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The *Caenorhabditis elegans* Ldb/NLI/Clim Orthologue *ldb-1* Is Required for Neuronal Function

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LIM homeodomain (LIM-HD) and nuclear LIM-only proteins play important roles in a variety of developmental processes in animals. In some cases their activities are modulated by a nuclear LIM binding protein family called Ldb/NLI/Clim. Here we characterize the Ldb/NLI/Clim orthologue *ldb-1* of the nematode *Caenorhabditis elegans*. Two alternatively spliced variants exist, which differ in their amino-termini. The *ldb-1* orthologue of *Caenorhabditis briggsae* has the same structure as that of *C. elegans* and is highly conserved throughout the open reading frame, while conservation to fly and vertebrate proteins is restricted to specific domains: the dimerization domain, the nuclear localization sequence, and the LIM interaction domain. *C. elegans ldb-1* is expressed in neurogenic tissues in embryos, in all neurons in larval and adult stages, and in vulval cells, gonadal sheath cells, and some body muscle cells. *C. elegans* LDB-1 is able to specifically bind LIM domains in yeast two-hybrid assays. RNA inactivation studies suggest that *C. elegans ldb-1* is not required for the differentiation of neurons that express the respective LIM-HD genes or for LIM-HD gene autoregulation. In contrast, *ldb-1* is necessary for several neuronal functions mediated by LIM-HD proteins, including the transcriptional activation of *mec-2*, the mechanosensory neuron-specific stomatin. © 2000 Academic Press

***Key Words:* *Caenorhabditis elegans; Caenorhabditis briggsae; ldb-1;* LDB/NLI/CLIM; neuronal expression; LIM interaction; neuronal function; RNAi; *ceh-14; mec-3; mec-2*.**

INTRODUCTION

The LIM homeodomain (LIM-HD) transcription factors contain two amino-terminal LIM domains and a carboxy-terminal, DNA-binding homeodomain. In contrast, the LIM-only proteins (LMO) contain only LIM domains with no additional domains (for review see Dawid *et al.*, 1995, 1998; Gill, 1995). In *Caenorhabditis elegans*, the expression and function of several LIM-HD genes has been examined. *mec-3*, the first described LIM-HD gene, is important for touch neuron function (Way and Chalfie, 1988). *ceh-14*, *ttx-3*, *lin-11*, *lim-4*, and *lim-6* function in a variety of different neuronal, gonadal, and vulval cells (Hobert *et al.*, 1997, 1998, 1999; Sagasti *et al.*, 1999; Cassata *et al.*, 2000). All of these genes have been shown to be important for the differentiation and function of the cells expressing them.

Two different functions have been attributed to the LIM domains of LIM-HD genes. First, the LIM domains in some LIM-HD proteins have been proposed to be intramolecular repressors. The LIM domain in Isl-1 inhibits binding of the homeodomain to its DNA target *in vitro*. This inhibition was abolished by the truncation of the LIM domains (Sanchez-Garcia *et al.*, 1993). Similar conclusions were drawn from *in vivo* experiments showing an enhancement of Xlim-1-mediated induction of neural and muscular tissue in *Xenopus laevis* embryos after injections of LIM-domain mutant versions of Xlim-1 (Taira *et al.*, 1994). Second, more recent experiments suggest that the LIM domain is required for the proper function of LIM-HD proteins. Experiments in *Drosophila melanogaster* showed that deletions of the LIM domains in the gene *apterous* caused loss-of-function phenotypes, implying that the LIM domains are necessary for *apter-*

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ous gene function. Furthermore, the LIM domains of *apterous* confer specificity, as they cannot be exchanged by the LIM domains of *lim3* (O'Keefe *et al.*, 1998).

These experiments together with the evidence that the LIM domain is a modular protein-binding interface (Schmeichel and Beckerle, 1994; Arber and Caroni, 1996) led to the search for possible LIM interactors which might modulate the LIM-HD function *in vivo*. Two types of LIM interacting factors were found. The first one is represented by specific interactors such as the bHLH transcription factor E47, which binds the LIM domains of Lmx-1 (Johnson *et al.*, 1997), and the POU homeodomain factor Pit-1, which binds the LIM domains of Lhx-3 (Bach *et al.*, 1995). This specificity can be very restricted, since E47 binds to Lmx-1 but not to Isl-1 (Johnson *et al.*, 1997). The second type is represented by NLI/Ldb1/CLIM2, which are more general factors that display high-affinity interactions. These factors bind to LIM domains of LIM-HD proteins but also to nuclear LMO proteins (Agulnick *et al.*, 1996; Bach *et al.*, 1997; Jurata *et al.*, 1996). Additional studies revealed that NLI/Ldb1/CLIM2 can form homodimers and that the protein contains two distinct domains, a LIM binding domain and a homodimerization domain. The homodimerization domain is not required for the binding to LIM domain. Thus, NLI/Ldb1/CLIM2 was hypothesized to regulate transcriptional activity of LIM-HD proteins by determining specific partner interactions (Jurata and Gill, 1997). This hypothesis was confirmed by showing that Chip, the fly orthologue of NLI/Ldb1/CLIM2, physically interacts with Apterous to form a functional complex during *Drosophila* wing development and that the composition and activity of this complex might be modulated by the temporarily dynamic expression of dLMO (Milan and Cohen, 1999; van Meyel *et al.*, 1999).

NLI/Ldb1/CLIM2 is ubiquitously expressed in developing *Xenopus*, mouse, and zebrafish embryos. In mouse and zebrafish the expression in the developing central nervous system is stronger than in other tissues (Agulnick *et al.*, 1996; Jurata *et al.*, 1996; Toyama *et al.*, 1998). In *Drosophila*, NLI/Ldb1/CLIM2 is expressed ubiquitously during embryogenesis and larval development and is required for accurate expression of segmentation genes, wing development, and axon guidance (Morcillo *et al.*, 1997; van Meyel *et al.*, 2000). While axon guidance and wing development defects can be clearly attributed to the failure of NLI/Ldb1/CLIM2 to bind Apterous via its LIM domain, the segmentation problems cannot be attributed to a LIM-HD gene, since no LIM-HD gene is known to be required for segmentation. Therefore, the *Drosophila* NLI/Ldb1/CLIM2 gene Chip may interact with factors other than LIM-HD proteins and thereby cause a wider range of phenotypes than that caused by LIM-HD genes (reviewed in Dorsett, 1999).

In this study we characterize the expression and function of *ldb-1*, the only *C. elegans* orthologue of NLI/Ldb1/CLIM2. *ldb-1* is expressed in almost all tissues during early embryogenesis. The expression becomes restricted to neuronal, gonadal, vulval, and some somatic muscle tissue later in development. RNAi experiments suggest that *ldb-1* may be required for the function of postmitotic neurons rather than embryonic development.

