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The *Caenorhabditis elegans* Locus *lin-15*, a Negative Regulator of a Tyrosine Kinase Signaling Pathway, Encodes Two Different Proteins

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Manuscript received February 14, 1994
Accepted for publication April 18, 1994

ABSTRACT

The *Caenorhabditis elegans* locus *lin-15* negatively regulates an intercellular signaling process that induces formation of the hermaphrodite vulva. The *lin-15* locus controls two separate genetic activities. Mutants that lack both activities have multiple, ectopic pseudo-vulvae resulting from the overproduction of vulval cells, whereas mutants defective in only one *lin-15* activity appear wild-type. *lin-15* acts non-cell-autonomously to prevent the activation of a receptor tyrosine kinase/*ras* signaling pathway. We report here the molecular characterization of the *lin-15* locus. The two *lin-15* activities are encoded by contiguous genomic regions and by two distinct, non-overlapping transcripts that may be processed from a single mRNA precursor by *trans*-splicing. Based on the DNA sequence, the 719- and 1,440-amino acid *lin-15* proteins are not similar to each other or to known proteins. *lin-15* multivulva mutants, which are defective in both *lin-15* activities, contain deletions and insertions that affect the *lin-15* genomic region.

THE vulva of the *Caenorhabditis elegans* hermaphrodite is formed by the 22 descendants of three ectodermal blast cells, P5.p, P6.p and P7.p (SULSTON and HORVITZ 1977) (Figure 1A). Three other cells, P3.p, P4.p and P8.p, also have the potential to produce vulval cells. Since all six of these cells are able to express any of three alternative cell lineages (referred to as 1°, 2° and 3°) and are equivalent in their developmental potential, they are considered to define the vulval equivalence group (SULSTON and WHITE 1980; KIMBLE 1981; STERNBERG and HORVITZ 1986). Cells that adopt the 1° and 2° cell fates generate eight and seven descendants, respectively, that together form the vulva, whereas those that express the 3° fate generate two non-vulval descendants that fuse with the syncytial hypoderm that envelops the animal.

Cell interactions determine the fates of the cells of the vulval equivalence group (see HORVITZ and STERNBERG (1991) for review). A signal from the gonadal anchor cell induces the nearest Pn.p cells to express vulval cell lineages: P6.p adopts a 1° cell fate, while P5.p and P7.p adopt 2° cell fates (Figure 1B). The more distant cells P3.p, P4.p and P8.p adopt 3° cell fates. The elimination of the anchor cell causes all six cells to express a non-vulval 3° fate, resulting in a vulvaless (Vul) phenotype (KIMBLE 1981) (Figure 1C). Genetic experiments suggest that an inhibitory signal from the syncytial hypoderm prevents the expression of vulval cell fates (HERMAN and HEDGECOCK 1990). The removal of the hypodermal inhibitory signal allows all six cells to express vulval cell fates, resulting in a multivulva (Muv) phenotype (Figure 1D). These results suggest that during wild-type development, the anchor cell signal promotes the expression of vulval cell fates by overcoming the hypodermal inhibitory signal. In addition, interactions among the induced Pn.p cells prevent adjacent cells from both expressing a 1° fate (STERNBERG 1988).

Many mutants with altered vulval cell lineages have been characterized (HORVITZ and SULSTON 1980; FERGUSON and HORVITZ 1985, 1989; BEITEL *et al.* 1990; HAN *et al.* 1990; KIM and HORVITZ 1990; AROIAN and STERNBERG 1991; CLARK *et al.* 1992; HAN *et al.* 1993; MILLER *et al.* 1993). Some mutations cause all six cells P3.p–P8.p to express a 3° cell lineage, and no vulva is formed. As this Vul phenotype is identical to that of animals lacking the anchor cell, these mutations define genes that could be involved in the signaling process required for vulval induction. Five such Vul genes encode proteins similar to those involved in intercellular signaling in other organisms: *lin-3* encodes a molecule related to TGFα (HILL and STERNBERG 1992), *let-23* encodes a receptor tyrosine kinase (AROIAN *et al.* 1990), *sem-5* encodes an adaptor protein with SH2 and SH3 domains (CLARK *et al.* 1992), *let-60* encodes a *ras* protein (HAN and STERNBERG 1990) and *lin-45* encodes a *raf* serine/threonine kinase (HAN *et al.* 1993). Recently, mutations in the *mpk-1/sur-1* gene, which encodes a mitogen-activated protein (MAP) kinase, have been identified as suppressors of activated *let-60 ras* mutations, suggesting that *mpk-1/sur-1* also functions in the signaling pathway required for vulval induction (LACKNER *et al.* 1994; WU and HAN 1994). Other mutations, including some *lin-15* mutations, cause all six cells P3.p-P8.p to express 1° and 2° cell lineages, resulting in a multivulva phenotype (FERGUSON *et al.* 1987).

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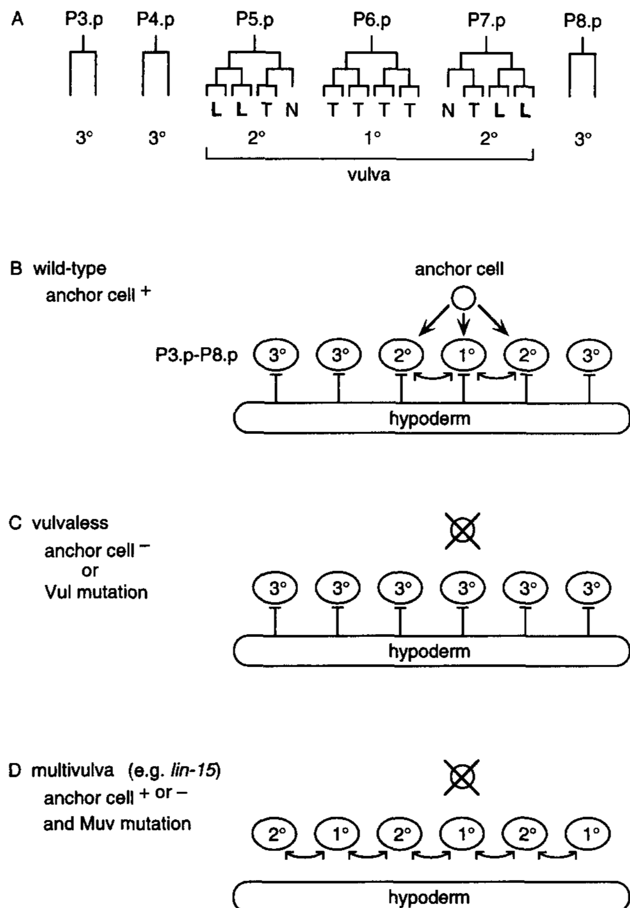

FIGURE 1.—Vulval cell lineages and models for vulval formation (adapted from SULSTON and HORVITZ 1977; BEITEL *et al.* 1990). (A) Cell lineages that generate the vulva. Each P3.p–P8.p cell expresses one of three lineages, referred to as 1°, 2° and 3°. P6.p normally expresses a 1° cell lineage, which produces eight descendants; P5.p and P7.p normally express a 2° cell lineage, which produces seven descendants; and P3.p, P4.p and P8.p normally express a 3° cell lineage, which produces two descendants. The vulva is formed by the 22 descendants of the 1° and 2° cell lineages, while the 3° cell lineage generates non-vulval hypodermal cells. The lineages are, in part, defined by the axis of the final cell division; L, longitudinal, T, transverse, N, non-dividing (STERNBERG and HORVITZ 1986). (B) Wild-type. An inductive signal from the gonadal anchor cell causes the nearest Pn.p cells to express a 1° or 2° cell lineage, while the more distant Pn.p cells express a 3° cell lineage. An inhibitory signal from the hypoderm prevents the expression of the 1° and 2° vulval cell lineages and is overridden by the anchor cell inducing signal. Interactions among the induced cells prevent adjacent Pn.p cells from both expressing a 1° cell lineage. (C) Vulvaless. In the absence of the anchor cell signal or of the response to that signal, the hypodermal inhibitory signal prevents all six Pn.p cells of the vulval equivalence group from expressing a 1° or 2° cell fate, so that no vulva is formed. (D) Multivulva. In the absence of the hypodermal inhibitory signal, all six Pn.p cells express a 1° or 2° cell lineage, and multiple vulva-like ventral protrusions are formed. Even after the elimination of the anchor cell, multivulva mutants such as *lin-15* have a multivulva phenotype.

