(aa 1–386) fusion protein. The crude antisera were subsequently affinity purified against the GST-LIN-8 (aa 1–386) fusion protein as described by Koelle and Horvitz (1996) and then preadsorbed against an acetone precipitate of proteins prepared from *lin-8(n2731)* mixed-stage worms, essentially as described by Harlow and Lane (1988). Affinity-purified and preadsorbed anti-LIN-8 antibodies HM2247 were used at a dilution of 1:1000 for Western blots. Samples for Western analysis were prepared by Dounce homogenization of mixed-stage worms in hypotonic lysis buffer (10 mm KCl, 1.5 mm MgCl2, 1 mm EDTA, 1 mm EGTA, 1 mm DTT, 250 mm sucrose) containing 1× protease inhibitor cocktail (800 μg/ml benzamidine HCl, 500 μg/ml phenanthroline, 500 μg/ml aprotinin, 500 μg/ml leupeptin, 500 μg/ml pepstatin A, 50 mm phenylmethylsulfonyl fluoride; BD Biosciences, Franklin Lakes, NJ) as well as phosphatase inhibitors (0.2 mm sodium orthovanadate, 50 mm sodium fluoride, 1 μm microcystin-LR).

Affinity-purified and preadsorbed anti-LIN-8 antibodies HM2247 were used at a dilution of 1:100 for immunocytochemistry. Anti-α-tubulin mouse monoclonal antibodies DM1A (Sigma, St. Louis) and MH27 (Francis and Waterston 1991), which recognizes the apical borders of *C. elegans* epithelial cells, were used as positive controls for immunocytochemistry at 1:100 and 1:1000 dilutions, respectively. Embryos were fixed in 0.8% paraformaldehyde for 20 min as described by Guenther and Garriga (1996). Larvae and adults were fixed in 2% paraformaldehyde for 15 min, essentially as described by Finney and Ruvkun (1990). Images were obtained using a Zeiss LSM510 laser confocal microscope and software and processed using Adobe Photoshop software.

Two-hybrid and *in vitro* binding experiments: The yeast two-hybrid screen of a *C. elegans* cDNA library was performed as described by Walhout and Vidal (2001). Full-length *lin-35* Rb was used as bait. A total of 1.4×10^6 colonies of the *C. elegans* AD-wrmcDNA library (Walhout *et al.* 2000b) were screened. Interaction of LIN-35 Rb and LIN-8 could not be tested in the reverse orientation, as LIN-8 was found to self-activate when fused to the Gal4 DNA-binding domain.

The full-length and partial MBP-LIN-8 constructs were made by subcloning appropriate portions of the *lin-8* cDNA into vector pMAL-c2 (NEB, Beverly, MA). The GST-LIN-8 (aa 175–285) construct was made by subcloning the appropriate portion of the *lin-8* cDNA into vector pGEX-2T (Amersham Biosciences, Piscataway, NJ). MBP and GST fusion constructs were expressed in *E. coli* BL21(DE3) cells (Studier *et al.* 1990) and purified using amylose resin (NEB) or glutathione Sepharose 4B (Amersham Biosciences), respectively, as recommended by the manufacturers. The constructs encoding LIN-35 Rb (aa 1–555) and LIN-35 Rb (aa 270–961) have been described previously (Lu and Horvitz 1998) and were used as templates for *in vitro* synthesis of ^{35}S-labeled protein (TNT-coupled reticulocyte lysate system, Promega, Madison, WI). *In vitro* binding experiments were otherwise performed as described by Reddien and Horvitz (2000), and formation of protein complexes was analyzed by SDS-PAGE and autoradiography.

RESULTS

LIN-8 defines a family of novel *C. elegans* proteins: The class A synMuv gene *lin-8* was originally identified through the chance recovery of a *lin-8(lf); lin-9(lf)* double mutant in a screen by S. Brenner (personal communication) for animals abnormal in morphology or behavior; the Muv phenotype of this strain was later shown to be synthetic, as it required the presence of two unlinked mutations, *lin-8(n111)* and *lin-9(n112)* (Horvitz and Sulston 1980). An additional eight alleles of *lin-8* have since been identified in two independent screens for synMuv A genes (Thomas *et al.* 2003). *lin-8* was previously mapped to the 7-MU interval between *sup-9* and *lin-31* on chromosome II (Ferguson and Horvitz 1985). We used the deficiencies *ccDf1*, *ccDf2*, and *ccDf11*, each of which deletes *lin-31* (Chen *et al.* 1992), to more precisely locate *lin-8* on the physical map. We performed complementation tests and found that of these three deficiencies, only *ccDf11* deletes the *lin-8* locus. As the *sup-9* locus resides in cosmid F34D6 (Perez de la Cruz *et al.* 2003) and the left endpoints of *ccDf1*, *ccDf2*, and *ccDf11* had been defined approximately by experiments using PCR (data concerning the left endpoints of *ccDf2* and *ccDf11* were generously provided to us by C. A. Spike and R. K. Herman), we placed *lin-8* between the cosmids F34D6 and M151 (Figure 1A). Further mapping using the polymorphism *pPK5363* placed *lin-8* between cosmids F34D6 and C17F4. We injected cosmids from this interval into *lin-8(n111); lin-15B(n744)* animals and obtained rescue of their synMuv phenotype with cosmid C03E12 as well as with a 7.5-kb subclone of C03E12 (Figure 1B). This minimal rescuing fragment contains a single intact predicted gene, B0454.1 (*C. elegans* Sequencing Consortium 1998). We determined the sequences of the nine alleles of *lin-8* and found all to contain mutations within the B0454.1 open reading frame (ORF) (Table 1). Furthermore, RNA-mediated interference (RNAi) of B0454.1 in *lin-15B(n744)* animals resulted in a synMuv phenotype, and expression of the B0454.1 ORF under the control of the two *C. elegans* heat-shock promoters (Stringham *et al.* 1992) shortly after L1 lethargus efficiently rescued the