METHODS

Sequence Analysis

C. elegans database searches were performed at the Sanger Center in Cambridge and the Genome Sequencing Center in St. Louis (http://www.sanger.ac.uk/Projects/C_elegans/, http://genome.wustl.edu/gsc/) using their Web implementation of Blast (Altschul *et al.*, 1990). Genomic organization of the gene structures was visualized using the ACeDB database on a local workstation (Eeckman and Durbin, 1995). Sequence comparisons were performed with an interactive dot matrix program on the Macintosh (Bürglin, 1998). GenBank searches were performed using Netblast at NCBI (Altschul *et al.*, 1990). Phylogenetic analysis was carried out using ClustalX 1.6 (Thompson *et al.*, 1997) and NJPLOT by M. Gouy (in ClustalX).

Molecular Biology Methods and Cloning

Yeast two-hybrid constructs were made as follows. Two cDNAs for *ldb-1a* and *ldb-1b* were a kind gift from Dr. Y. Kohara (yk238d10 and yk391b9, respectively). They were cloned by PCR into the GAL4 activation domain vector pGAD424. The CEH-14 LIM domain region and point-mutated versions thereof were cloned by PCR into the vector pGBT9 (both vectors from Clontech). The *mec-3* cDNA was cloned into the LexA DNA-binding domain vector pBTM116 (Bartel *et al.*, 1993). Furthermore, we used the Xldb-1 cDNA clone in a GAL4 activation domain vector, which is described in Agulnick *et al.* (1996).

Expression constructs were made according to Cassata *et al.* (1998), without using the second PCR step mentioned therein, but by direct cloning instead. As template genomic DNA was used. The reporter construct for *ldb-1b*, *ldb-1b::gfp*, contains 7 kb of upstream sequence, the construct for *ldb-1a* contains 3.7 kb of intronic sequence. For all the *gfp* constructs the vector pPD95.67 was used.

All the sequences of the primers used for cloning or to introduce point mutations in the CEH-14 LIM domain are available on request.

Double-stranded RNA was synthesized from the clone yk391b9, which has a Bluescript backbone that allows synthesis of sense and antisense RNA from T3 and T7 promoters. Synthesis was performed using a transcription kit from Stratagene, according to the instructions of the manufacturer.

Yeast Two-Hybrid Assay

Yeast two-hybrid assays were performed according to the instructions of the manufacturer (Clontech) following the principles described by Fields and Song (1989). Liquid β-galactosidase assays were performed using the CPRG method according to instructions (Roche Molecular Biochemicals).

Worm Strains, Transgenic Worm Lines, and RNAi Experiments

For all the experiments wild-type *C. elegans* strain N2 was used. Transgenic lines were established following Mello *et al.* (1991).