The multivulva phenotype of many mutants requires mutations in two genes (HORVITZ and SULSTON 1980; FERGUSON and HORVITZ 1985, 1989; J. THOMAS and H. R. HORVITZ, unpublished results). Over 70 such "synthetic Muv" mutations have been identified and divided into two classes, referred to as A and B. Hermaphrodites carrying a class A and a class B mutation have a multivulva phenotype, while hermaphrodites carrying only one mutation or two mutations of the same class have a wild-type vulval phenotype. Thus, these class A and class B genes appear to define two functionally redundant genetic pathways both of which must be disrupted to cause the expression of vulval cell fates.

The first synthetic Muv mutations were discovered when the Muv phenotype of the strain CB1322 was found to require mutations in two unlinked genes, *lin-8 II* and *lin-9 III* (HORVITZ and SULSTON 1980). Five additional synthetic Muv mutations were fortuitously obtained after mutagenesis of a strain that contained an undetected class A mutation (FERGUSON and HORVITZ 1989). Subsequent mutageneses of existing class A and class B single mutants, which are phenotypically wild-type, allowed the systematic isolation of further synthetic Muv mutants (FERGUSON and HORVITZ 1989; J. THOMAS and H. R. HORVITZ, unpublished results). Two genes, *lin-8* and *lin-38*, are defined only by class A mutations, and six genes, including *lin-35*, *lin-36* and *lin-37*, are defined only by class B mutations (FERGUSON and HORVITZ 1989).

Of the mutations that caused a synthetic Muv phenotype, six mapped to the same small interval as the gene *lin-15*, which had been defined previously by single mutations that cause a Muv phenotype (FERGUSON and HORVITZ 1985, 1989). Three of these mutations were in class A, and three were in class B. Complementation tests established that both the class A and class B mutations failed to complement *lin-15* multivulva mutations, indicating that *lin-15* is a complex locus with three distinct types of alleles: Muv, class A synthetic Muv and class B synthetic Muv. These observations indicate that the *lin-15* locus controls two separate activities, one that acts in the class A pathway and one that acts in the class B pathway, and that both of these activities are disrupted in the *lin-15* Muv mutants.

A *lin-15* Muv mutant lacking an anchor cell still has a multivulva phenotype, suggesting that its Muv phenotype does not result from the overproduction of the anchor cell signal and that *lin-15* functions elsewhere than in the anchor cell to regulate the expression of vulval cell lineages (FERGUSON *et al.* 1987). Genetic mosaic analysis indicates that *lin-15* acts non-cell-autonomously and likely functions in the syncytial hypoderm that surrounds the Pn.p cells (HERMAN and HEDGECOCK 1990). These observations suggest that *lin-15* acts in the hypoderm to inhibit the expression of vulval cell lineages and that the absence of *lin-15* function allows the six cells P3.p–P8.p to express vulval cell lineages. Loss-of-function mutations in the receptor tyrosine kinase *let-23* gene, the SH3-SH2-SH3 adaptor protein *sem-5* gene, *let-341*, *let-60 ras* and *lin-45 raf* suppress the Muv phenotype conferred by *lin-15* mutations, indicating that *lin-15* acts upstream of these five genes in the genetic

pathway for vulval induction (FERGUSON *et al.* 1987; BEITEL *et al.* 1990; HAN *et al.* 1990; CLARK *et al.* 1992, 1993; HAN *et al.* 1993). The action of *lin-15* within the surrounding hypodermis may negatively regulate the *let-23* receptor tyrosine kinase signaling pathway.

We cloned the *lin-15* locus by identifying polymorphisms associated with *lin-15* mutations and by rescuing the *lin-15* mutant phenotype by germline transformation. Two adjacent transcripts of 2.3 and 4.6 kb encode the *lin-15* class A and class B genetic activities, respectively. We show that these transcripts are *trans*-spliced and provide evidence that supports the hypothesis (SPIETH *et al.* 1993) that these transcripts are processed from a single mRNA precursor. The 719- and 1,440-amino acid *lin-15* proteins are not highly similar to any known proteins and thus may represent new types of molecules involved in intercellular signaling.

MATERIALS AND METHODS

Standard methods for culturing and handling *C. elegans* were used (BRENNER 1974).

Strains: The following mutations were used in our studies. *Linkage group (LG) II: lin-8(n111).*

LGIII: lin-9(n112), lin-36(n766).

LGX: lin-15(e1763, n309, n374, n433, n743, n744, n749, n765, n767, n1139, n1296, n1331, n1344, n2141) *n1296*, *n1331* and *n1344* were identified as spontaneous Muv mutants (KIM and HORVITZ 1990) in the mutator strain TR679 (COLLINS *et al.* 1987). *n1139* was isolated after γ-irradiation (FERGUSON and HORVITZ 1985). *n2141* (C. BARGMANN, personal communication) as well as the other nine *lin-15* mutations (FERGUSON and HORVITZ 1985, 1989) were recovered following treatment with ethyl methanesulfonate (EMS). The *n433*, *n749* and *n767* mutations belong to the A class and cause a Muv phenotype in the presence of a class B mutation, such as *lin-9(n112)* or *lin-36(n766)*. The *n374*, *n743* and *n744* mutations belong to the B class and cause a Muv phenotype in association with a class A mutation, such as *lin-8(n111)*. *n765*ts mutants have a temperature-sensitive Muv phenotype because they lack class B function and have a temperature-sensitive defect in class A function. The remaining *lin-15* mutants lack both class A and B function and have a Muv phenotype that is independent of other synthetic Muv mutations.

Analysis of *lin-15*-associated polymorphisms: Genomic DNA was prepared from *lin-15* mutants raised on 1.5% agarose NGM plates seeded with HB101 essentially as described (SULSTON and HODGKIN 1988). Approximately 5 μg of genomic DNA from each mutant was digested with *Eco*RI, separated by agarose gel electrophoresis and blotted to nylon filters. The filters were probed with cosmid or plasmid ^{32}P-labeled DNA. All eight *lin-15* multivulva mutants examined and one *lin-15* class A mutant, *n767*, contained *Eco*RI restriction fragment length polymorphisms. Based on our *Eco*RI restriction map, we determined the approximate positions and lengths of the deletions and insertions within each *lin-15* mutant (see Figure 5).

Germline transformation and analysis of *lin-15* genomic region: Germline transformation (MELLO *et al.* 1991) of *lin-8* and *lin-15*, of *lin-9* and *lin-15*, of *lin-36* and *lin-15* and of *lin-15* Muv mutants was performed by coinjecting test DNA (10–50 μg/ml) and the dominant *rol-6(su1008)* roller marker (plasmid pRF4 at 80 μg/ml). Transgenic animals typically carry coinjected DNAs as extrachromosomal arrays (MELLO *et al.* 1991) and are identified by the roller phenotype conferred by pRF4. A rescued F_2 population was defined as an F_2 roller population (derived from F_1 rollers) in which at least 50% of the animals were not multivulva in phenotype. The overlapping cosmids C29B12 and ZC436 each rescued the *lin-15(n765)* Muv phenotype, suggesting that the *lin-15* gene is located in the region common to these cosmids. A number of genomic DNA fragments were subcloned from C29B12 and were similarly tested for the rescue of the Muv phenotype of *lin-15(n765*ts), of *lin-8(n111); lin-15(n374)* and of *lin-9(n112); lin-15(n433)*, *lin-9(n112); lin-15(n749)* or *lin-36(n766); lin-15(n767)* (see Figure 2B). We determined the DNA sequences of both strands of the 11.7-kb *Eag*I-*Eco*RI fragment (see Figure 2B) of C29B12 using Sequenase 2.0 (U.S. Biochemical Corp.) or using the ABI Prism cycle sequencing kit and an ABI 373A DNA Sequencer (Applied Biosystems, Inc.), essentially according to the manufacturers' instructions. Based on the sequences of the two cDNAs (see below), the *lin-15* coding regions are completely contained in this genomic DNA fragment.