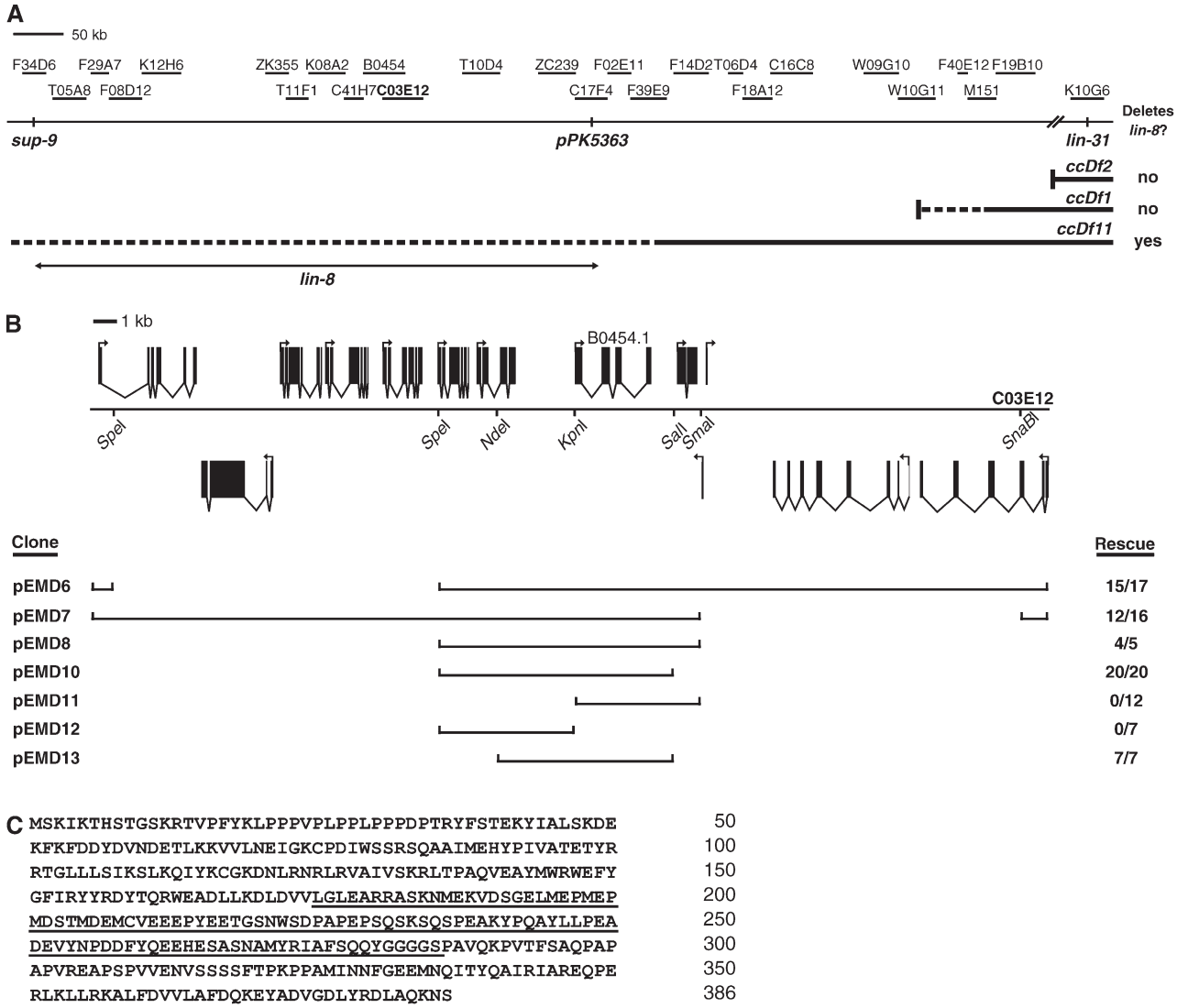


FIGURE 1.—Cloning of *lin-8*. (A) Physical map of the genomic region containing the *lin-8* locus. Deficiency and polymorphism mapping placed *lin-8* between cosmids F34D6 and C17F4. *lin-8* rescuing cosmid C03E12 is shown in boldface type. Solid lines indicate regions known to be deleted by the deficiencies; dashed lines indicate regions that may be deleted by the deficiencies. (B) Transformation rescue of *lin-8*. (Top) The predicted open reading frames within rescuing cosmid C03E12 and (bottom) the subclones derived from this cosmid. (C) Sequence of the LIN-8 protein. The region sufficient for interaction with LIN-35 Rb is underlined.

synMuv phenotype of *lin-8(n2731); lin-15B(n744)* animals (data not shown). We conclude that *lin-8* and B0454.1 are equivalent.

lin-8 encodes a novel, acidic protein of 386 amino acids (Figure 1C). Sequencing of six *lin-8* cDNA clones (courtesy of Yuji Kohara) verified the exon/intron junctions predicted by GENEFINDER (EDGLEY *et al.* 1997). Extensive database searches [using PSI-BLAST (ALTSCHUL *et al.* 1997), PROSITE (FALQUET *et al.* 2002), Pfam (BATEMAN *et al.* 2002), and SMART (SCHULTZ *et al.* 2000)] with the LIN-8 protein sequence have revealed no significant canonical motifs and no apparent sequence homologs in other species. LIN-8 is, however, a member of a family of 17 *C. elegans* proteins (Figure 2). This family was independently detected by the Pfam protein families database and is referred to there as DUF278 (BATEMAN *et al.* 2002). While the scores are very weak, LIN-8 identifies the most distant family member in BLAST searches and vice versa. The biological roles of the remaining 16 family members are not known. Several of the family members, including LIN-8, possess an N-terminal proline-rich region (Figure 2) containing at least one PxxP motif, the core sequence to which SH3 domains can bind (KAY *et al.* 2000).

Characterization of *lin-8* alleles: To identify null alleles of *lin-8* as well as residues important for LIN-8 function, we characterized all nine independently isolated *lin-8* alleles (Tables 1 and 2). Three *lin-8* alleles—*n2731*, *n2738*, *n2739*—contain nonsense mutations (Table 1). The remaining six *lin-8* alleles—*n111*, *n2376*, *n2378*, *n2403*, *n2724*, *n2741*—contain missense mutations (Table 1). Two of the missense alleles, *n2403* and *n2724*

TABLE 1

Sequences of *lin-8* mutations

Allele	Wild-type codon	Mutant codon	Substitution
n111	CTG	CCG	L20P
n2741	GTG	ATG	V68M
n2376	GAG	AAG	E148K
n2378	CGC	TGC	R154C
n2403	GAG	AAG	E164K
n2724	GAG	AAG	E164K
n2738	TGG	TAG	W79amber
n2731	CAA	TAA	Q113ochre
n2739	AGA	TGA	R304opal

Amino acid substitutions are indicated as wild-type residue, residue number, and mutant residue. Mutated bases are underlined.

(Thomas *et al.* 2003), contain the identical nucleotide change; only *n2403* was subsequently used for quantitative studies. Four of the five amino-acid residues altered in the missense alleles are conserved in several LIN-8 family members (Figure 2).

The nine *lin-8* alleles are not easily distinguishable in combination with the strong canonical synMuv B allele *lin-15B(n744)* (data not shown). The synMuv phenotype is inherently temperature sensitive: both its penetrance and its expressivity are usually greater in mutants raised at 20° than at 15° (Ferguson and Horvitz 1989). We therefore quantitated the penetrance of the synMuv phenotype associated with each *lin-8* allele in combination with two weak synMuv B alleles, *lin-15B(n2245)* (Thomas *et al.* 2003) and *lin-52(n771)* (Ferguson and Horvitz 1989), at both 15° and 20° (Table 2). The missense alleles can be placed in three categories on the basis of phenotypic strength: weak (*n2741*), intermediate (*n111*), and strong (*n2376*, *n2378*, *n2403*). The three strong missense alleles all mutate charged amino acids in a cluster of residues conserved in many members of the LIN-8 family. Of the nonsense mutations, *n2731* and *n2738* appeared to be substantially stronger than *n2739* when tested in combination with *lin-52(n771)*. Both *n2731* and *n2738* are predicted to truncate more than two-thirds of the wild-type LIN-8 protein, whereas *n2739* is predicted to leave more than two-thirds of the wild-type LIN-8 protein intact.

