The Caenorhabditis elegans lim-6 LIM homeobox gene regulates neurite outgrowth and function of particular GABAergic neurons

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SUMMARY

We describe here the functional analysis of the C. elegans LIM homeobox gene lim-6, the ortholog of the mammalian Lmx-1a and b genes that regulate limb, CNS, kidney and eye development. lim-6 is expressed in a small number of sensory-, inter- and motoneurons, in epithelial cells of the uterus and in the excretory system. Loss of lim-6 function affects late events in the differentiation of two classes of GABAergic motorneurons which control rhythmic enteric muscle contraction. lim-6 is required to specify the correct axon morphology of these neurons and also regulates expression of glutamic acid decarboxylase, the rate limiting enzyme of GABA synthesis in these neurons. Moreover, lim-6 gene activity and GABA signaling regulate neuroendocrine outputs of the nervous system. In the chemosensory system lim-6 regulates the asymmetric expression of a probable chemosensory receptor. lim-6 is also required in epithelial cells for uterine morphogenesis. We compare the function of lim-6 to those of other LIM homeobox genes in C. elegans and suggest that LIM homeobox genes share the common theme of controlling terminal neural differentiation steps that when disrupted lead to specific neuroanatomical and neural function defects.

Key words: LIM homeobox, Caenorhabditis elegans, Axogenesis, GABA, Uterus

INTRODUCTION

Dedicated neural circuits which mediate specific behaviors have been defined in Caenorhabditis elegans. The description of the complete neural connectivity of C. elegans as well as the behavioral defects induced by laser or genetic ablation of specific neurons has led to the definition of neural circuits that mediate, for example, mechanosensory, locomotory, thermosensory and rhythmic defecation behaviors (Avery and Thomas, 1997; Chalfie et al., 1985; Hart et al., 1995; McIntire et al., 1993a,b; Miller et al., 1992; Mori and Oshisma, 1995; Thomas, 1990; White et al., 1992). Not surprisingly, specific neurotransmitter synthesis, synaptic signaling, and receptor signaling pathways have been implicated in the function of most of the pathways (Bargmann and Kaplan, 1998). But genetic analysis has also revealed that transcriptional control mechanisms regulate both early stages of neural cell fate determination and events late in neural differentiation and in the mature nervous system. For example, the unc-4 homeobox gene determines the pattern of interneuron connectivity to motor neurons from its site of expression within motoneurons (Miller et al., 1992; White et al., 1992), and the unc-55 nuclear hormone receptor is required for the correct wiring of VD type motoneurons (Zhou and Walthall, 1998). The analysis of several LIM homeobox genes also revealed that they regulate terminal steps in the differentiation of specific components of the mechanosensory (Way and Chalfie, 1988, 1989) or thermosensory circuits (Hobert et al., 1998, 1997).