Isolation and characterization of *lin-15* cDNAs: The 10.5-kb *Bam*HI-*Eco*RI fragment from C29B12 (see Figure 2B) was used to screen approximately 400,000 plaques of a λZAP cDNA library derived from mixed-stage poly(A)$^+$ RNA (BARSTEAD and WATERSTON 1989). Eleven positive clones were identified; four were from the *Eag*I-*Sal*I region, and seven were from the *Sal*I-*Eco*RI region. We determined the complete DNA sequences of one strand of the longest clone derived from the *Eag*I-*Sal*I region as well as partial sequences of the remaining 10 clones. As none of the cDNAs was full length, we used the method of reverse transcription-polymerase chain reaction (RT-PCR) to clone the 5′ regions of the *lin-15* transcripts. To amplify the 5′ region of the 2.3-kb transcript, we used a primer corresponding to sequences of the *lin-15* A cDNA (5′CAGATTCCATTGACTGGGTAGG) and a primer corresponding to sequences of either the *C. elegans trans*-spliced leaders SL1 (5′GTTTAATTACCCAAGTTTGAG) (KRAUSE and HIRSH 1987) or SL2 (5′GGTTTTAACCCAGTTACTCAAG) (HUANG and HIRSH 1989). DNA was amplified from 250 ng of mixed-stage RNA using the GeneAmp Thermostable r*Tth* Reverse Transcriptase RNA PCR Kit (Perkin Elmer) and then cloned using the TA Cloning Kit (Invitrogen). We determined the DNA sequences of both strands of two independent clones obtained using the SL1 primer and one strand of three independent clones recovered using the SL2 primer. These RT-PCR-derived clones contained the *trans*-spliced leader sequences and lacked putative intron sequences, indicating that they were derived from a processed transcript and not from genomic DNA. The DNA sequence of the 5′ region of the 4.6-kb *lin-15* B transcript was assembled from the sequences of four overlapping DNA fragments (referred to as B1, B2, B3 and B4) amplified using multiple sets of primers. Fragments B2 and B3 were amplified directly from mixed-stage RNA using RT-PCR, whereas fragments B1 and B4 were generated by a second round of PCR amplification from DNA produced by RT-PCR of RNA. The following primers were used: B1, 5′CACACGATGCCTTGCAAACG (1st round), 5′CATTGATCGAAGAAGGTGCTCC (2nd round) and 5′GCAGGTTCACCTTGGTCTTTATGC (both rounds); B2, 5′GTGGAATGTCATAGTTTGCAACTG and 5′CAAATTTAGAAGAATGCAAGTT; B3, 5′CATGATGGCTGGCACAACTTGAG and 5′ATCTGCGGAAATTGCTACTTACC; B4, 5′GTTTGTGAGGAGACTGTTGCT (1st round), 5′GCAATTTCCGCAGATAAATCGC (2nd round) and SL1 primer (both rounds). We determined the complete DNA sequences of one strand of one independent clone for each fragment as well as partial sequences of additional clones.

Construction of frameshift mutation clones: To establish that the *lin-15* A and B activities are encoded by the two identified genes, we introduced a frameshift mutation into the predicted coding region of each gene within the *Spe*I-*Nru*I

FIGURE 2.—Genetic and physical maps of the *lin-15* region of the *X* chromosome. (A) Genetic map with cosmid clones representing this region shown below. Cosmids were tested for the rescue of the Muv phenotype of the *lin-15(n765ts)* mutant when maintained as extrachromosomal arrays after germline transformation. The fraction of independently derived transformed F_2 populations rescued for the Muv phenotype of *lin-15(n765)* animals is listed for each clone. (B) Restriction map of the central region of C29B12 and structures of genomic subclones. Genomic subclones were tested for rescue of the Muv phenotype of *lin-15(n765)* animals; a subclone containing either *lin-15* A or *lin-15* B function could rescue the Muv phenotype of *lin-15(n765)*. Subclones were also tested for the rescue of the Muv phenotype of strains defective in either *lin-15* A activity [*lin-9(n112); lin-15(n433)*, *lin-9(n112); lin-15(n749)* or *lin-36(n766); lin-15(n767)*] or *lin-15* B activity [*lin-8(n111); lin-15(n374)*]. Specifically, we used *lin-9(n112); lin-15(n433)* for the *Eag*I-*Nru*I and *SnaBI-Nru*I fragments, *lin-9(n112); lin-15(n749)* for the *Eag*I-*Nhe*I and *Sph*I-*Sal*I fragments, and *lin-36(n766); lin-15(767)* for the *Sph*I-*Nru*I, *Spe*I-*Nru*I and *Spe*I-*Sal*I fragments. The fraction of independently derived transformed F_2 populations rescued for the Muv phenotype of *lin-15(n765)* animals (A or B Rescue), for *lin-15* A activity (A Rescue) and for *lin-15* B activity (B Rescue) are listed for each clone. +, rescue of *lin-15* B activity was observed in 32/33 transformed F_1 animals, but no stably transformed F_2 animals were recovered. −, 0/20 transformed F_1 animals were rescued for *lin-15* A activity, and no stably transformed F_2 animals were obtained. Frameshift mutations (denoted by ×) were introduced in the coding regions of the 2.3- and 4.6-kb transcripts present in the *Spe*I-*Nru*I genomic fragment (see MATERIALS AND METHODS), and these clones were then tested for the rescue of *lin-15* A and *lin-15* B function. Arrows represent the lengths and directions of transcription of the *lin-15* A and B transcripts. Abbreviations are as follows: Se (*Spe*I), Sp (*Sph*I), E (*Eag*I), B (*Bam*HI), Sn (*SnaBI*), S (*Sal*I), Nh (*Nhe*I), N (*Nru*I) and R (*Eco*RI).

genomic DNA fragment and tested these altered clones for the rescue of *lin-15* A activity in *lin-9(n112); lin-15(n433)* and B activity in *lin-8(n111); lin-15(n374)* or *lin-8(n111); lin-15(n744)* by germline transformation. For the putative *lin-15* A coding region, an 8-bp *Nco*I linker (5′CCCATGGG) was ligated into a *Msc*I site corresponding to nucleotide 757 of the 2.3-kb cDNA (see Figure 4A). This alteration is predicted to produce a truncated *lin-15* A protein consisting of residues 1–243 of the native protein and an additional 18 amino acids (PWATLEWQLQRKRFVMRG) derived from the linker sequences and the altered reading frame. For the putative *lin-15* B coding region, the 8-bp *Nco*I linker was ligated into a *Sca*I site corresponding to position 2352 of the 4.6-kb cDNA (see Figure 4B). This alteration is expected to generate a truncated *lin-15* B protein containing residues 1–775 of the native protein and an additional 32 amino acids (PWDWPQNYPRRKAHHHQLQLLHKLDPMLPLRN) derived from the linker sequences and the altered reading frame.

To verify that the failure to rescue the *lin-15* gene activity resulted from the frameshift mutation, we restored the reading frame in each modified clone by digesting with *Nco*I and then filling in the 5′ overhang using DNA polymerase I large (Klenow) fragment. This treatment produced a 12-bp insertion (CCCATGCATGGG) at the original site and is predicted to result in the insertion of 4 amino acids (PMHG). The constructs containing the restored reading frames rescued *lin-15* gene activity. We confirmed the DNA sequences of the modified region for all four clones.