On the basis of their molecular lesions, we considered *n2731* and *n2738* to be candidate null alleles of the *lin-8* locus. Neither nonsense allele, however, inactivated the synMuv A pathway to the same extent as did loss of the class A synMuv gene *lin-56* in the *lin-15B(n2245)* mutant background (Table 2). We also observed that the strong missense mutations *n2376*, *n2378*, and *n2403* appeared more penetrant for the synMuv phenotype than either *n2731* or *n2738* in combination with *lin-15B(n2245)* (Table 2). To more stringently determine if *n2731* and *n2738* were null alleles of the *lin-8* locus, we compared the penetrances of the synMuv phenotype of hermaphrodites homozygous for each allele with hermaphrodites heterozygous for each allele and for *ccDf11*, a deficiency that deletes the *lin-8* locus (see Figure 1A). The penetrances of the synMuv phenotype for both *lin-8(n2731); lin-15B(n2245)* and *lin-8(n2738); lin-15B(n2245)* homozygotes were weaker than those of *lin-8(n2731)/ccDf11; lin-15B(n2245)* and *lin-8(n2738)/ccDf11; lin-15B(n2245)* heterozygotes, respectively (Table 2). One possible interpretation of these observations is that neither *n2731* nor *n2738* completely eliminates *lin-8* function. However, the *ccDf11* deficiency also eliminates several other LIN-8 family members: B0454.9, C41H7.3, C41H7.4, C41H7.5, C41H7.6, F14D2.2, F54D10.3, K08A2.1, and K08A2.4. We suspect that *n2731* and/or *n2738* are null alleles of *lin-8* and that the penetrances of their synMuv phenotypes are enhanced by a decrease in the dosage of one or more of the *lin-8* family genes deleted by *ccDf11*. This latter hypothesis is supported by the observation that no LIN-8 protein is detected in *n2738* protein extracts (see below).

Unlike mutants carrying the nonsense mutations *n2731* and *n2738*, mutants carrying the missense mutation *n2376* had an equally penetrant phenotype when homozygous as when heterozygous over the *ccDf11* deficiency (Table 2), suggesting that *n2376* may be a null allele of *lin-8*. However, *n2376* results in the production of stable LIN-8 protein at least in extracts from mixed-stage animals (see below). Furthermore, *lin-8(n2376)* retains wild-type *lin-8* function in another assay (H. T. Schwartz and H. R. Horvitz, unpublished results). Thus, it is likely that the *n2376* allele is not a null allele of *lin-8* but is instead specifically defective for *lin-8* synMuv A function.

LIN-8 is a nuclear protein expressed in many cells: To determine the expression pattern and localization of the LIN-8 protein, we generated a rabbit polyclonal antibody against a fusion of full-length LIN-8 with maltose-binding protein (MBP-LIN-8). The affinity-purified and preadsorbed antibody recognized an apparent doublet of ~50 kD in wild-type but not in *lin-8(n2731)* or *lin-8(n2738)* protein extracts analyzed by Western blots (Figure 3A); the predicted size of the LIN-8 protein is 44 kD. As the two LIN-8 proteins are approximately equal in their levels and the six *lin-8* cDNA clones (courtesy of Yuji Kohara) that we analyzed are identical in sequence, we suspect that the LIN-8 protein may be post-translationally modified. The *n2376* missense mutation does not destabilize the full-length LIN-8 protein (Figure 3A).

Since *lin-8* functions with *lin-15A*, *lin-38*, and *lin-56* to inhibit vulval development (Ferguson and Horvitz 1989; Thomas *et al.* 2003), we analyzed the impact of loss-of-function mutations in these class A synMuv genes on the LIN-8 protein. By Western blot analysis, neither the levels nor the electrophoretic mobility of LIN-8 appears to be altered in *lin-56(lf)*, *lin-15A(lf)*, or *lin-38(lf)* mutants (Figure 3A). This result contrasts with that for the class A synMuv proteins LIN-15A and LIN-56, which are dependent on each other, but not on *lin-8* or

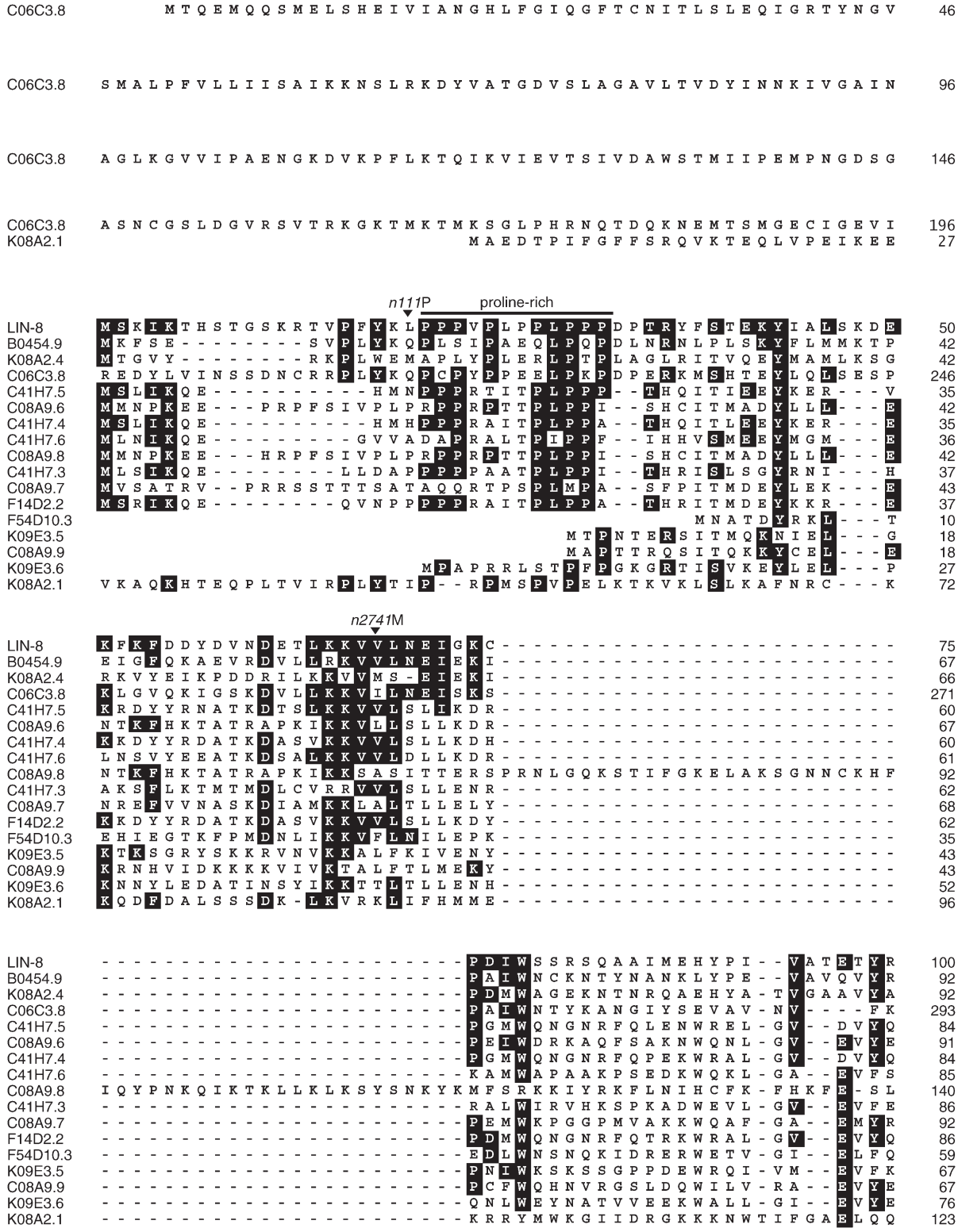


FIGURE 2.—LIN-8 defines a family of *C. elegans* proteins. Alignment of LIN-8 with the other 16 members of the LIN-8 family; all of these proteins are from *C. elegans*. Solid background indicates identity with LIN-8 in at least three additional family members. Arrowheads indicate positions of missense mutations found in the indicated *lin-8* alleles. The proline-rich N-terminal motif and the region of LIN-8 sufficient for interaction with LIN-35 Rb are indicated by solid lines.