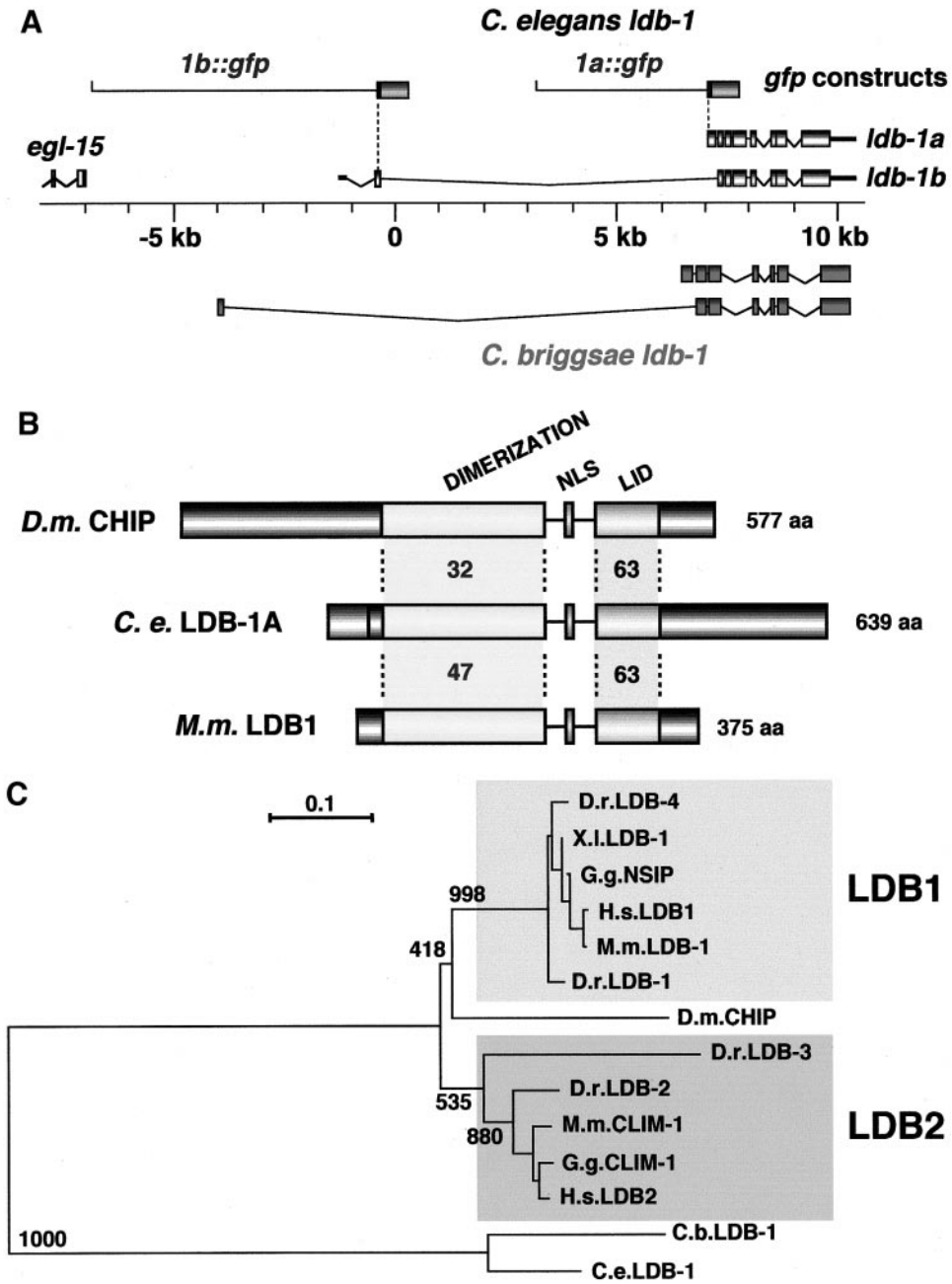
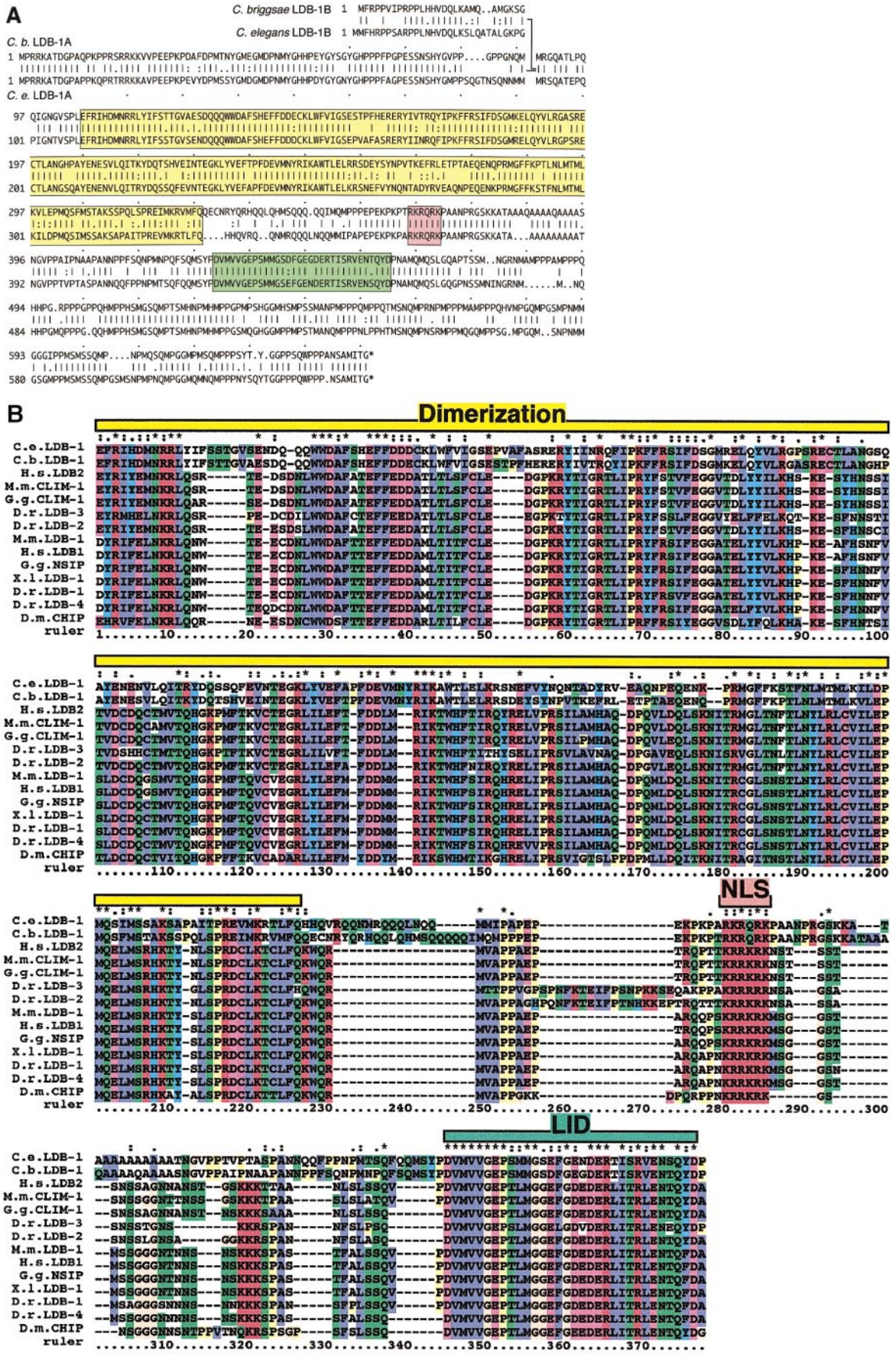


FIG. 1. *ldb-1* gene structure and sequence similarities. (A) Top shows the structure of the two *C. elegans ldb-1* isoforms, the bottom those of *C. briggsae*. *gfp* constructs are indicated. (B) Structures and percentages of identities between *D. melanogaster* (*D.m.* CHIP), *C. elegans* (*C.e.* LDB-1A), and mouse (*M.m.* LDB1) homologues. The dimerization domains and the positions of the NLS and the LIM interacting domain (LID) are indicated. (C) Phylogenetic tree constructed from the aligned dimerization, NLS, and LID domains of the respective genes. In vertebrates, two distinct LDB families that we term LDB1 and LDB2 can be recognized. Abbreviations: H.s., *Homo sapiens;* M.m., *Mus musculus;* X.l., *X. laevis;* D.r., *Danio rerio;* G.g., *Gallus gallus;* D.m., *D. melanogaster;* C.e., *C. elegans;* C.b., *C. briggsae.*

RNAi experiments were performed according to Fire *et al.* (1998). Ten animals were injected. The offspring of all 10 showed the phenotypes described under Results. The transgenic integrated line for *ttx-3::gfp mgIs18; him-5(el467)*was kindly provided by Dr. O. Hobert (Columbia, NY). The *mec-3::gfp* transgenic strain was created using the construct pPD118.17 kindly provided by Dr. A.


A
C. briggsae LDB-1B
C. elegans LDB-1B
C. b. LDB-1A
C. e. LDB-1A
B
Dimerization
NLS
LID
C.e.LDB-1
C.b.LDB-1
H.s.LDB2
M.m.CLIM-1
G.g.CLIM-1
D.r.LDB-3
D.r.LDB-2
M.m.LDB-1
H.s.LDB1
G.g.NSIP
X.l.LDB-1
D.r.LDB-1
D.r.LDB-4
D.m.CHIP
ruler

Fire. The transgenic integrated line for *mec-2::gfp* was kindly provided by Dr. M. Chalfie (Columbia, NY).