RESULTS

Identification of *lin-15*-specific polymorphisms: We cloned the *lin-15* gene on the basis of its map position near the *sdc-1* gene on the *X* chromosome (Figure 2A). Based upon a physical map of the *C. elegans* genome consisting of overlapping cosmids and yeast artificial

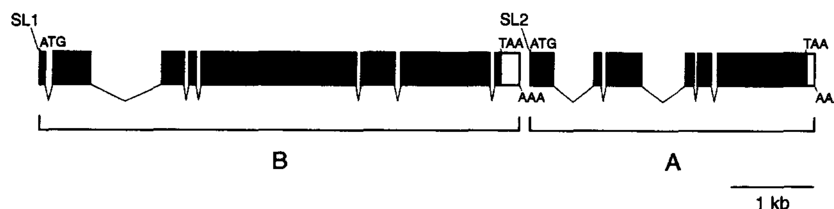

FIGURE 3.—*lin-15* genomic structure deduced from cDNA and genomic DNA sequences. The protein coding regions are denoted as solid boxes and the 3′ untranslated regions are shown as open boxes. SL1 and SL2, the *trans*-spliced SL1 and SL2 leader sequences, respectively. The downstream *lin-15* A cDNA is also processed with SL1 (not shown). ATG and TAA, the predicted start and stop sites of translation, respectively. AAA, poly(A) addition sites.

chromosomes (YACs) (COULSON *et al.* 1986, 1988; A COULSON and J. SULSTON, personal communication), we obtained cosmids that covered a small region left of the *sdc-1* gene (NONET and MEYER 1991). Genomic DNA prepared from four *lin-15* Muv mutants (*n1139, n1296, n1331, n1344*) was probed with these cosmids to detect possible *lin-15*-associated DNA rearrangements (see MATERIALS AND METHODS). *n1139* was recovered following mutagenesis by γ-irradiation (FERGUSON and HORVITZ 1985), and the other three alleles were isolated as spontaneous mutants in the mutator strain TR679 (KIM and HORVITZ 1990). As γ-irradiation often causes chromosomal rearrangements (ROSENBLUTH *et al.* 1985) and TR679-derived mutants often result from transposon insertions (COLLINS *et al.* 1987), these four mutants seemed likely to have readily detectable *lin-15* polymorphisms. All four *lin-15* Muv strains were found to contain *Eco*RI restriction fragment length polymorphisms (RFLPs) in a region covered by two overlapping cosmids, C29B12 and ZK662 (see below). Subsequently, five *lin-15* strains (*e1763, n309, n765, n767, n2141*) that were recovered following treatment with EMS were also shown to have *Eco*RI RFLPs in this region (see below and Figure 5 for description). These results suggest that at least part of the *lin-15* gene is located within the region shared by these two cosmids.

Germline rescue of *lin-15* mutants: We tested C29B12, ZK662 and a third cosmid, ZC436, in germline transformation experiments (MELLO *et al.* 1991) and found that C29B12 and ZC436 each rescued the Muv phenotype of *lin-15(n765*ts) animals (Figure 2A). A 14-kb genomic fragment (*Sph*I-*Nru*I) as well as shorter fragments derived from the region common to C29B12 and ZC436 also rescued the Muv phenotype of *lin-15(n756*ts) mutants (Figure 2B). As the Muv phenotype of *lin-15* mutants requires the loss of both A and B activities, the recovery of either A or B activity alone would be sufficient to rescue the Muv phenotype of *lin-15(n765*ts) animals or other *lin-15* Muv mutants. To assay specifically for *lin-15* A function, genomic DNA subclones were tested for the rescue of the Muv phenotype of a double mutant strain containing a class B mutation, *lin-9(n112)* or *lin-36(n766)*, and a *lin-15* A mutation. As these strains do not lack *lin-15* B function, the rescue of the Muv phenotype would indicate that the tested genomic DNA fragment had *lin-15* A rescuing activity. We found that the 11-kb *Eag*I-*Nru*I and longer fragments contained *lin-15* A activity because they rescued the Muv phenotype of the *lin-9(n112); lin-15(n433), lin-9(n112); lin-15(n749)* or *lin-36(n766); lin-15(n767)* double mutant (Figure 2B). No rescued transformants were recovered using a fragment (*Sph*I-*Sal*I or *Spe*I-*Sal*I) that lacked the 3.8-kb *Sal*I-*Nru*I region, suggesting that this region is needed for the rescue of *lin-15* A function. However, this region alone is not sufficient for *lin-15* A rescue, because no rescued transformants were obtained using the 4.9-kb *Sna*BI-*Nru*I fragment. To assay specifically for the rescue of *lin-15* B function, we used a double mutant strain containing a class A mutation, *lin-8(n111)*, and a *lin-15* B mutation. In contrast to the results for *lin-15* A rescue, the *Eag*I-*Nhe*I and *Sph*I-*Sal*I fragments rescued the Muv phenotype of the *lin-8(n111); lin-15(n374)* double mutant (Figure 2B). These results indicate that the 7.2-kb region common to these two fragments is sufficient for the rescue of the *lin-15* B defect in the *lin-8(n111); lin-15(n374)* double mutant and, by inference, in the *lin-15(n765*ts) Muv mutant. Together, these data indicate that the 11-kb *Eag*I-*Nru*I genomic DNA fragment contains both *lin-15* A and B gene activities. While the *Eag*I-*Sal*I region is needed for the rescue of both *lin-15* functions, the 3.8-kb *Sal*I-*Nru*I region is essential for only the rescue of *lin-15* A activity.

***lin-15* encodes two different polypeptides:** We determined the DNA sequences of the 11.7-kb *Eag*I-*Eco*RI genomic fragment and of multiple cDNAs derived from this region (see MATERIALS AND METHODS). As none of the cDNAs isolated from a mixed-stage cDNA library was full length, the DNA sequences of cDNAs obtained by the method of RT-PCR were also determined (see MATERIALS AND METHODS). From our analysis of the cDNA and genomic DNA sequences, we determined that there are two separate, similarly oriented transcripts that are 2.3 and 4.6 kb in length (Figures 3 and 4). cDNAs for the downstream 2.3-kb transcript were generated by RT-PCR using an oligonucleotide primer for either the SL1 or SL2 *trans*-spliced leader (KRAUSE and HIRSH 1987; HUANG and HIRSH 1989). These results show that SL1 and SL2 are used for the processing of the 2.3 kb transcript, but do not indicate the relative abundances of