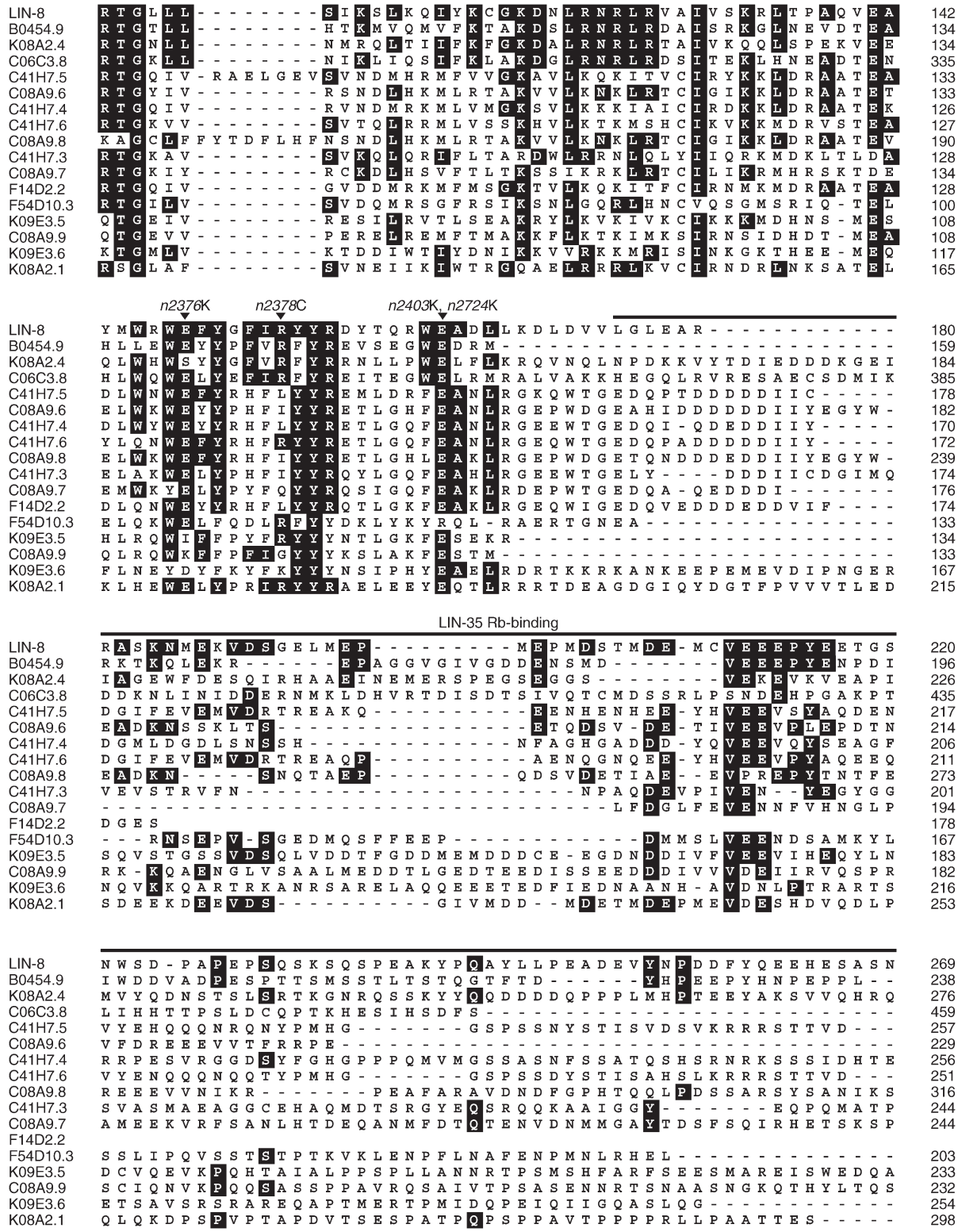


Figure 2.—*Continued.*

lin-38, for wild-type levels (E. M. Davison, A. M. Saffer, L. S. Huang, J. DeModena, P. W. Sternberg and H. R. Horvitz, unpublished results).

We used the same anti-LIN-8 antibody for whole-mount staining of worms. LIN-8 appeared to be predominantly localized to nuclei (Figure 3, B–D). We observed LIN-8 expression in many cells in embryos, larvae, and adults, and LIN-8 staining was particularly prominent in the germline as well as in neuronal nuclei of the head (Figure 3, B–D). Although diffuse within the