RESULTS

ldb-1 Gene Structure

Searches of the *C. elegans* genome sequence revealed a single locus on chromosome X (cosmid F58A3) with significant similarity to *Xenopus ldb-1*. This *C. elegans* locus, termed *ldb-1,* is defined not only by sequence similarity, but also by several ESTs showing that there are two isoforms, which differ in their amino-terminal sequences (Fig. 1A). The first two exons of *ldb-1b,* defined by EST yk89b5, are separated from the main part of the gene by a large intron of 7.6 kb. The first exon of *ldb-1a* (exon 3, defined by its longest EST yk61f12) is separated by a small intron from the remainder of the gene. The longest predicted open reading frame (ORF) of *ldb-1a* encodes 639 amino acids, while that of *ldb-1b* encodes 579 amino acids (Fig. 2A). The presumptive start methionine of *ldb-1b* is encoded in the second exon, as the junction of exon 1 and exon 2 produces an upstream in-frame stop codon. Two shorter ESTs have been sequenced and confirm the gene structure through the remainder of the coding sequence [yk238d10 (U80220) and yk391b9 (U80221); O. Hobert, Y. Kohara, and G. Ruvkun, pers. comm.].

Blast searches also revealed the orthologue of *ldb-1* in *C. briggsae* on clone G36H10 (Genome Sequencing Center, St. Louis, pers. comm.). The analysis of the *C. briggsae ldb-1* locus shows the same type of 5′ alternative splicing as *C. elegans ldb-1* (Fig. 1A) and the sequence similarity begins at the predicted methionine residues (Fig. 2A). The two gene structures are similar, with the exception that the first exon common to both splice variants in *C. briggsae* is split into two exons in *C. elegans.* The *C. briggsae* ORF LDB-1A (647 aa long) is 78% identical to *C. elegans* LDB-1A over the whole ORF, while LDB-1B (588 aa long) is 77% identical (Fig. 2A).

The two *Caenorhabditis* sequences help define conserved elements in the LDB genes. Sequence alignments over the whole length of nematode, fly, and vertebrate sequences show a single central conserved region that can be subdivided into three functional domains: the amino-terminal region involved in dimerization, a nuclear localization sequence (NLS), and the LIM interaction domain (LID) (Figs. 1B and 2B). The sequence identity is about 32–47% in the dimerization domain between worm and fly/vertebrate proteins and 63% in the LID (Fig. 1B). Phylogenetic analysis using the central conserved domains reveals that the nematode sequences are the most divergent (Fig. 1C).

C. elegans ldb-1::gfp Constructs Are Expressed in the Nervous System, in the Reproductive System, and in Muscles

The expression pattern of construct *ldb-1b::gfp* (Fig. 1A) is shown in Fig. 3. *ldb-1b::gfp* is expressed throughout the nervous system in larvae and in adults (Figs. 3A, 3F, 3H, 3I, and 3J). Moreover it is expressed in developing and adult vulval cells (Figs. 3M, 3O, and 3Q) and in adjacent developing and adult uterine cells (Figs. 3M, 3O, and 3S). In adult male animals, all the neurons of the tail region also express *ldb-1b::gfp.* Expression analysis of the construct *ldb-1a::gfp* (Fig. 1A) revealed a temporal and spatial overlapping expression pattern with *ldb-1b::gfp.* Additional expression is seen in all gonadal sheath cells and adult dorsal body muscle cells and vulval muscles (data not shown; Fig. 3Q).

Embryonic expression of *ldb-1* was detected first in 200- to 260-min-old embryos, in which all but the hyp-7 cells express the *gfp* construct (Fig. 3B). This quite ubiquitous expression becomes more restricted to the anterior, ventral, and posterior part in the comma stage embryo, in which all the cells but hyp-7 and the gut express the construct (Fig. 3D).

LDB-1 Interacts with C. elegans LIM Domains in Yeast Two-Hybrid Assays

To test whether *C. elegans* LDB-1 can interact with LIM domains like its vertebrate orthologues, we performed yeast two-hybrid assays. Both isoforms of LDB-1 can interact with the LIM domains of the *C. elegans* homeodomain protein CEH-14 (Fig. 4). Only the LIM domains of CEH-14 were used since the entire protein autoactivates transcription (data not shown). To demonstrate that the interaction of the LIM domains with LDB is specific, we introduced point mutations into the codons for the first zinc finger of either LIM domain or both LIM domains simultaneously. For this purpose, we exchanged cysteine residues with serine residues to prevent the binding of zinc ions and thereby disrupting the secondary structure (Taira *et al.*, 1994). These point mutations were sufficient to abolish the interaction (Figs. 4A and 4B).

To examine whether this interaction is evolutionarily

FIG. 2. Sequence alignment of the LDB proteins. (A) Sequence comparison of *C. elegans* and *C. briggsae* LDB-1A and LDB-1B. Lines indicate the point where the two different amino-termini join to the common part. The conserved domains are boxed in color. (B) Alignment of the central region of the different Ldb/NLI/Clim proteins. The three conserved regions are indicated by colored bars: the dimerization domain, the nuclear localization signal (NLS), and the LIM interacting domain (LID). Color coding scheme of the residues according to the default settings of ClustalX.

conserved, we also tested *X. laevis* LDB-1 and found that it can interact with the CEH-14 LIM domain with a similar specificity (Fig. 4B).

To quantitate the differences in activation between the two isoforms of LDB-1, we performed β-galactosidase activity assays of yeast extracts. However, we did not quantify the CEH-14 LDB-1 interaction, since it does not reflect the interaction with the entire CEH-14 protein. Instead, we decided to test the interaction with another full-length LIM-HD protein, MEC-3, also to demonstrate that LDB-1 can interact with LIM-HD proteins other than CEH-14. The MEC-3 LDB-1b complex was found to stimulate transcription by more than 7-fold compared to MEC-3 in which the LIM domains were deleted (MEC-3 ΔLIM, Fig. 4C). The MEC-3 LDB-1a complex has a very potent activation potential, stimulating by about 200-fold (Fig. 4C). Thus, the two isoforms of LDB-1 have clearly distinct properties in yeast two-hybrid assays and can interact with different LIM-HD proteins.

The ldb-1 RNAi Worms Display Mechanosensory Defects and Are Uncoordinated

Since no *ldb-1* mutant is available, we performed a series of RNAi experiments to transiently abolish *ldb-1* expression. First, young adult wild-type hermaphrodites were injected with *ldb-1* double-strand RNA and the offspring were analyzed. No lethality was observed. Instead, we observed a strong uncoordinated phenotype (Unc) in L1 and L2 larval stage animals, reminiscent of *lim-4* animals, which show a somewhat loopy movement (Sagasti *et al.*, 1999). The animals were partially lethargic, probably due to the loss of mechanosensation, and loopy (Fig. 5). The *ldb-1*(RNAi) in L1 to L2 stage animals are also Mec, reminiscent of *mec-3* animals that do not react to light touch and are lethargic (Way and Chalfie, 1988). These phenotypes became weaker in later stages and eventually disappeared in young adults. The transient phenotypes indicate that the affected cells, which cause the Unc phenotype, regain their function when the RNAi effect is lost and that therefore they must still be present in adult animals. The motility and mechanosensation defects are summarized in Table 1. Interestingly, most of the animals are normal for nose touch, which is also not affected in *mec-3*.