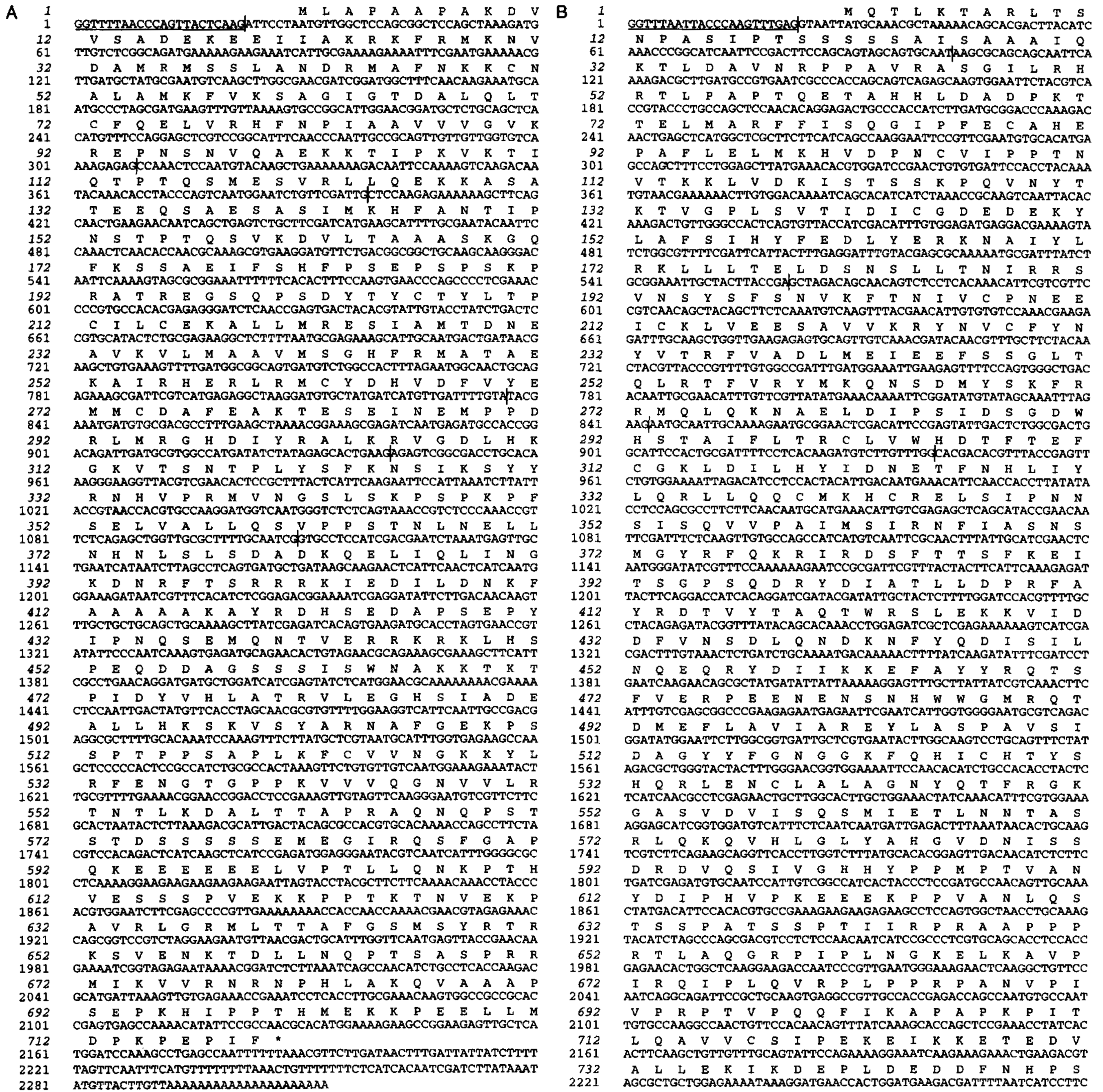

FIGURE 4.—Nucleotide and deduced amino acid sequences (single-letter code) corresponding to *lin-15* cDNAs. (A) Nucleotide sequence of a composite *lin-15* A cDNA assembled from sequences of cDNAs obtained by RT-PCR (nucleotides 1–337) and cDNAs isolated from a λ cDNA library (nucleotides 338–2,313) (see MATERIALS AND METHODS). The nucleotides derived from the 22-nucleotide *trans*-spliced leader SL2 (HUANG and HIRSH 1989) are underlined. Nucleotides are numbered on the left beginning with the SL2 sequences. Amino acids are numbered on the left in italics beginning with the first predicted methionine. The positions of splice sites as determined by comparison with genomic sequences are marked with vertical lines. The ochre termination codon is denoted as an asterisk. (B) Nucleotide sequence of a composite *lin-15* B cDNA assembled from sequences of cDNAs generated by RT-PCR (nucleotides 1–2554) and cDNAs isolated from a λ cDNA library (nucleotides 2554–4623) (see Materials and Methods). The nucleotides derived from the 22-nucleotide *trans*-spliced leader SL1 (KRAUSE and HIRSH 1987) are underlined. The ochre termination codon is denoted as an asterisk. (C) Nucleotide sequence of the *lin-15* A and B intragenic region. The sequence shown begins at the polyadenylation site of the *lin-15* B transcript and ends at the splice leader acceptor site of the *lin-15* A transcript.

SL1- and SL2-containing transcripts. cDNAs for the upstream 4.6 kb transcript were generated using an oligonucleotide primer for SL1 but could not be recovered using a primer for SL2, suggesting that this transcript is processed with SL1 and not with SL2. There are 110 nucleotides between the polyadenylation site of the 4.6-kb transcript and the splice-leader acceptor site of the 2.3-kb downstream transcript (Figure 4C).

```
752   T  D  P  V  P  N  R  T  T  A  S  S  Q  G  P  S  S  Y  P  R
2281  AACTGATCCGGTGCCAAACCGAACGACTGCTTCTTCACAAGGACCATCTTCGTATCCACG
772   K  I  V  V  L  A  S  K  L  P  T  S  Q  S  S  S  P  S  T  A
2341  GAAAATTGTAGTACTGGCCTCAAAACTACCCACGTCGCAAAGCTCATCACCATCAACTGC
792   T  S  A  Q  A  R  S  H  V  T  T  A  Q  L  I  R  C  G  P  S
2401  AACTTCTGCACAAGCTAGATCCCATGTTACCACTGCGCAACTGATCCGATGTGGGCCAAG
812   E  G  T  V  P  Q  K  I  H  S  H  N  F  V  Q  K  F  A  Q  K
2461  TGAAGGCACCGTTCCACAAAAAATTCATTCACACAACTTTGTGCAGAAGTTTGCTCAAAA
832   Q  N  F  V  H  K  Y  A  L  N  S  Q  D  H  T  G  R  L  N  Q
2521  GCAAAATTTTGTTCACAAGTATGCACTGAACTCACAAGACCACACAGGCAGGCTCAACCA
852   T  V  P  M  R  A  A  L  R  L  P  N  S  E  Q  K  S  G  A  P
2581  AACTGTACCCATGAGAGCTGCGCTGAGGCTTCCAAACAGCGAACAAAAGTCAGGAGCACC
872   S  S  I  N  G  K  V  Q  R  D  D  F  K  L  E  P  L  D  D  F
2641  TTCTTCGATCAATGGAAAAGTACAGAGGGATGACTTCAAATTGGAACCACTGGACGATTT
892   N  G  E  P  D  Y  D  N  L  I  G  A  Q  R  L  M  Y  S  D  N
2701  CAACGGAGAACCCGACTATGACAATTTAATTGGGGCTCAACGGCTTATGTACAGTGATAA
912   L  N  D  A  S  A  E  D  A  F  A  R  H  R  V  T  M  E  F  Q
2761  TCTCAATGACGCATCGGCAGAAGATGCGTTTGCAAGGCATCGTGTGACTATGGAATTCCA
932   K  R  R  A  C  N  R  R  C  A  V  C  G  H  L  E  I  H  E  R
2821  GAAGCGCAGAGCTTGCAACCGTCGCTGTGCAGTATGTGGTCACTTGGAAATCCACGAGCG
952   L  K  N  V  T  I  E  N  E  K  L  L  I  M  L  G  C  I  Y  R
2881  TCTGAAGAATGTGACGATTGAAAATGAAAAGCTGTTGATTATGTTGGGCTGTATTTATCG
972   G  E  F  T  L  G  Q  A  Q  L  F  M  A  R  E  S  K  T  Y  I
2941  CGGAGAATTTACTCTAGGACAGGCACAGCTTTTCATGGCGCGTGAGAGCAAAACGTACAT
992   C  R  L  H  F  L  E  T  L  D  E  I  Y  Q  M  L  R  L  K  S
3001  TTGCCGTCTCCATTTCCTGGAGACCCTCGACGAGATCTACCAGATGCTACGTCTGAAATC
1012  A  D  D  I  L  I  C  P  L  D  L  I  Q  N  A  L  I  T  V  S
3061  TGCCGATGATATTCTGATTTGCCCATTGGACTTGATTCAGAACGCATTGATCACTGTTTC
1032  A  L  R  P  H  I  I  A  S  Q  L  R  K  I  L  H  D  F  A  E
3121  CGCCCTCCGTCCACACATTATTGCTTCACAACTTCGAAAGATTCTTCACGATTTTGCCGA
1052  R  N  N  H  L  R  E  T  P  A  E  L  K  K  L  G  Q  Q  Y  F
3181  AAGAAATAATCATCTTCGTGAAACGCCAGCGGAATTGAAGAAACTTGGACAACAATACTT
1072  D  Y  R  E  P  E  P  E  P  E  R  N  D  V  D  E  Q  E  I  I
3241  TGACTACCGCGAGCCCGAACCTGAGCCAGAGAGAAACGATGTGGATGAGCAAGAAATCAT
1092  P  K  L  F  R  Q  P  R  K  Q  V  L  E  A  D  Q  H  D  G  T
3301  ACCGAAACTCTTTCGCCAGCCACGCAAGCAAGTTCTGGAAGCTGATCAGCATGACGGAAC
1112  V  K  V  I  E  Q  E  D  F  K  L  P  T  V  K  P  S  E  N  E
3361  TGTCAAGGTTATAGAGCAGGAAGATTTCAAGCTCCCAACTGTCAAACCGTCTGAAAATGA
1132  E  C  D  N  P  G  V  C  C  F  C  S  K  R  G  D  R  G  G  M
3421  GGAATGCGACAATCCGGGCGTTTGCTGCTTCTGTTCGAAACGTGGCGACCGAGGTGGAAT
1152  L  R  V  P  R  S  E  E  R  L  A  R  W  V  D  K  L  G  P  E
3481  GCTTCGTGTCCCGAGAAGTGAGGAACGTTTGGCTAGATGGGTGGATAAGCTTGGACCAGA
1172  F  E  A  R  L  H  T  N  T  E  N  L  I  C  R  S  H  F  P  D
3541  ATTTGAAGCCCGACTTCACACCAATACGGAAAACCTAATTTGCCGAAGCCATTTCCCAGA
1192  A  A  F  S  S  R  G  R  L  L  K  G  M  I  P  D  A  A  P  E
3601  TGCAGCGTTCAGCAGTCGTGGTAGACTTTTGAAAGGAATGATTCCAGATGCTGCTCCAGA
1212  K  V  E  T  T  Y  I  I  Q  G  N  N  F  L  K  L  K  E  R  K
3661  AAAAGTTGAGACCACGTATATCATTCAAGGGAACAACTTCCTCAAACTCAAAGAGCGCAA
1232  S  G  T  D  K  N  S  A  I  D  L  A  N  M  L  N  P  D  G  V
3721  GTCTGGAACTGATAAGAATTCAGCCATTGATTTGGCGAATATGTTAAACCCCGACGGTGT
1252  E  Y  T  Q  E  E  E  E  E  E  E  Y  E  E  M  S  R  S  P  T
3781  TGAATATACTCAGGAAGAAGAAGAAGAAGAGGAATATGAAGAAATGTCAAGATCCCCAAC
1272  E  E  T  S  D  D  E  P  S  Q  A  A  V  Y  N  N  A  P  V  I
3841  GGAAGAGACTTCAGACGACGAGCCCAGTCAAGCAGCAGTTTACAACAATGCTCCAGTGAT
1292  K  R  T  Y  R  K  R  E  L  S  N  E  D  G  P  L  N  L  V  T
3901  AAAACGTACCTATAGAAAGCGGGAGTTGTCAAACGAGGATGGACCTCTTAACTTGGTAAC
1312  P  P  A  H  T  P  N  P  R  G  R  P  R  K  Y  P  K  N  S  V
3961  ACCACCTGCTCATACTCCGAACCCAAGAGGGCGCCCAAGGAAGTATCCCAAGAATAGTGT
1332  T  P  E  A  E  K  S  L  T  D  Y  D  Y  N  P  G  T  S  Q  R
4021  AACACCAGAAGCTGAAAAAAGCCTCACCGACTACGACTACAATCCGGGAACATCTCAACG
1352  R  A  L  K  K  G  Y  V  Q  L  E  D  G  E  I  V  G  E  D  C
4081  ACGTGCACTTAAGAAAGGTTACGTACAACTCGAAGACGGAGAAATTGTCGGCGAAGATTG
1372  E  Y  V  P  E  K  T  P  S  G  R  L  I  R  Q  A  V  A  R  R
4141  CGAATACGTGCCTGAAAAAACACCAAGTGGAAGATTGATTCGACAAGCTGTTGCTAGACG
1392  S  F  A  F  A  D  E  E  E  E  E  E  E  Y  E  E  S  P  I  V
4201  AAGCTTTGCTTTTGCTGATGAGGAAGAGGAGGAGGAAGAGTATGAAGAATCTCCAATTGT
1412  K  K  P  K  I  A  G  R  P  V  G  R  P  R  K  D  A  N  K  L
4261  TAAAAAGCCCAAGATTGCTGGAAGACCAGTCGGACGACCGCGCAAAGATGCTAATAAGCT
1432  P  T  P  T  P  P  S  N  E  *
4321  GCCAACCCCCACACCACCCAGCAACGAATAAGTGGAAATATTTTCATCCACCGTTCCTGA
4381  TTGTTGTTTTTATATATAATTTTTTCTCCATTGATGTTTTTGATGATTAAGTCATACGCA
4441  TAGTTTTACCCTGTTACTTTGATTGCCCTTAAAATTACCACTTTTATTTTACCTATTTAT
4501  TTTAATCATCATTCATCTGAAACCTCACAATGATGAGATGATCTCATCATTTTTATATTT
4561  TGTAGTTTTATATCAGTTTTATAAATGTTCACCGAAAAAAAAAAAAAAAAAAAAAAAAAA
4621  AAA
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C    1 TTTGATGCCA ACTGGTTTTT TTCTAATTCT TTCCAAATGG TTTCACATCA
    51 CCAAATGACA ATTTTCCTTT ACTTTCCTAT CTTGCTTATC ATACACTATC
   101 ACTTATTCAG
```