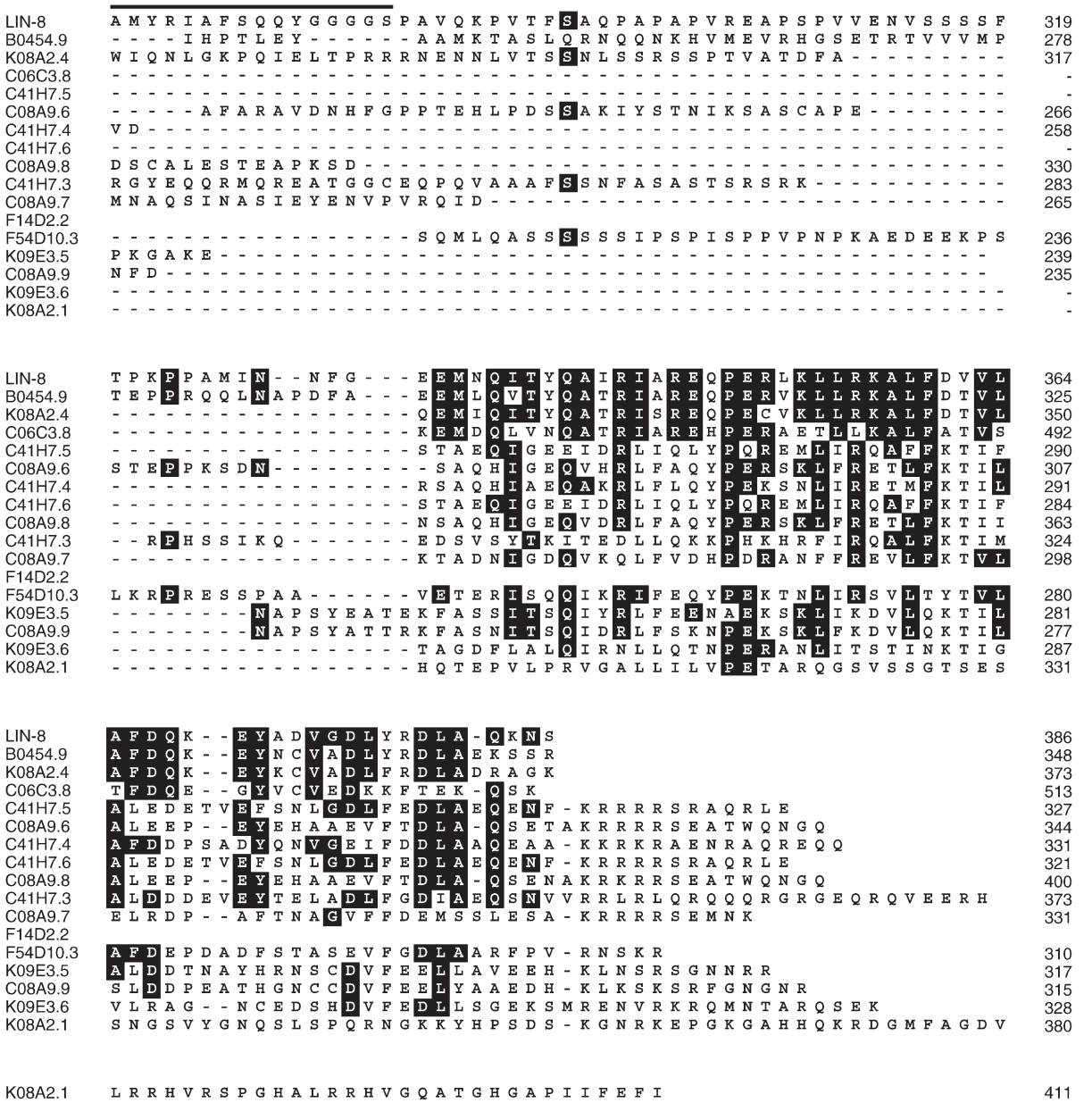


FIGURE 2.—*Continued.*

syncytium of the distal gonad arms, LIN-8 was specifically associated with germ cell nuclei during the pachytene stage and was also localized to oocyte nuclei (Figure 3C). No anti-LIN-8 staining was observed in any of these somatic or germ cell nuclei in *lin-8(n2731)* animals at any stage (Figure 3, B and C; data not shown). Background staining in the larval midbody of *lin-8(n2731)* animals was too high to examine LIN-8 expression in vulval cells.

LIN-8 interacts with LIN-35 Rb *in vitro*: We performed a yeast two-hybrid analysis of a *C. elegans* cDNA library using full-length *lin-35* Rb as bait and identified LIN-8 as a potential LIN-35 Rb interactor (data not shown). The Gal4-based screen made use of three reporter genes: *GAL1::HIS3*, *GAL1::lacZ*, and *SPAL10::URA3* (FIELDS and SONG 1989; WALHOUT and VIDAL 2001). Of 1.4×10^6 transformants, we identified 11 clones that grew on selective medium in the presence of 3-aminotriazole. Further analysis revealed that 6 of these 11 clones also expressed β-galactosidase and were able to grow in the absence of uracil. All 6 clones that tested positive for expression of all three reporter genes were found to contain the B0454.1 open reading frame encoding LIN-8. Neither LIN-35 nor LIN-8 interacted in the yeast two-hybrid system with any of 29 other vulval proteins tested (WALHOUT *et al.* 2000a; data not shown). Western blot analyses (data not shown) indicate that LIN-35 is expressed at wild-type levels in *lin-8(n2731)* worms and that LIN-8 is expressed at wild-type levels in *lin-35(n745)* worms, which lack LIN-35 protein (LU and HORVITZ 1998).

TABLE 2

***lin-8* allele strengths**

	Penetrance of Muv phenotype (%)			
	lin-15B(n2245)		*lin-52(n771)*	
Genotype[a,b]	15° (*n*)	20° (*n*)	15° (*n*)	20° (*n*)
lin-8(n111)	29 (94)	100 (109)	49 (98)	99 (143)
lin-8(n2741)	1 (98)	100 (101)	15 (96)	99 (146)
lin-8(n2376)	98 (99)	100 (103)	80 (96)	100 (155)
lin-8(n2378)	99 (98)	100 (101)	88 (97)	100 (142)
lin-8(n2403)[c]	92 (101)	100 (104)	98 (100)	100 (107)
lin-8(n2738)	68 (96)	100 (103)	96 (97)	100 (139)
lin-8(n2731)	77 (74)	100 (104)	99 (97)	100 (146)
lin-8(n2739)	76 (100)	100 (102)	63 (95)	97 (119)
lin-8(n2376) dpy-10(e128)/ccDf11	96 (135)	ND	ND	ND
lin-8(n2738) dpy-10(e128)/ccDf11	95 (127)	ND	ND	ND
lin-8(n2731) dpy-10(e128)/ccDf11	94 (183)	ND	ND	ND
lin-56(n2728)[d]	100 (127)	100 (127)	ND	ND
lin-56(n3355)[d]	100 (124)	100 (100)	ND	ND

Penetrance of Muv phenotype (%), percentage of animals with at least one pseudovulva on their ventral sides. *n*, number of animals scored. ND, not determined.

[a] Animals homozygous for either a *lin-8(lf)* or a *lin-56(lf)* allele were raised at either 15° or 20° for at least three generations before scoring.

[b] To generate *lin-8(lf)/ccDf11; lin-15B(n2245)* animals, *dpy-10(e128); lin-15B(n2245)* hermaphrodites were mated with *ccDf11/nIs128* males. The resulting non-Dpy non-GFP *ccDf11/dpy-10(e128); lin-15B(n2245)* male offspring were then crossed with *lin-8(lf) dpy-10(e128); lin-15B(n2245)* hermaphrodites, and the Muv phenotype of any non-Dpy cross progeny of this mating was scored. All crosses were carried out at 15°.

[c] Since *n2403* and *n2724* contain the same amino acid change, only *n2403* was analyzed.

[d] The *n2728* allele contains a deletion of the entire *lin-56* locus. The *n3355* allele contains an early nonsense mutation within the *lin-56* coding sequence (E. M. Davison, A. M. Saffer, L. S. Huang, J. DeModena, P. W. Sternberg and H. R. Horvitz, unpublished results).

To test the hypothesis that LIN-8 and LIN-35 Rb can interact and, if so, to identify the region of LIN-35 Rb required for the interaction, we sought to determine if LIN-8 could associate *in vitro* with two different fragments of LIN-35 Rb. The A/B pocket domain of mammalian pRb, p107, and p130 mediates association with many interacting proteins (Morris and Dyson 2001), and in *C. elegans* is contained within a portion of LIN-35 Rb sufficient for interaction in *in vitro* pull-down experiments with LIN-53 RbAp48, HDA-1 HDAC, DPL-1 DP, and EFL-1 E2F (Lu and Horvitz 1998; Ceol and Horvitz 2001). An [^{35}S]methionine-labeled N-terminal LIN-35 Rb fragment (aa 1–555), which lacks an intact pocket domain, failed to show any significant association with a full-length MBP-LIN-8 fusion protein in *in vitro* pull-down assays (Figure 4A). By contrast, an [^{35}S]methionine-labeled C-terminal LIN-35 Rb fragment (aa 270–961), which contains the pocket domain, interacted with MBP-LIN-8 (Figure 4A). LIN-8 and LIN-35 Rb are thus capable of interacting in both yeast two-hybrid and *in vitro* pull-down assays. Furthermore, these observations suggest that, as with its other *C. elegans* interactors (Lu and Horvitz 1998; Ceol and Horvitz 2001), LIN-35 Rb associates with LIN-8 through its C terminus, possibly via the pocket domain.

We next identified the domain of LIN-8 required for interaction with the C-terminal LIN-35 Rb fragment using an *in vitro* pull-down assay. Progressive deletions of N- and C-terminal LIN-8 residues revealed that amino acids 170–359 of LIN-8 were necessary for interaction with LIN-35 Rb (data not shown; Figure 4C). Furthermore, amino acids 175–285 of LIN-8 were sufficient for interaction with the C-terminal LIN-35 Rb fragment (Figure 4, B and C). Several viral proteins interact with the pocket domain of pRb via an LXCXE motif (Harbour and Dean 2000); this sequence is not contained within amino acids 175–285 of LIN-8. Amino acids 175–285 of LIN-8 share a small region of similarity with other LIN-8 family members (Figure 2). None of the *lin-8* missense mutations resides within the interaction domain, and thus no existing mutation compromises the predicted association between LIN-35 Rb and LIN-8 by directly affecting the interaction domain.