Another set of experiments was performed by injecting L1/L2 animals to test whether *ldb-1* RNAi would cause any defects in vulval and gonad development, since it is expressed in these tissues (see Fig. 2). None of the injected animals ($n = 10$) showed any detectable defects (data not shown).

Finally we tested whether *ldb-1* affects *lim-6* function. Among the defects in *lim-6* mutants two obvious ones are defective defecation (*lim-6* controls the function of the neuron DVB) and altered uterine lumen development (Hobert *et al.*, 1999). The progeny of dsRNA-injected animals was first scored for the Unc/Mec phenotype as an internal control and subsequently tested for the two *lim-6* phenotypes mentioned above. L2/L3 animals were analyzed by differential interference contrast (DIC) microscopy but none showed the typical constipated phenotype (data not shown). Some animals were kept until young adults and analyzed for defects in the uterine lumen. DIC microscopy analysis of these animals revealed no obvious defects in the uterine structure (data not shown).

ldb-1 Regulates Expression of mec-2::gfp but Not mec-3::gfp Constructs in Vivo

The LIM-HD genes proteins MEC-3 and TTX-3 are known to autoregulate their own transcription (Way and Chalfie, 1989; Hobert *et al.*, 1997). It is possible that LDB-1 is a cofactor for these proteins and loss of LDB-1 might interfere with their autoregulation. We tested this hypothesis by performing *ldb-1* RNAi experiments on transgenic lines carrying *gfp* reporters that have been shown to be downregulated in *mec-3* and *ttx-3* null mutants (Way and Chalfie, 1989; Hobert *et al.*, 1997). No downregulation of *gfp* expression was seen in injected animals or in their offspring. All cells expressing *mec-3* or *ttx-3* were present and showed no apparent defects (Figs. 6A and 6B). All animals tested for MEC-3::GFP expression were Mec. We conclude from this experiment that *ldb-1* is necessary for touch neuron function, but not involved in the generation of these cells. We also performed *ldb-1* RNAi on animals transgenic for *lin-11::gfp* constructs. In contrast to *mec-3* and *ttx-3, lin-11* transcription is not autoregulated (Hobert *et al.*, 1998). Like in *mec-3* and *ttx-3* animals, all neurons are apparently present in *lin-11* animals and display normal morphology after *ldb-1* RNAi experiments (Fig. 6C).

To analyze the Mec phenotype in more detail we performed RNAi of *ldb-1* in lines transgenic for the mechanosensory-specific reporter gene *mec-2::gfp*. Strains with integrated constructs were used. We tested the offspring of injected animals by performing mechanosensory tests and only mechanosensation-defective animals were scored. We observed a clear downregulation of *mec-2::gfp* expression in these animals (Figs. 6D and 6E). Thirty Mec animals were analyzed, all of which showed no or strongly reduced *gfp* expression. As a control 30 worms from uninjected animals were analyzed. *mec-2* is a stomatin-like gene necessary for mechanosensation in *C. elegans* (Huang *et al.*, 1995). Downregulation was also reported in *mec-3* mutant animals (Huang *et al.*, 1995) and in *unc-86(u5)* and in *unc-86(168)* animals (Röhrig *et al.*, 2000) and might explain the Mec phenotype of the *ldb-1*(RNAi) animals.

DISCUSSION

Both *C. elegans* and *D. melanogaster* have only one member of the Ldb/NLI/Clim family (Fig. 1C), while