FIGURE 4.—*continued*

DNA sequences encoding the 4.6-kb transcript are located within the 7.2-kb *Eag*I-*Sal*I fragment sufficient for rescue of *lin-15* B function, while DNA sequences encoding the 2.3-kb transcript, except for the first exon and part of the first intron, are present within the 3.8-kb *Sal*I-*Nru*I fragment required only for rescue of *lin-15* A activity. In addition, the *lin-15* A mutant *n767* contains a 300-bp deletion within the 3-kb *Eco*RI fragment that contains part of the coding region for the 2.3-kb transcript (see below). These observations suggest that the 2.3-kb transcript encodes *lin-15* A gene activity and that the 4.6-kb transcript encodes *lin-15* B activity.

To confirm that these two transcripts encode the two *lin-15* proteins, we introduced frameshift mutations into the putative *lin-15* coding regions within the *Spe*I-*Nru*I genomic DNA clone and then tested these modified clones for the rescue of *lin-15* A and B function by germline transformation (see MATERIALS AND METHODS). We found that a *Spe*I-*Nru*I genomic clone containing a frameshift mutation within the predicted coding region for the downstream 2.3-kb transcript rescued *lin-15* B but not *lin-15* A function (Figure 2B). A clone altered to restore the appropriate reading frame rescued *lin-15* A function in 6/6 transformed F_2 populations, indicating that the introduced frameshift mutation within the coding region of the 2.3-kb transcript caused the loss of *lin-15* A rescuing activity. By contrast, a *Spe*I-*Nru*I genomic clone possessing a frameshift mutation within the coding region for the 4.6-kb transcript rescued *lin-15* A but not *lin-15* B activity. A clone modified to restore the proper reading frame rescued *lin-15* B function in 16/16 transformed F_2 populations, demonstrating that the frameshift mutation within the coding region of the 4.6-kb transcript eliminated the *lin-15* B rescuing activity. Together, these results establish that the 2.3-kb transcript encodes *lin-15* A activity and that the 4.6-kb transcript encodes *lin-15* B activity.

Based on the DNA sequences, the 2.3-kb *lin-15* A cDNA encodes a 719-amino acid protein and the 4.6-kb *lin-15* B cDNA encodes a 1,440-amino acid protein (Figure 4, A and B). The two *lin-15* proteins are not obviously similar in sequence to each other, and a search of the GenBank, PIR and SWISS-PROT databases using BLAST (ALTSCHUL and LIPMAN 1990) failed to identify any protein highly similar to either. Thus, the *lin-15* proteins define new types of molecules involved in intercellular signaling.

***lin-15* Muv mutations are rearrangements:** To investigate the molecular basis of the different classes of *lin-15* mutations, we analyzed genomic DNA prepared from *lin-15* Muv mutants as well as from *lin-15* mutants defective for either class A or class B activity (see MATERIALS AND METHODS). All eight *lin-15* Muv mutants analyzed, including four carrying EMS-induced alleles, had chromosomal rearrangements in the *lin-15* coding regions: seven mutants (*e1763*, *n309*, *n765*ts, *n1139*, *n1296*, *n1331*, *n1344*, *n2141*) contained deletions and one, *n1296*, had a deletion and a 1.6-kb insertion (Figure 5). The lengths of the deletions varied from about 200 bp in the *n765* mutant to at least 15 kb in the *e1763*, *n309* and *n1344* mutants. The *e1763*, *n309*, *n1139*, *n1344* and *n2141* Muv mutants had deletions that affected all or most of the genomic region encoding the two *lin-15* transcripts.