DISCUSSION

The class A synMuv genes function redundantly with the *C. elegans* homolog of the mammalian tumor

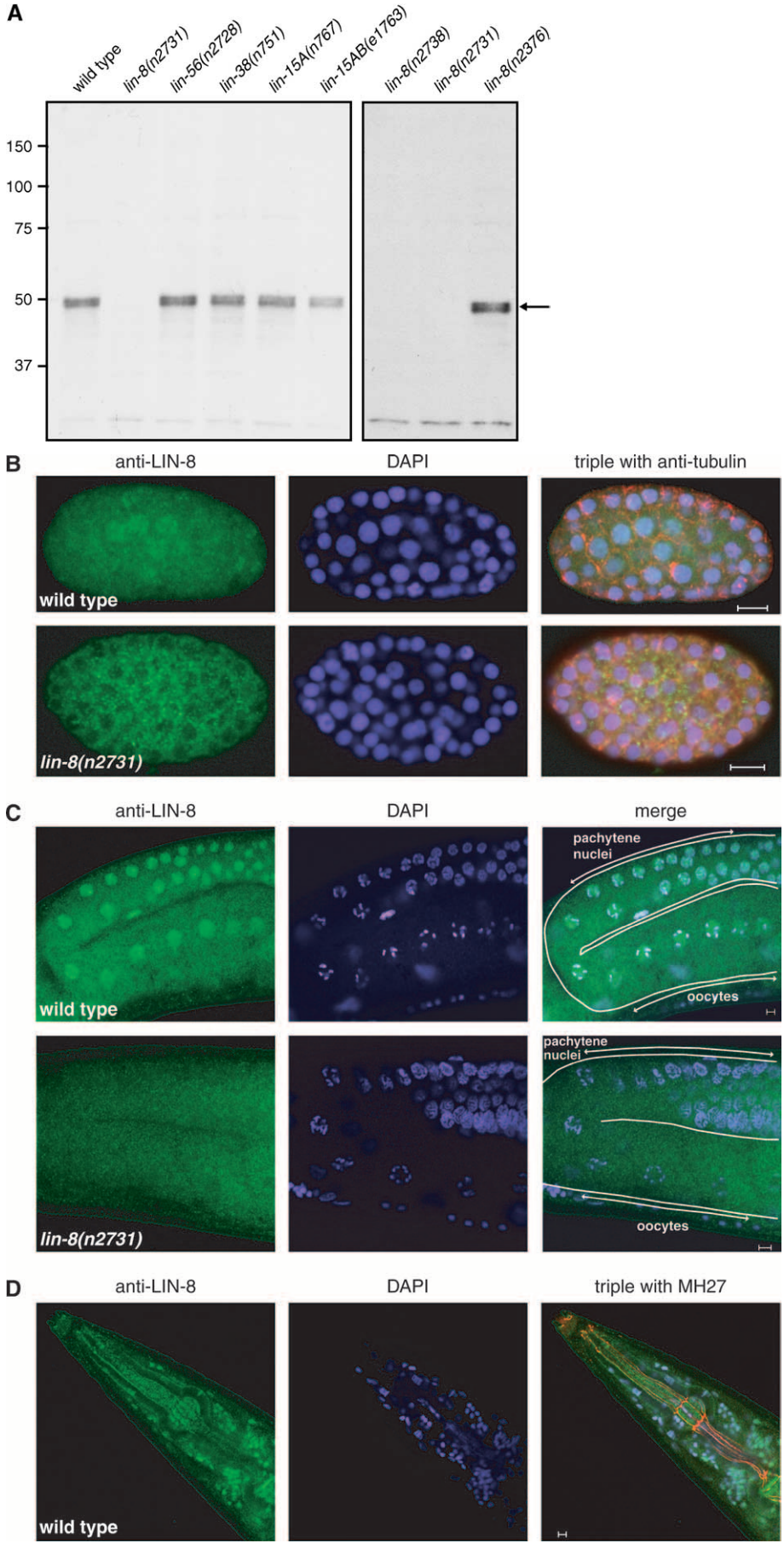
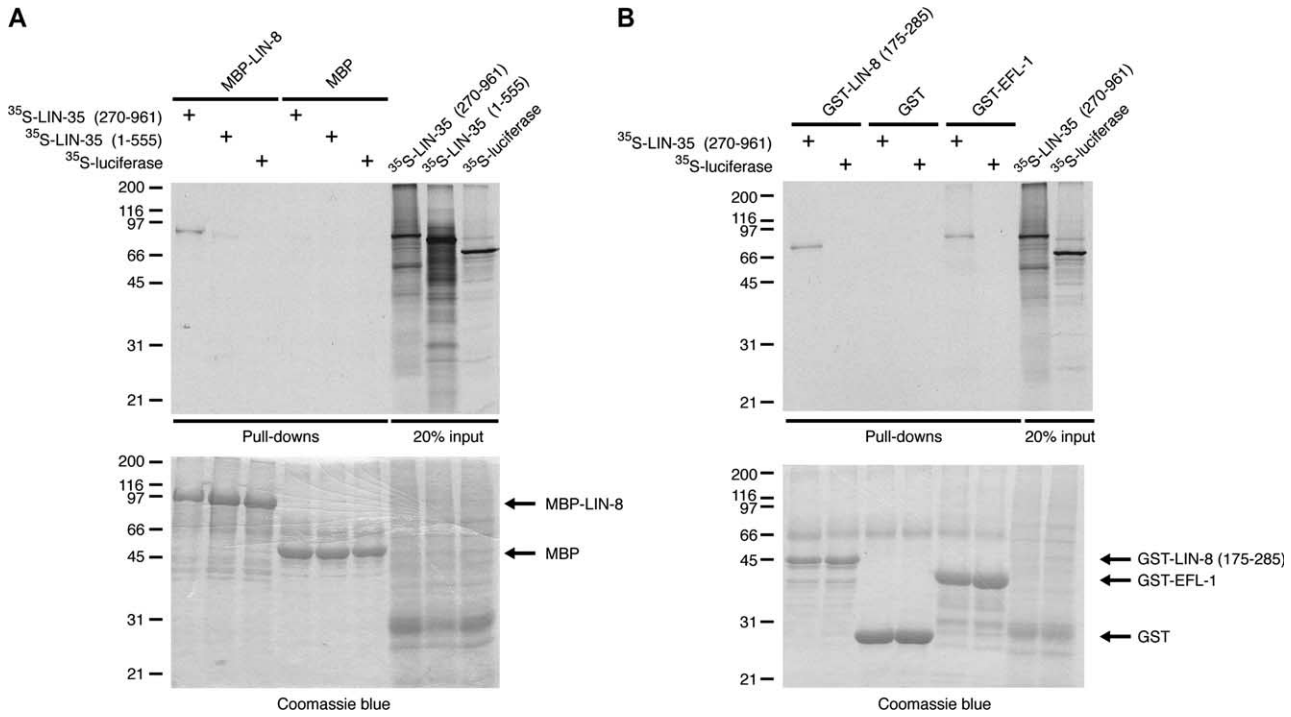


FIGURE 3.—LIN-8 protein is expressed broadly and localized in nuclei. (A) Western analysis of protein extracts from wild-type, *lin-8(lf)*, *lin-56(n2728)*, *lin-38(n751)*, *lin-15A(n767)*, and *lin-15AB(e1763)* mixed-stage worms probed with affinity-purified and preadsorbed anti-LIN-8 antibody. The position of the apparent LIN-8 protein doublet is indicated by the arrow. The molecular weights of marker proteins are indicated at left in kilodaltons. (B–D) Whole-mount staining of wild-type and *lin-8(lf)* animals with affinity-purified and preadsorbed anti-LIN-8 antibodies (green), as well as DAPI (blue) to visualize DNA. Staining with antitubulin antibody (red) is shown as a fixation control in embryos. Staining with the MH27 antibody (red), which recognizes the apical borders of *C. elegans* epithelial cells, is shown as a fixation control in adults. Bars, 5 μm. (B) LIN-8 staining is observed in multiple nuclei in the wild-type but not the *lin-8(n2731)* embryo. (C) LIN-8 staining is present in the wild-type but not in the *lin-8(n2731)* gonad in pachytene nuclei and in oocytes. (D) LIN-8 staining is observed in multiple nuclei in the wild-type adult head.