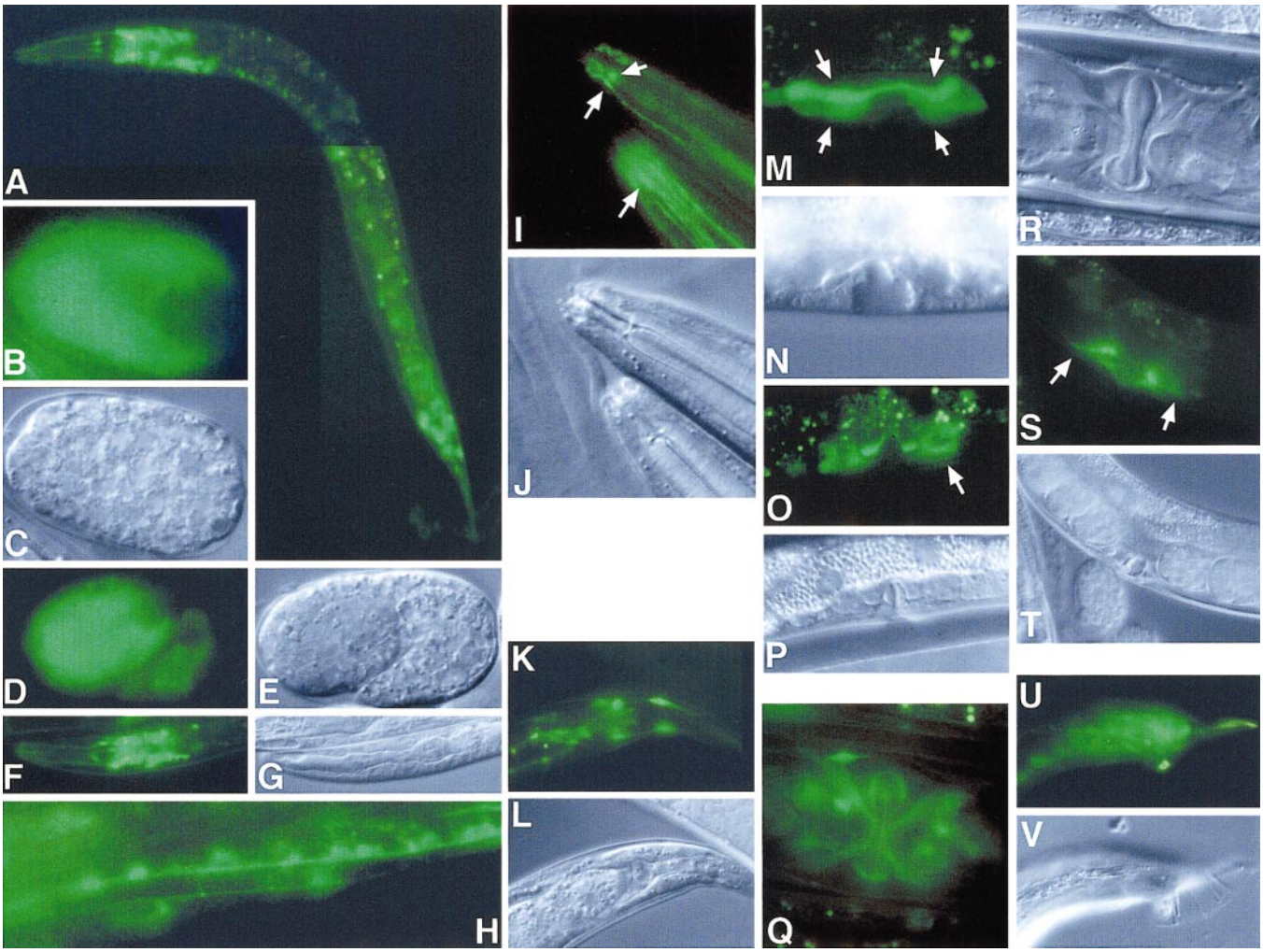


FIG. 3. Expression pattern of the reporter construct *ldb-1b::gfp*. (A) Overview of a L2 larva expressing the construct in all neurons. (B, C) Early embryo (200–260 min). Expression is seen in all cells but the centrally located hyp-7 precursors (mostly Cp lineage and some deriving from AB). The lateral hyp-7 precursors show *ldb-1b* expression. (D, E) Comma stage embryo. Expression is seen in all cells but hyp-7 and gut. (F, G) Head region of L4 larva. Expression is seen in neurons in the head ganglia. (H) Ventral cord neurons. Expression is also seen in the commissures. (I, J) Sensory endings of amphids in adult animals. The upper right arrow indicates the sensory ending of the thermosensory cell AFD. The upper left arrow indicates the sensory endings of other amphid neurons. The lower arrow shows the dendritic processes of additional sensory cells. (K, L) Tail of adult animal. Mosaic expression is seen in tail neurons. (M, N) L3 larva, approximately 40 h of age. Vulval (lower arrows) and overlying gonadal cells (upper arrows) show expression. (O, P) Vulval region of young adult. Expression is seen in vulval and uterine cells (arrow). (Q, R) Ventral surface view of an adult vulva. Expression is seen in vulval cells, vulval muscle, and neurons. (S, T) Adult vulva. Expression is seen in uterine cells. (U, V) Adult male tail. All tail neurons express *ldb-1b*. (C), (E), (G), (J), (L), (N), (P), (R), (T), and (V) are DIC micrographs corresponding to the *gfp* fluorescence micrographs.

there are two distinct LDB families in vertebrates and four genes in zebrafish (Fig. 1C). This is consistent with the notion of genome duplication in vertebrate evolution and an additional duplication event in zebrafish (Meyer and Schartl, 1999). From sequence comparisons (Fig. 2B) it is clear that the most conserved parts of the LDB proteins coincide with the domains which were biochemically characterized for being responsible for dimerization, nuclear localization, and LIM domain binding (Jurata and Gill, 1997). Our analysis suggests that the dimerization domain is confined to about 220 amino acids. Regions in the *C. elegans* Ldb proteins are present with extra insertions or deletions compared to the fly and vertebrate LDB proteins. This may indicate loop regions