LIM homeobox genes represent a large subfamily of homeobox genes; although they act in a variety of different developmental contexts, a common theme is their regulatory role in neural development (Dawid et al., 1998). In Drosophila, a requirement for LIM homeobox genes in axonal fasciculation, pathfinding and neurotransmitter choice was demonstrated for the apterous, lim3 and islet genes, respectively (Benveniste et al., 1998; Lundgren et al., 1995; Thor and Thomas, 1997; Thor et al., 1999). In vertebrates, LIM homeobox genes appear to play a similar role in terminal steps of neural differentiation, although it is clear that they have also been recruited to several additional functions outside the nervous system, such as early embryonic inductions or limb patterning (Dawid et al., 1998). In the vertebrate spinal cord, the temporal and spatial patterns of LIM homeobox gene expression implicates them in control of motoneuron differentiation (Tsuchida et al., 1994). Loss of function of the mouse Isl-1 gene leads to a failure of motoneuron differentiation (Pfaff et al., 1996), Lhx-3 and Lhx-4 determine motoneuron subtype identities (Sharma et al., 1998) whereas loss of function of the Lhx-2 gene causes a variety of brain developmental defects (Porter et al., 1997).
Here we analyse the function of the C. elegans LIM homeobox gene \textit{lim-6} and compare it with its mammalian orthologs \textit{Lmx-1a} and \textit{Lmx-1b}. The \textit{Lmx-1a} LIM homeodomain protein was originally identified as a regulator of insulin gene expression (Geman et al., 1992). The closely related \textit{Lmx-1b} gene (100\% identity in homeodomain) is expressed in the central nervous system, including regions of the hindbrain and in particular domains in the spinal cord (Chen et al., 1998; Matise and Joyner, 1997; Riddle et al., 1995). \textit{Lmx-1b} mutant mice display CNS patterning defects (R. Johnson, personal communication). However, the analysis of \textit{Lmx-1b} has so far been focused on its function outside the nervous system, mainly in limb and kidney development, where it is required for specific patterning and differentiation events (Chen et al., 1998; Dreyer et al., 1998; Riddle et al., 1995; Vogel et al., 1995). Moreover, Nail Patella Syndrome, an autosomal dominant disorder characterized by renal defects and various forms of skin and limb hypoplasias is caused by a heterozygous null mutation in human \textit{Lmx-1b} (Dreyer et al., 1998). Thus the normal two copy gene dosage of \textit{Lmx-1b} is essential for these patterning events. We show that the single \textit{C. elegans Lmx-1a/b} ortholog \textit{lim-6} is expressed in particular neurons, many of which are GABAergic, in the excretory system and in epithelial cells of the uterus. Using a \textit{lim-6} deletion mutant we show that \textit{lim-6} is required for the terminal differentiation of sensory- and motoneurons, and for morphological aspects of uterine development. The behavioral and neuroendocrine defects of the \textit{lim-6} mutant animals are mostly due to defects in maturation of GABAergic neurons that depend on \textit{lim-6} function. The neuronal defects of \textit{lim-6} mutant animals resemble those of other LIM homeobox genes in \textit{C. elegans}, thus pointing to a common theme in the function of this gene family in \textit{C. elegans}. Moreover, the sites of \textit{Lmx-1} and \textit{lim-6} expression also suggest that specific functions of these genes may be conserved during evolution.

**MATERIALS AND METHODS**

**Strains and transgenic lines**

Wild type were N2 Bristol, \textit{lim-6(nr2073), unc-25(e156), unc-30(e191), daf-7(e1372), unc-1(e719), unc-33(e204), unc-36(e251), unc-73(e936), unc-76(e911), egl-38(n578), lin-11(n389), lin-17(n671), che-3(e1379), pha-1(e2123ts), aex-2(sa3), sem-4(n2654).

Transgenic lines were either created in a wild-type background using pRF4 (rol-6(su1000)) at 100 ng/\mu l or mec-7::GFP at 50 ng/\mu l as an injection marker or in a \textit{pha-1(e2123ts)} mutant background using pBX (pha-1 wild-type expression construct) at 100 ng/\mu l as the rescuing construct for the \textit{pha-1} lethality (Granato et al., 1994). The lines are:

- \textit{pha-1(e2123ts)}; mgEx[lim-6r::GFP; pBX] (4 independent lines)
- \textit{pha-1(e2123ts)}; mgEx[lim-6prom::GFP; pBX] (3 independent lines)
- \textit{pha-1(e2123ts)}; mgEx[lim-6::GFP; pBX] (8 independent lines)
- \textit{pha-1(e2123ts)}; mgEx[lim-6::GFP; pBX] (1 line)
- \textit{N2; mgEx[GAD::GFP; pRF4]} (8 independent lines)
- \textit{mgEx446, mgEx447} (2 independent lines)
- \textit{mgEx446, mgEx447} = \textit{N2; mgEx[fl-lim-6-2; mec-7::GFP]} (2 independent lines)
- \textit{mgEx408, mgEx409} = \textit{N2; mgEx[lim-6VP16-2; mec-7::GFP]} (2 independent lines)
- \textit{juIs8}; integrated pSC381 (Jin et al., 1999)
- \textit{oxIs12}; McIntire et al. (1997)

**Plasmid construction**

Unless noted otherwise below, the expression plasmids were constructed by amplifying the respective sequences either from wild-type genomic DNA or the K03E6 cosmid and subcloning the PCR products into the pPD95.75 expression vector. The respective constructs contain the following sequences (the numbering originates from an arbitrary numbering of the K03E6 cosmid; the ATG startcodon is at position 14254, the stop codon ends at position 10541).