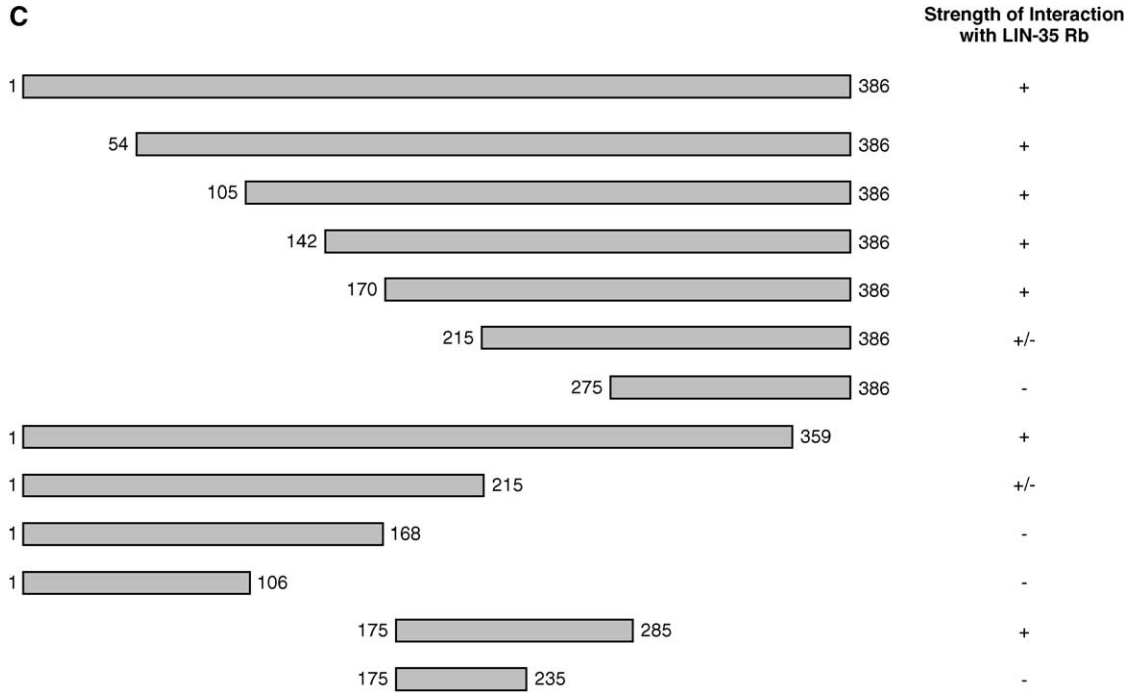


FIGURE 4.—LIN-8 interacts with LIN-35 Rb *in vitro*. (A) LIN-35 Rb (aa 270–961) but neither LIN-35 Rb (aa 1–155) nor luciferase interacts with full-length MBP-LIN-8 fusion protein. None of the constructs interacts with MBP alone. Twenty percent of the ^{35}S-labeled proteins used in the binding reactions are shown. Coomassie blue staining indicates that approximately equal amounts of full-length MBP-LIN-8 and MBP were used in the binding reactions. The molecular weights of marker proteins are indicated at the left in kilodaltons. (B) LIN-35 Rb (aa 270–961) but not luciferase interacts with GST-LIN-8 (aa 175–285) and GST-EFL-1. Neither construct binds to GST alone. Twenty percent of the ^{35}S-labeled protein used in the binding reactions are shown. Coomassie blue staining indicates that approximately equal amounts of MBP, GST, and fusion proteins were used in the binding reactions. The molecular weights of marker proteins are indicated at the left in kilodaltons. (C) Summary of the LIN-8 fragments used for *in vitro* pull-down experiments and of their ability to interact with LIN-35 Rb (aa 270–961). +, wild-type interaction; +/−, interaction detected but weaker than that of wild type; −, no interaction.

suppressor pRb to inhibit Ras-mediated vulval development. We have shown that the class A synMuv gene *lin-8* encodes a novel nuclear protein that not only functions redundantly but also physically interacts with *C. elegans* Rb. Given these observations, we propose that the class A synMuv genes act in transcriptional regulation. Further characterization of the mechanism by which the proteins of the class A synMuv pathway act may well reveal molecular processes that interact with the mammalian Rb tumor-suppressor pathway in both the regulation of cell fate and the prevention of tumorigenesis.

Class A synMuv genes may regulate transcription: The two previously cloned synMuv A genes—*lin-15A* and *lin-56*—encode novel nuclear proteins that share a novel C2CH motif also found in the synMuv B proteins LIN-15B and LIN-36, as well as in HIM-17, a protein required for meiotic recombination and histone H3 lysine-9 methylation in the germline (Reddy and Villeneuve 2004; E. M. Davison, A. M. Saffer, L. S. Huang, J. DeModena, P. W. Sternberg and H. R. Horvitz, unpublished results). This C2CH motif is likely related to the THAP domain (Roussigne *et al.* 2003; Clouaire *et al.* 2005) and has been proposed to mediate interaction with chromatin or chromatin-associated proteins (Reddy and Villeneuve 2004; E. M. Davison, A. M. Saffer, L. S. Huang, J. DeModena, P. W. Sternberg and H. R. Horvitz, unpublished results). The THAP domain of the human protein THAP1 has been shown to possess zinc-dependent sequence-specific DNA-binding activity *in vitro* (Clouaire *et al.* 2005). It has therefore been proposed that the synMuv A proteins inhibit vulval development through the regulation of transcription (E. M. Davison, A. M. Saffer, L. S. Huang, J. DeModena, P. W. Sternberg and H. R. Horvitz, unpublished results).

The nuclear localization of LIN-8 and the physical association between LIN-8 and LIN-35 Rb is consistent with the hypothesis that LIN-8 is present at the sites of transcriptional repressor complexes. How might the proteins encoded by these three synMuv A genes modulate transcriptional activity? Three general mechanisms seem possible. First, like some synMuv B proteins (Lu and Horvitz 1998; Solari and Ahringer 2000; von Zelewsky *et al.* 2000; Ceol and Horvitz 2001; Couteau *et al.* 2002), the synMuv A proteins may impact chromatin structure. Second, the synMuv A proteins may have more direct functions in regulating the initiation, elongation, or termination of transcription. Third, the synMuv A proteins may mediate the localization of target genes to nuclear subdomains where their transcription could be coordinately and efficiently regulated. The localization of genes to the vicinity of centromeric heterochromatin, for example, may contribute to transcriptional repression in Drosophila and mammals (Gasser 2001).