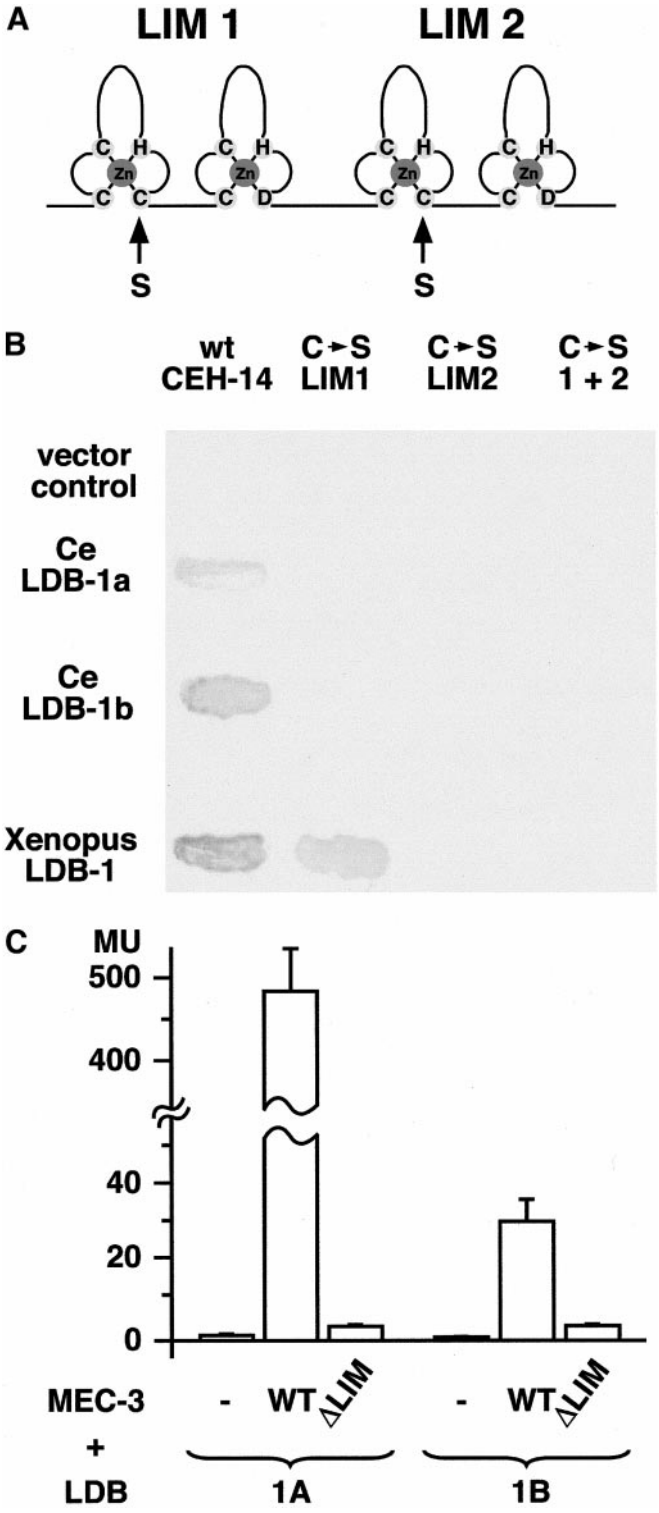


FIG. 4. Yeast two-hybrid assay. (A) Scheme of the LIM domain of CEH-14. The two domains LIM1 and LIM2 are indicated. The conserved cysteine and histidine residues complexing with Zn^{2+} that form the typical four Zn fingers are highlighted. The arrows indicate the substitution of single residues which were introduced to abolish the Zn-finger structure of the two domains. (B) Yeast two-hybrid assay showing the specificity of the interaction of the CEH-14 LIM domain with either LDB-1A or LDB-1B. β-Galactosidase assay using X-Gal of a filter lift with streaks of different yeast transformants. The LIM domain fused to the DNA binding domain of the GAL4 yeast transcriptional activator coexpressed with the GAL4 activation domain alone (vector control) is not sufficient to trigger the expression of the β-galactosidase reporter (yeast remain white). Interaction between the CEH-14 LIM domain and LDB-1 is necessary for reconstitution of the GAL4 complex which triggers β-galactosidase expression. Positive interactions (shown by blue color) are wt CEH-14 with CeLDB-1a, wt CEH-14 with CeLDB-1b, wt CEH-14 with *Xenopus* LDB-1, and CEH-14(C→SLIM1) with *Xenopus* LDB-1. This interaction is generally abolished when point mutations are introduced in the LIM domain of CEH-14. Interestingly, the interaction is evolutionarily conserved and can be demonstrated for CEH-14 with *Xenopus* LDB-1. (C) Quantitative liquid β-galactosidase interaction assays of MEC-3 with the two different isoforms of LDB-1. The full-length cDNA (WT) or a partial cDNA lacking the region encoding the LIM domains (ΔLIM) of *mec-3* was fused to the DNA-binding domain of the bacterial LexA repressor as described (Röckelein *et al.,* 2000). The *ldb-1a/b* cDNAs were fused to the activation domain of yeast GAL4 protein. The interaction of MEC-3 with LDB-1A/B is dependent on the LIM domains. The LDB-1A interaction is approximately 15-fold stronger than the interaction with LDB-1B. β-Galactosidase activity is shown in Miller units (MU).

of the protein that do not have to be absolutely conserved in length.

The highest degree of sequence conservation is found in the LID. This is consistent with the observation that *Xenopus* Ldb-1 can also bind *C. elegans* LIM domains in a yeast two-hybrid assay (Fig. 4). Remarkably, a point mutation within the first LIM domain cannot completely abolish this interaction. This phenomenon is similar to the results of Arber and Caroni (1996), who showed that the second Lim domain of MLP (a cytoplasmic LIM only protein) is more important for the interaction with its partner (actin) than the first. Thus one LIM domain might contribute to LDB binding while the second domain might act synergistically. This model is supported by the finding that LIM domains of LIM-HD proteins can dimerize via LDB, but can bind additional transcription factors, for example, the bHLH transcription factor E47 (Johnson *et al.,* 1997) or RLIM, which is responsible for repression by recruiting a histone deacetylase complex (Bach *et al.,* 1999). Probably LIM-HD, LMO, and LDB proteins plus other factors form large complexes that participate in transcriptional regulation.

To address the function of *C. elegans ldb-1 in vivo,* we performed RNAi. We were able to identify two prominent phenotypes: mechanosensory defects and uncoordinated movements. These phenotypes are reminiscent of the phe-

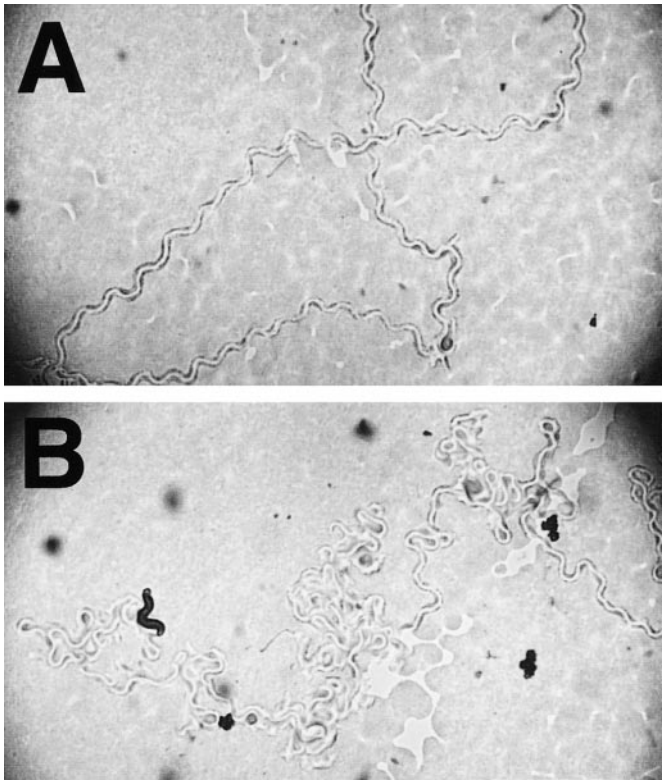


FIG. 5. Worm tracks on bacterial lawns showing the *ldb-1*(RNAi) Unc phenotype. (A) Track of a N2 wild-type L2 larva. (B) Typical track of a L2 larva descendent from an *ldb-1* RNAi-treated parent. The animals, in general, did not move much, but when moving they showed "loopy," exaggerated sinus wave movements.

notypes observed in *mec-3* (Way and Chalfie, 1988) and *lim-4* (Sagasti *et al.,* 1999) mutant animals, respectively. Since we can demonstrate protein interactions of LDB-1 with MEC-3 and CEH-14, we suggest that the phenotypes observed may be caused by the loss of LDB-1/LIM-HD protein interactions.

We analyzed a possible involvement of *ldb-1* in LIM-HD gene function by assaying the autoregulatory function of *mec-3* and *ttx-3* in *ldb-1* RNAi animals. Since no downregulation of these genes was detected, *ldb-1* does not seem to be required for LIM-HD gene autoregulation. However, we cannot exclude that our reporter assays missed subtle changes in the expression patterns of *ttx-3* and *mec-3*. Our data, on the other hand, suggest that *ldb-1* is necessary for LIM-HD protein functions. This is difficult to address in the case of TTX-3, which is required for correct morphogenesis of the AIY interneurons involved in thermotaxis (Hobert *et al.,* 1997). While it is not feasible to perform isothermal tracking experiments on RNAi-treated animals, the RNAi experiments of the *gfp* transgenic lines allowed us to confirm that all neurons expressing *ttx-3* (and also *mec-3*) are made and that they show no apparent morphological abnormalities. *ldb-1*(RNAi) downregulated the expression of *mec-2*, encoding a stomatin that is specifically expressed in the touch neurons. We recently demonstrated that UNC-86/MEC-3 heterodimerization is a prerequisite of *mec-2* transcription (Röhrig *et al.,* 2000). This interaction of both transcription factors synergistically enhances the transcriptional activation mediated by UNC-86 (Röckelein *et al.,* 2000). Thus, we propose that the main role of *ldb-1* in touch neurons may be to modulate the protein interactions of a LIM-HD factor with other proteins. Since all RNAi animals displayed a wild-type phenotype as adults, either *ldb-1* function is no longer required in adult animals or the effect of RNAi is lost with time due to degradation and/or dilution of the dsRNA.