\begin{itemize}
  \item \textit{lim-6r::GFP}: bp 18290-10544
  \item \textit{lim-6prom::GFP}: bp 18290-12846
  \item \textit{lim-6int3::GFP}: bp 12785-11584
  \item \textit{lim-6up::GFP}: bp 2019-16193
  \item \textit{fl-lim-6-2::GFP}: bp 18290-10544 (\textit{unc-54} 3’UTR, without GFP)
  \item \textit{lim-6VP16-2}: As \textit{fl-lim-6-2}, except that the acidic activation domain of VP16 (aa 411-aa 490) was cloned into a BamHI site that had been engineered at the C terminus of \textit{lim-6}.
\end{itemize}

**GADcompl::GFP**

The GADunc-25 gene is from YAC y3748contig.03366. Its structure was predicted by Genefinder. The GFP fusion was generated by PCR amplification of the complete gene including 1846 bp of upstream sequence (up to the preceding predicted gene) and the full coding sequence. The amplification product was fused to GFP using a PCR fusion approach with overlapping PCR primers (O. H. and G. R., unpublished). The fusion amplification product was directly injected into adult animals. The sites of expression of this \textit{GADcompl::GFP} construct was similar to the expression construct pSCh31/juls8 described by Jin et al. (1999).

**Isolation and rescue of \textit{lim-6(nr2073)}**

\textit{lim-6(nr2073)} was kindly provided by NemaPharm, Inc. It was isolated from an EMS-induced \textit{C. elegans} deletion library which included approximately 400,000 mutagenized chromosomes using a PCR based sib-selection procedure (Jansen et al., 1997). The mutant strain was backcrossed five times. Its genotype was confirmed using a triplex PCR with 3 primers, two of which flank the deletion (yielding a PCR product of 0.9 kb on the deleted chromosome), the third is located within the deletion (yielding a PCR product of 1.1 kb on the wild-type chromosome). The exact deletion point was determined by DNA sequencing. All the phenotypes reported here were linked with the \textit{lim-6 deletion nr2073} through the five backcrosses. Moreover, the \textit{nr2073} mutant phenotypes were complemented by a wild type \textit{lim-6(+)} transgene and phenocopied by expression of a dominant negative \textit{lim-6} gene product (described below). For the rescuing approach, we introduced two independent extrachromosomal arrays, \textit{mgEx446} and \textit{mgEx447}, which contain a wild-type copy of \textit{lim-6}, termed \textit{fl-lim-6-2} into wild-type animals. \textit{fl-lim-6-2} contains the same genomic region as the GFP construct \textit{lim-6r::GFP} shown in Table 1. To cross these arrays into \textit{lim-6(nr2073)} the \textit{mgEx446} and \textit{mgEx447} transgenes were mated into \textit{unc-1(e719)}. Then these animals were crossed with the \textit{lim-6(nr2073)} strain and the closely linked \textit{unc-1} chromosome was segregated away to yield a homozygous \textit{lim-6} mutant carrying the transgene. The genotype was verified by PCR. The resultant strain \textit{lim-6(nr2073)} \textit{mgEx446/447} at least partially rescues the uterine defects and defection defects. We noted that the same \textit{mgEx446/447} arrays cause moderate defection defects in some wild-type animals, suggesting that either overexpression of \textit{lim-6} or promoter titration effects cause similar defects to its loss of function. This observation is a potential explanation for why not all \textit{lim-6(nr2073)} \textit{mgEx446} animals are completely rescued. We also constructed a \textit{lim-6::VP16} expression construct \textit{(lim-6VP16-2)}, which by increasing transactivation function of \textit{LIM-6} was expected to enhance rescue (as
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lim-6 in neuronal differentiation

exemplified by ttx-3::VP16, O. H., I. Mori and G. R., unpublished data). Extrachromosomal arrays expressing the VP16 fusion constructs (mgEx408 and mgEx409) indeed completely rescued the lim-6 mutant phenotype; 20/20 animals showed intact uterine lumens; 19/20 animals restored gcy-5 expression in ASEr; 3/3 showed intact EMC cycles.

The second approach to show linkage of the defects with lim-6 consisted of expressing a dominant negative derivative of lim-6 in which we deleted from the fl-lim-6-2 expression construct the C-terminal 72 amino acids (including parts of the homeodomain) and replaced it by GFP. Expression was similar to that seen for lim-6r::GFP (Table 1). We observed strong and highly penetrant constipation defects and brood size reductions, which thus phenocopies the lim-6(nr2073) defects.