Physical interaction between synMuv A and B proteins: Indirect physical interaction between class A and class B proteins was proposed by Walhout *et al.* (2000a), who found that the synMuv A protein LIN-15A shared interactors with the synMuv B proteins LIN-36 and LIN-37 in the yeast two-hybrid system. As RNAi analysis has thus far not revealed a role for any of these shared interactors in the class A or class B synMuv pathways or in antagonism of these pathways (C. J. Ceol and H. R. Horvitz, unpublished results), the significance of this observation remains unclear.

The direct interaction between LIN-8 and LIN-35 Rb *in vitro* described in this article suggests that LIN-8 and LIN-35 Rb may associate *in vivo*. The biological role of such an interaction is unclear. If the putative interaction between LIN-8 and LIN-35 Rb were to facilitate *lin-8* and/or *lin-35* Rb function in vulval development, then one might expect to observe both synMuv A and synMuv B activity associated with one or both of these genes. However, a reduction of *lin-8* function does not result in a synMuv phenotype in combination with a loss of the function of the synMuv A genes *lin-15A*, *lin-38*, or *lin-56* (Ferguson and Horvitz 1989; Thomas *et al.* 2003), suggesting that *lin-8* does not possess class B synMuv activity. Similarly, a loss of *lin-35* Rb function does not result in a synMuv phenotype in combination with a loss of the function of the synMuv B genes *lin-36*, *lin-37*, or *lin-15B* (Ferguson and Horvitz 1989), suggesting that *lin-35* Rb does not possess class A synMuv activity.

Proteins that physically interact often work together directly in the same biological process. By contrast, synthetic genetic interactions between null alleles of two genes usually indicate that the genes affect a biological process through separate mechanisms. Although *lin-8* and *lin-35* Rb function in the parallel synMuv A and synMuv B pathways, respectively, the proteins that they encode physically interact *in vitro*. If the interaction between LIN-8 and LIN-35 Rb is biologically important, then three models could explain why neither *lin-8* nor *lin-35* Rb appears to possess both synMuv A and synMuv B activity. First, the functional consequence of the LIN-8/LIN-35 Rb interaction may be redundant with another process in vulval development. For example, LIN-8 may be independently localized to the promoters of vulval specification genes by both LIN-35 Rb and another protein. Second, LIN-8 and LIN-35 Rb may function together in the vulva, but in some process not required for vulval development. Third, *lin-8* and *lin-35* Rb may act together but not in the vulva. The widespread expression of *lin-8* and *lin-35* Rb (Lu and Horvitz 1998) indicates that they could function together in other tissues. Mutation of *lin-35* Rb has indeed been shown to result not only in the synMuv B phenotype but also in defects in cell cycle progression (Boxem and van den Heuvel 2001; Fay *et al.* 2002; Garbe *et al.* 2004), in defects in pharyngeal morphogenesis (Fay *et al.* 2003), and in severely reduced expression of a muscle-cell-specific *gfp* reporter gene from repetitive transgene arrays (the Tam phenotype) (Hsieh *et al.* 1999).

Although current evidence suggests that *lin-8* does not function with *lin-35* Rb in the regulation of either cell cycle progression or transgene expression (Hsieh *et al.* 1999; Boxem and van den Heuvel 2002; Garbe *et al.* 2004; E. C. Andersen and H. R. Horvitz, unpublished observations), the possibility remains that *lin-8* and *lin-35* Rb act together in the developing pharynx or in processes not yet analyzed.

Partial redundancy in the LIN-8 family: The nonsense alleles *n2731* and *n2738* appeared to be null alleles on the basis of their molecular lesions and lack of LIN-8 protein, yet, by comparison to loss of the class A synMuv gene *lin-56* and by comparison to a deficiency that removes the *lin-8* locus, did not appear to have lost all synMuv A pathway activity. By contrast, three of the *lin-8* missense alleles impaired synMuv A activity almost to the extent seen upon loss of *lin-56*. One of these three *lin-8* missense alleles, *n2376*, acted like a null by deficiency analysis but did not destabilize full-length LIN-8 protein (at least in extracts from mixed-stage animals). One hypothesis to account for these observations is that LIN-8 normally functions as part of a protein complex and that other family members can partially replace LIN-8 activity within the complex in its absence. Specifically, in mutants that lack LIN-8 protein, closely related family members may partially substitute for LIN-8. *ccDf11*, the deletion used for deficiency analysis, removes both *lin-8* and several *lin-8* family members. The predicted partial replacement of LIN-8 by other LIN-8 family members may therefore be reduced in *lin-8(null)*/cc*Df11* heterozygotes as compared to *lin-8(null)* homozygotes. The strong missense alleles may encode stable LIN-8 proteins that inactivate other family members either by direct interaction or by competition with a partner. A similar phenomenon has been observed in *Saccharomyces cerevisiae* for the MAP kinases Fus3 and Kss1, which in wild-type yeast regulate the mating pheromone response and filamentation pathways, respectively (Madhani *et al.* 1997). In the complete absence of Fus3, Kss1 provides substitute MAPK activity for the mating pheromone response pathway and thus only a slight reduction in mating efficiency is observed. By contrast, when Fus3 is rendered catalytically inactive by a missense mutation, Kss1 cannot substitute in the mating pheromone response pathway and a much stronger mating defect results. A comparable model has been proposed for the histone deacetylase family in Drosophila, as missense but not null mutations of the histone deacetylase HDAC1 dominantly suppress silencing caused by position-effect variegation (Mottus *et al.* 2000).

The biological roles of the remaining 16 *lin-8* family members are not known. Their similarity to *lin-8* suggests that these genes are also likely to have roles in transcriptional regulation, perhaps with other components or in other cells. The high degree of similarity shared by the *lin-8* family members also suggests that redundancy may have prevented their identification in genetic screens: more than one family member may have to be inactivated for a mutant phenotype to be apparent.

Interactions within the class A synMuv pathway: LIN-56 and LIN-15A are dependent on each other for wild-type protein levels and likely form a functional complex *in vivo* (E. M. Davison, A. M. Saffer, L. S. Huang, J. DeModena, P. W. Sternberg and H. R. Horvitz, unpublished results). Mutation of *lin-8* or *lin-38* does not perturb the expression or localization of either LIN-56 or LIN-15A, indicating that neither *lin-8* nor *lin-38* is normally required for formation or stability of the putative LIN-56/LIN-15A complex. In this article, we demonstrate that *lin-56*, *lin-15A*, and *lin-38* do not appear to be required for expression of LIN-8. These results form a basis upon which our understanding of both the roles of individual components of the class A synMuv pathway and the interactions among these components can be further expanded.

Implications for human cancer: Mammalian tumorigenesis requires deregulation of cell proliferation, cell differentiation, and apoptosis and is thus an inherently synthetic process requiring multiple mutations in the proto-oncogene and tumor-suppressor pathways controlling these biological activities (Hanahan and Weinberg 2000). The Rb tumor-suppressor pathway likely plays a critical role in preventing oncogenic transformation, as its inactivation is observed in many human cancers (Nevins 2001). That the class A synMuv genes function redundantly with the *C. elegans* Rb pathway suggests that mammalian counterparts of the synMuv A genes may well possess tumor-suppressor activity. We hope that characterization of the mechanism by which the class A synMuv genes function will lead to greater understanding of processes that act with the mammalian Rb pathway both in cell-fate determination and in protection from oncogenic transformation and that the redundancy of the class A, B, and C synMuv genes in regulating *C. elegans* vulval cell fates will serve as a model for the etiology of other synthetic processes, such as tumorigenesis, the manifestation of which requires multiple mutations.

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