ldb-1 is expressed in all neurons and some other tissues of the larval and adult nematode. The combined expression patterns of all the LIM-HD genes are more restricted than that of *ldb-1*. Therefore, the function of *ldb-1* may go beyond the possible regulation of this particular family of transcription factors. This is similar to flies, in which Chip (the *ldb-1* orthologue) is important for the correct expression of segmentation genes, whereas LIM-HD genes are not involved in segmentation. Furthermore, Chip also interacts with several other homeodomain proteins (Dorsett, 1999). Moreover, the vertebrate homologue has been shown to

TABLE 1
Phenotype of *ldb-1*(RNAi)

	Phenotype				
	WT	Mec+Unc	Mec only	Nose touch abnormal	*n*
ldb-1(RNAi)	14%	84%	2%		44
ldb-1(RNAi)*		—	—	25%	20

Note. ldb-1(RNAi)* shows the percentage of Mec+Unc animals which additionally display abnormal nose touch. The Mec phenotype was scored as described in Hobert *et al.* (1999). Control *ceh-14*(RNAi) animals were wildtype in coordination and mechanosensation (Cassata *et al.,* 2000, not shown).

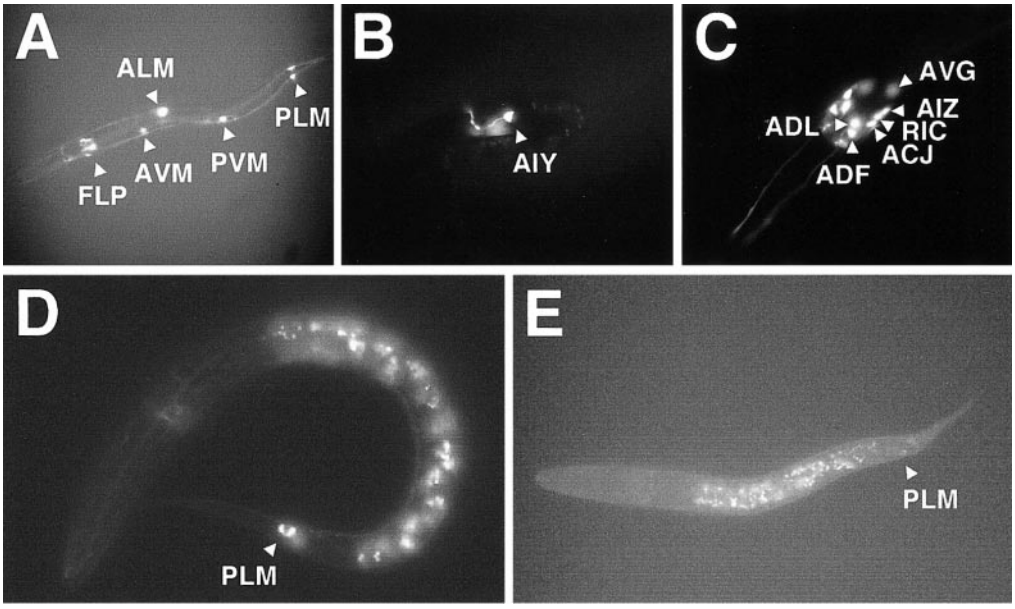


FIG. 6. Expression of neuronal markers in *ldb-1*(RNAi) larvae. (A) *ldb-1*(RNAi) larvae that display a Mec phenotype, but express the *mec-3::gfp* construct in all predicted neurons (FLP, AVM, ALM, PLM, PVM indicated) with the same intensity as uninjected animals (not shown). All the cells are present and display wild-type morphology. Thus, *ldb-1* does not play an eminent role in the autoregulation of *mec-3*, nor can the Mec phenotype be attributed to non- or malformation of the touch neurons. (B) *ldb-1*(RNAi) larva with Mec phenotype expressing *ttx-3::gfp* construct in the interneuron AIY (indicated) with the same intensity as in uninjected animals. The morphology of these interneurons appears indistinguishable from that of wildtype. As for *mec-3*, the LIM-HD gene *ttx-3* regulates its own expression (see text), therefore *ldb-1* is not required for this positive feedback. (C) Normal expression of the *lin-11::gfp* construct in the *ldb-1*(RNAi) background. Expression of the LIM-HD gene *lin-11* is normally not prone to autoregulation (Hobert *et al.*, 1998). Nevertheless, position and morphology of all neurons expressing *lin-11::gfp* are normal [ADL, AVG, AIZ, RIC, ACJ, ADF indicated, others (posterior cells) are not shown]. (D) The stomatin-like gene *mec-2* is required for mechanosensation and is expressed in touch neurons (Huang *et al.*, 1995). A wild-type larva is shown with expression of *mec-2::gfp* in the touch neurons (PLM is indicated). (E) *mec-2::gfp ldb-1*(RNAi) larva. All Mec animals of *ldb-1* dsRNA-injected worms show little or no *mec-2::gfp* expression. The picture was taken with a longer exposure time than (D).

interact with Ptx-1 (P-Otx), a paired-like homeodomain protein that lacks LIM domains (Bach *et al.*, 1997). We can assume that in *C. elegans* LDB-1 does not only bind to LIM-HD proteins. While we found only a very limited set of phenotypes with the RNAi, we cannot exclude that *ldb-1* may function in many processes that are not revealed by RNAi.

Transcriptional regulation is controlled by a concert of proteins that form large complexes and differ in function within these complexes. While some factors bind DNA and/or recruit other regulatory proteins, others, like LDB-1, are most likely general coupling factors or perhaps enhancer facilitators (Dorsett, 1999). The fact that *C. elegans* LDB-1 contains several domains and is present in two alternatively spliced isoforms differing in their amino-terminus might be indicative of complex protein–protein interactions and also of fine modulation of transcriptional regulation. This view is underlined by the differential affinity of the two isoforms in yeast two-hybrid assays. It will be interesting to analyze whether the different isoforms of LDB-1 in *C. elegans* serve distinct roles in the animal, thereby possibly compensating for the higher number of LIM domain binding proteins found in vertebrates.

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