Antibody staining

FMRFamide antibody staining was performed as previously described by Schinkmann and Li (1992).

Behavioral assays

The defecation assay was described by J. Thomas (1990). Well fed animals were scored as young adults. Muscle contractions of individual animals were scored using a stereomicroscope at 50x magnification. Each animal was observed for 5 to 15 defecation cycles. The time elapsed between the pBoc and the EMC and the intercycle time was recorded. An EMC was only scored as complete if the release of gut contents was observed.

Egg laying behavior was scored by picking single L4 staged animals to a fresh plate and transferring them every day to a fresh plate until no further progeny was produced. The total amount of progeny was counted.

Dauer arrest was scored with non-starved, non-crowded animals. Adult animals of the respective genotype were allowed to lay eggs for 4-12 hours; those eggs were kept at the respective temperature and the larvae hatching from these eggs were scored for dauer characteristics 3-7 days after the egg lay.

RESULTS

lim-6, the C. elegans homolog of Lmx-1a/b, is expressed in the developing nervous system, uterus, and excretory system

lim-6 is one of seven LIM homeobox genes revealed by the C. elegans genome sequence. Three of the LIM homeobox genes emerged from genetic analysis of development and neural function, mec-3, lin-11, and ttx-3 (Freyd et al., 1990; Hobert et al., 1997; Way and Chalfie, 1988). Most of the C. elegans LIM homeobox genes detect clear vertebrate orthologs in the database (Chen et al., 1998; German et al., 1992; Iannotti et al., 1997). The degree of similarity between lim-6 and the Lmx-1 genes in the homeodomain as well as the LIM domains suggests that lim-6 and Lmx-1a/b were derived from a common ancestor and are orthologous genes (Fig. 1B,C). lim-6 is located on cosmid K03E6 which maps to a region of the X chromosome bearing no obvious genetic candidates for mutations in lim-6, based on the function of its vertebrate orthologs or its expression pattern in C. elegans (see below).

Fig. 1. Genomic structure of lim-6 and sequence comparisons. (A) Predicted exon/intron structure of lim-6. The location of the nr2073 deletion mutation is schematically shown; the deletion starts at amino acid position 65 and deletes the C-terminal 213 amino acids of the predicted LIM-6 protein. (B) Alignment of the homeodomains of representative proteins from different LIM homeodomain classes. The homeodomain sequences of all vertebrate Lmx-1 proteins identified so far are 100% identical. (C) Sequence relationship of representative members of several LIM homeobox gene classes. The dendrogram was constructed with the homeodomains of the respective proteins from a distance matrix created with the Jukes-Cantor method using the neighbor-joining method (GCG software package); Prd-type homeodomains were used as an outgroup to root the tree (not shown). C. elegans proteins are shaded. The accession number for LIM-6 is U55375. All other sequences were retrieved by their sequence names from GenBank.
Table 1. Expression of \textit{lim-6} reporter gene constructs in neuronal and non-neuronal cells*

<table>
<thead>
<tr>
<th>Neuronal</th>
<th>Non-neuronal</th>
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<tbody>
<tr>
<td>ASEL</td>
<td>PVT</td>
</tr>
<tr>
<td>RIS</td>
<td>AVL</td>
</tr>
<tr>
<td>RMEL/R</td>
<td>RIGL/R</td>
</tr>
<tr>
<td>EG</td>
<td>uterus</td>
</tr>
</tbody>
</table>

\begin{tabular}{l|l|l|l|l}
\hline
K03E6.4 & \textbf{lim-6} (K03E6.1) & LIM & LIM homeobox & \textbf{sequential} + & + + + +
\hline
fl-lim-6-2 & \textbf{lim-6r::GFP} & + & + & +
\hline
\textbf{lim-6prom::GFP} & + & + & + & +
\hline
\textbf{lim-6up::GFP} & + & + & + & +
\hline
\textbf{lim-6int3::GFP} & + & + & + & +
\hline
\end{tabular}

*Multiple independent transgenic lines were analyzed and found to have identical patterns of expression (see Materials and Methods).

(+): indicates very faint expression.