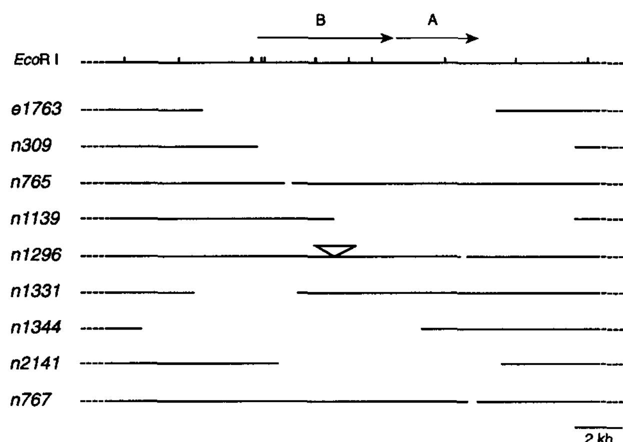

FIGURE 5.—*lin-15* Muv mutants contain chromosomal rearrangements that affect both the *lin-15* A and B coding regions. Based on the analysis of *Eco*RI restriction fragment length polymorphisms, the approximate position of the deletion and insertion is shown for each Muv mutant and for the *lin-15* A mutant *n767*. For example, the *n765*ts mutant contains a 200-bp deletion within the 2.1-kb *Eco*RI fragment; a 200-bp gap is shown within the center of the region, as the precise location within the 2.1-kb fragment was not determined. The genomic region not analyzed is depicted as a dotted line.

The 4-kb deletion present in the *n1331* mutant removed part of the *lin-15* B coding region and sequences 5′ of the *lin-15* B gene. Unless a second, undetected mutation exists in the *n1331* strain, this alteration caused the loss of *lin-15* A and *lin-15* B function. The requirement for the region deleted in the *n1331* strain for the expression of both *lin-15* activities is consistent with our germline transformation results, which indicate that this region is essential for the rescue of both *lin-15* A and B function. The *n1296* mutant had a 1.6-kb insertion within a region encoding the *lin-15* B gene and a 200-bp deletion in the 3-kb *Eco*RI fragment that contains part of the *lin-15* A gene. *n1296* was identified as a spontaneous Muv mutant in the mutator strain TR679 (KIM and HORVITZ 1990). The 1.6-kb insertion might be either a *Tc1* or a *Tc4* transposon, both of which are about 1.6 kb in size (ROSENZWEIG *et al.* 1983; YUAN *et al.* 1991).

The *n765*ts mutant has a temperature-sensitive defect in *lin-15* A activity and lacks *lin-15* B activity, resulting in a temperature-sensitive Muv phenotype (FERGUSON and HORVITZ 1989). The 200-bp deletion in the *n765*ts mutant is within the genomic region encoding the *lin-15* B transcript and likely disrupts *lin-15* B function; it is unclear whether this deletion or another lesion, perhaps a point mutation within the *lin-15* A gene, confers the temperature-sensitive defect in *lin-15* A function. This deletion might lead to a temperature-dependent loss of *lin-15* A function if it caused the elimination of the *lin-15* B protein and if the *lin-15* A protein were unstable at higher temperatures in the absence of *lin-15* B protein. If so, the three *lin-15* class B mutations (*n374*, *n743*, *n744*) must not eliminate the *lin-15* B protein, as they do not cause a temperature-dependent loss of *lin-15* A function.

In short, the alterations within all *lin-15* Muv mutants examined, except possibly *n765*ts, affected regions defined by germline rescue experiments to be required for both *lin-15* A and B activity. No obvious chromosomal alterations were found in two of three EMS-induced *lin-15* class A mutants (*n433*, *n749*) or in three EMS-induced *lin-15* class B mutants (*n374*, *n743*, *n744*) (data not shown). The *lin-15* class A mutant *n767* had a 300-bp deletion within the 3-kb *Eco*RI fragment that encodes part of the *lin-15* A gene (Figure 5).

DISCUSSION

lin-15 is a complex locus that encodes two separate genetic activities involved in the negative regulation of vulval induction (FERGUSON and HORVITZ 1989). We identified an 11-kb genomic fragment that complemented the loss of both of these activities when maintained as an extrachromosomal array following germline transformation. The analysis of cDNAs and genomic DNA sequences indicated that two distinct, non-overlapping and similarly oriented transcripts of lengths 2.3 and 4.6 kb encode the two *lin-15* gene activities. The 4.6-kb transcript encodes *lin-15* B function and is processed by the addition of the SL1 *trans*-spliced leader. The 2.3-kb transcript encodes *lin-15* A function, is processed by the addition of the SL1 and SL2 *trans*-spliced leaders and is located 110 bp downstream of the polyadenylation site of the 4.6-kb transcript.

The mRNAs of six *C. elegans* genes are *trans*-spliced to SL2, and each of these genes is located between 96 and 294 bp downstream of a similarly oriented gene (SPIETH *et al.* 1993). In particular, SPIETH *et al.* reported the unpublished conclusions of L. HUANG and P. STERNBERG that the *lin-15* A gene is located downstream of the *lin-15* B gene and that the *lin-15* A transcript is *trans*-spliced to SL2. HUANG *et al.* (1994) independently cloned and characterized the *lin-15* locus and obtained results similar to ours. SPIETH *et al.* proposed that those gene clusters are transcribed as a single polycistronic mRNA precursor and that the mature transcript for the downstream gene is formed by cleavage at the polyadenylation site and by *trans*-splicing to SL2.

The results of our germline rescue experiments and the site of the deletion in the *lin-15* Muv mutant *n1331* are consistent with the cotranscription model proposed by SPIETH *et al.* for the expression of the two *lin-15* transcripts. For example, if the *lin-15* A and *lin-15* B genes were transcribed as a single mRNA precursor, the expression of the downstream *lin-15* A gene would require expression of the upstream *lin-15* B gene. We found that rescue of *lin-15* A gene activity required the 7-kb region upstream of the *lin-15* A coding region that alone rescued only *lin-15* B function and contained the *lin-15* B gene. A frameshift mutation within the *lin-15* B gene eliminated the rescue of only *lin-15* B gene function and not the rescue of *lin-15* A gene activity, suggesting that

the expression of the downstream *lin-15* A gene required the transcription but not the translation of the upstream *lin-15* B gene. The deletion present in the Muv mutant *n1331* removed part of the *lin-15* B coding region and sequences 5′ of the *lin-15* B gene. Unless another lesion exists in the *n1331* strain, this deletion confers the loss of both *lin-15* A and B activities, indicating that this region is required for the expression of both *lin-15* functions. Together, these results support the cotranscription model proposed by SPIETH *et al.* (1993) but do not exclude the possibility that the promoter for the *lin-15* A gene is present within the *lin-15* B gene.

Using the technique of RT-PCR, we recovered cDNAs for the *lin-15* A gene using primers for sequences of either SL1 or SL2. These results indicate that the downstream *lin-15* A transcript is processed by *trans*-splicing using SL1 and SL2, although the relative abundances cannot be estimated from our data. Similar results were reported for transcripts of the downstream *gpd-3* gene and were suggested to reflect a variability in the selectivity of the *trans*-splicing process for polycistronic precursors (SPIETH *et al.* 1993). The *lin-15* A transcript might also be alternatively *trans*-spliced in this way. On the other hand, the SL1-containing transcripts might originate from another promoter located within the *lin-15* B gene and not be derived from a polycistronic precursor.

The class A and class B synthetic multivulva genes define redundant genetic pathways involved in the negative regulation of vulval induction (FERGUSON and HORVITZ 1989). Although multiple class B genes are located on chromosome *III*, the class A and class B genes are not clustered, except for the *lin-15* locus. Our analysis provides a molecular basis for understanding the *lin-15* complex locus but fails to explain why the *lin-15* A and B genes are clustered and apparently cotranscribed. Perhaps the cotranscription of the *lin-15* A and B genes ensures that both genes are expressed within the same cell and at similar levels.