EG, excretory gland cells.

\(\text{\textasteriskcentered textasteriskcentered} = \text{unc-54 3^'UTR.}\)

To reveal the expression pattern of \textit{lim-6}, we fused \textit{lim-6} genomic regions to green fluorescent protein (GFP) and generated transgenic lines bearing these fusion genes (Table 1). One of these reporter genes, \textit{lim-6r::GFP}, contains precisely the same \textit{lim-6} non-coding and coding regions as a genomic region that complements the null phenotype of \textit{lim-6} (fl-lim-6-2; Table 1); moreover, most cells expressing \textit{lim-6} reporter genes show defects in \textit{lim-6} null mutant animals (described below). Thus, with the usual caveats of reporter gene expression studies, these observations indicate that the \textit{lim-6r::GFP} constructs reveals authentic sites of endogenous \textit{lim-6} expression.

The \textit{lim-6r::GFP} fusion gene reveals expression in a restricted set of neurons, epithelial cells of the uterus and the excretory system (Table 1; Fig. 2). Reporter gene expression in the nervous system begins late in embryogenesis at about 300 minutes of development, which is after these neurons have been generated and while they initiate neurite outgrowth (Sulston, 1983). After hatching, \textit{lim-6r::GFP} is expressed in one chemosensory neuron, ASEL, and in eight inter- and motoneurons (Fig. 2A-E). Most of these neurons are GABAergic, namely RMEL/R, AVL, RIS and DVB (McIntire et al., 1993b). RMEL/R, AVL and DVB are motoneurons which innervate specific sets of head and enteric muscles, respectively, whereas RIS is an interneuron (White et al., 1986). The other three neurons, PVT and RIGL/R, express the neuropeptide FMRFamide (Schinkmann and Li, 1992). The expression of \textit{lim-6} in all neurons continues throughout adulthood, indicating that \textit{lim-6} may continue to function in the mature nervous system.

The expression of \textit{lim-6} in the uterus is dynamic. \textit{lim-6} reporter gene constructs (\textit{lim-6prom::GFP}, \textit{lim-6up::GFP}, \textit{lim-6r::GFP}, Table 1) start to be expressed in two uterine cells during the late L3/early L4 stage and the expression widens during the L4 stage to include the uv2 and uv3 cells, several uterine toroid (ut) cells, which form the lumen of the uterus and at least one cell type (sujn) of the spermatheca-uterine junction (Fig. 2F,G). Occasionally, weaker and less consistent expression can be observed in some cells of the distal side of the spermatheca, which connect the spermatheca to the rest of the somatic gonad. Expression of \textit{lim-6} in the uterus is absent in adults suggesting that \textit{lim-6} function is specific for the stages of uterine development. Consistent with these uterine cell identities, we could not observe any major changes of \textit{lim-6} expression in animals that are mutant for genes that affect other cells of the developing uterus, such as \textit{egl-38}, which affects the fate of the uv1 cells (Chamberlin et al., 1997) or \textit{lin-11}, which affects the utse cells (Newman et al., 1996) (data not shown).

Considering the expression and function of vertebrate \textit{Lmx-1b} in the kidney, it is intriguing that \textit{lim-6} is expressed in the excretory system of \textit{C. elegans}. The \textit{C. elegans} excretory system is composed of four cell types (Nelson et al., 1983), one of which, the A-shaped excretory gland cell, expresses \textit{lim-6} from late embryogenesis throughout adulthood (Fig. 2B).

Analysis of \textit{lim-6} promoter deletion derivatives reveals that the \textit{lim-6} promoter is composed of separable regulatory elements that are specific for individual cell types as shown in Table 1. The regulatory element for uterine and sensory neuron expression is localized upstream of the transcriptional start site, while the regulatory element for \textit{lim-6} expression in several GABAergic neurons is located in the third intron.