The *lin-15* null phenotype is Muv: The phenotype caused by *lin-15* mutations in *trans* to a deficiency of the locus suggests that the known *lin-15* Muv alleles may not completely eliminate *lin-15* gene function (FERGUSON and HORVITZ 1985). Specifically, the phenotype of *lin-15(n765*ts*)/mnDf4* animals at 25° is more severe than that of *lin-15(n765*ts*)* homozygotes at 25°. *lin-15(n765*ts*)* animals raised at 25° have a highly penetrant Muv phenotype similar to that of *lin-15(n309)* and other strong Muv alleles; most *n765/mnDf4* animals raised at 25° grow to the size of L3 larvae and are sterile, which is a phenotype only occasionally exhibited by *lin-15(n765*ts*)* animals grown at 25°. At 20°, the deficiency does not enhance the *lin-15* phenotype. A null mutation in *trans* to a deficiency uncovering that locus is likely to cause the same phenotype as a homozygous null mutation, which suggested that the strong *lin-15* alleles may not be null. However, deficiencies often remove large chromosomal regions containing many genes, so haplo-insufficiency could cause strains heterozygous for a particular mutation and a deficiency to have a more severe phenotype than homozygous mutants even if the mutation is null. Our molecular analysis revealed that some Muv mutations delete the complete *lin-15* coding region and thus cannot possess any *lin-15* function. For example, the *e1763* mutation deletes the entire *lin-15* genomic region, while the *n309*, *n1139*, *n1344* and *n2141* mutations remove most of this region. Thus, *e1763* and most likely the other strong *lin-15* mutations are true null alleles.

Although the class A and class B pathways are redundant for the formation of the vulva, some mutations in the class B pathway affect viability and fertility in the presence of a functional class A pathway, indicating that some genes in the class B pathway may have unique functions outside of vulval formation (FERGUSON and HORVITZ 1989). In particular, three class B mutations *lin-9(n112)*, *lin-35(n745)* and *lin-37(n758)* cause a reduction in fertility and body size at 25°, and a double mutant carrying *lin-9(n112)* and *lin-35(n745)* is sterile. By contrast, the complete elimination of *lin-15* A and B activity causes few defects in fertility and body size at 25° and fewer defects at 20°. Although *lin-15* B activity is required in the class B pathway for vulval formation, *lin-15* B function is largely dispensable in the class B pathway for other processes.

Possible roles for the *lin-15* A and *lin-15* B proteins: The expression of vulval cell fates depends upon the activation of a tyrosine kinase/*ras* signaling cascade that during wild-type development is triggered by a signal from the gonadal anchor cell (for review, see HORVITZ and STERNBERG 1991) (Figures 1B and 6A). The removal of *lin-15* function causes the activation of this signaling pathway even in the absence of the anchor cell inductive signal. A signal involving *lin-15* from the surrounding hypoderm might normally prevent the activation of this signaling pathway, since mosaic analysis suggests that *lin-15* functions in the hypoderm (HERMAN and HEDGECOCK 1990) (Figures 1D and 6A). As loss-of-function mutations in the receptor tyrosine kinase *let-23* gene, the SH3-SH2-SH3 adaptor protein *sem-5* gene, *let-341*, *let-60 ras* and *lin-45 raf* suppress the Muv phenotype induced by *lin-15* mutations, *lin-15* acts upstream of these five genes in the genetic pathway for vulval induction (FERGUSON *et al.* 1987; BEITEL *et al.* 1990; HAN *et al.* 1990; CLARK *et al.* 1992, 1993; HAN *et al.* 1993). Preliminary data suggest that *let-23* and *let-60* are expressed in P3.p–P8.p (M. KOGA and Y. OHSHIMA; J. DENT, L. AVERY and M. HAN, personal communications), suggesting that these genes as well as *sem-5*, *let-341* and *lin-45* act in these cells to induce the differentiation of vulval cell types. Thus, the action of *lin-15* within

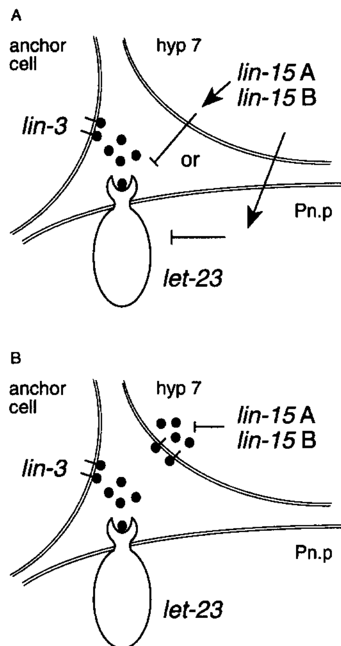

FIGURE 6.—Two models for the negative regulation of the tyrosine kinase/*ras* signaling cascade by *lin-15*. (A) A signal from the hypoderm involving the two *lin-15* proteins may inhibit the receptor tyrosine kinase *let-23* by direct interactions. The products of other class A and class B synthetic multvulva genes might mediate these intercellular interactions because the *lin-15* A and B proteins lack hydrophobic signal sequences. Alternatively, the hypoderm might signal to factors within the Pn.p cells that block the action of *let-23*. These factors may be encoded by other synthetic multivulva genes. (B) The action of the *lin-15* proteins may prevent the hypoderm from producing or releasing molecules related to the *lin-3* product that would inappropriately activate the vulval inductive pathway.

the hypoderm may negatively regulate the *let-23* receptor tyrosine kinase signaling pathway within the cells P3.p–P8.p.

The sequences of the 719- and 1,440-amino acid *lin-15* proteins do not suggest how *lin-15* A and *lin-15* B might function. One possibility is that a hypodermal signal involving *lin-15* directly inhibits the *let-23* receptor tyrosine kinase (Figure 6A). Since neither of the *lin-15* proteins contains a hydrophobic signal sequence, this hypothesis would suggest that products of other synthetic Muv genes of both the A and B classes directly mediate these intercellular interactions. The inhibition of *let-23* activity could be either directly from the hypoderm or indirectly from within the cells P3.p–P8.p, in which case we expect some synthetic Muv genes to act within these cells. The inhibitory signal from the hypoderm might be similar to the signal involving the Drosophila gene *argos* (FREEMAN *et al.* 1992). Mutations in the *argos* gene cause the overproduction of photoreceptors within the developing fly eye analogously to the proliferation of vulval cells that occurs in *lin-15* mutants. *argos* functions non-cell-autonomously, and, based upon its DNA sequence, is predicted to encode a secreted protein.

Alternatively, the *lin-15* A and B proteins might act in parallel to prevent the hypoderm from expressing a signal that inappropriately causes vulval induction. For example, in the absence of *lin-15* function, the hypoderm might release a factor similar to the *lin-3* protein, which is related to TGF-α (Figure 6B).

The *lin-15* A and B genes encode unfamiliar molecules involved in intercellular signaling. Receptor tyrosine kinase/*ras* signaling pathways highly conserved with the *C. elegans* vulval induction pathway have been identified in both Drosophila and mammals, suggesting that molecules similar to the *lin-15* A and B proteins exist in these organisms. The further study of *lin-15* and other synthetic Muv genes may lead to a greater understanding of *C. elegans* vulval induction as well as of the regulation of other tyrosine kinase/*ras* signaling pathways.

We are grateful to STUART KIM for providing the TR679-derived *lin-15* mutants, to MIKE NONET and BARBARA MEYER for sharing unpublished results regarding the location of the *sdc-1* gene and to TOM BLUMENTHAL for suggestions concerning possible *trans*-splicing of the *lin-15* transcripts. We thank CORI BARGMANN, ANDREW CHISHOLM, ERIK JORGENSEN, KERRY KORNFELD, VILLU MARICQ and PIALI SENGUPTA for critically reading the manuscript. This work was supported by U.S. Public Health Service grants GM24663 and GM24943 to H.R.H. and by the Howard Hughes Medical Institute. S.G.C. was a Research Associate of the Howard Hughes Medical Institute during part of this work. H.R.H. is an Investigator of the Howard Hughes Medical Institute. The Genbank accession numbers for the *lin-15* A cDNA, *lin-15* B cDNA and 11.7-kb *lin-15* genomic DNA fragment are U10411, U10412 and U10413, respectively.

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Communicating editor: R. K. Herman