Isolation of a \textit{lim-6} deletion mutant

To determine the function of \textit{lim-6}, a \textit{lim-6} deletion mutant was isolated and phenotypically characterized. This deletion allele, \textit{nr2073}, was isolated by NemaPharm, Inc. (Cambridge, MA) using a PCR screen of a mutagen induced deletion library. The \textit{nr2073} allele harbors a 1.7 kb deletion in the \textit{lim-6} gene which deletes three quarters of the \textit{lim-6} coding region, including the second LIM domain and the homeodomain (Fig. 1A). Since
the DNA binding homeodomain represents the crucial functional feature of LIM homeodomain proteins, it is very likely that nr2073 represents a strong loss-of-function allele, presumably a null allele. After extensive backcrossing of the mutant, we undertook a phenotypic analysis of lim-6(nr2073). lim-6(nr2073) animals are viable, move normally and show no gross morphological abnormalities. However, the animals do not expel the gut contents normally and have reproductive defects. In addition, upon a detailed characterization of particular neural markers, lim-6 mutant animals show defects in the pattern of gene expression and function of GABAergic neurons (see below). The defects of the mutant animals are summarized in Table 2. All of the cellular defects that we observe correspond to the defects expected based on the sites of lim-6 expression described above. Both the neural and non-neural defects of lim-6(nr2073) could be rescued with a genomic lim-6 transgene that contains all the regulatory elements described above in the lim-6r::GFP reporter gene construct (see Material and Methods). Moreover, most of the defects can be phenocopied by expressing, in wild-type animals, a dominant-negative version of the LIM-6 protein under its own promoter (data not shown; see Materials and Methods), further supporting that the nr2073 deletion allele causes the defects that we observe.

**Behaviors mediated by GABAergic neural circuits are defective in lim-6 mutants**

lim-6 mutant animals display defective defecation behavior, as revealed by the bloated appearance of their gut (Fig. 3A). Periodic contractions of the enteric muscle are required to release the gut content (Avery and Thomas, 1997). The enteric muscle contractions (EMC) are regulated by the motorneurons A VL and DVB which innervate the enteric muscles (Liu and Thomas, 1994; McIntire et al., 1993b). The expression of lim-6 in AVL and DVB and the bloated visible phenotype of the lim-6 mutant animals suggested that lim-6(nr2073) mutants may have defects in the rhythmically executed EMCs triggered by AVL and DVB. In wild-type animals, the EMCs are intricately linked to two other muscle contraction steps, posterior body wall contractions (pBoc) and anterior body wall contractions (aBoc). The activation of these muscle contractions is precisely

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**Table 2. Summary of defects caused by the lim-6 null mutation**

<table>
<thead>
<tr>
<th>Neural defects</th>
<th>Non-neural defects</th>
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<tbody>
<tr>
<td>EMC defects (Exp)</td>
<td>Egg-laying defective/low brood size (Egl)</td>
</tr>
<tr>
<td>DVB/AVL axonal defects</td>
<td></td>
</tr>
<tr>
<td>Asymmetric sensory receptor regulation</td>
<td></td>
</tr>
<tr>
<td>Foraging defective (Nup)</td>
<td></td>
</tr>
<tr>
<td>Dauer defects (SynDaf-c)</td>
<td></td>
</tr>
<tr>
<td>unc-25 misregulation</td>
<td></td>
</tr>
</tbody>
</table>

Phenotypes were analyzed with a 5x backcrossed strain. Linkage of these defects to lim-6 was demonstrated by rescue and by phenocopying of some of the defects with a dominant negative lim-6 expression construct. The phenotypes also correlate with the sites of lim-6 expression.
timed and represents an ultradian rhythm that is temperature compensated and can be entrained by external stimuli (Avery and Thomas, 1997; Liu and Thomas, 1994; Thomas, 1990). Significant amounts of gut contents were omitted from the count.

The EMC step of this motor program is activated 3-5 seconds after the pBoc step of the motor program; after a 40-55 second intercycle time, the pBoc (and its closely linked aBoc step) is activated again and is again followed by the EMC and so on. While in wild-type animals the EMC is activated in 88% (n=82) of all cycles with a correct timing of 3-5 sec after the pBoc, in \textit{lim-6} mutant animals the EMC is activated only in 25% (n=205) of the cycles; in 75% of the cycles, the EMC is either entirely absent (59%) or significantly delayed relative to the stereotyped pBoc step (16%) (Fig. 3). The overall cycle length, i.e. the time between the intact pBoc steps, is not affected.

The GABAergic AVL and DVB neurons are essential for the muscle contraction step of the defecation cycle and proteins involved in GABAergic signaling, such as the GABA-synthesizing enzyme UNC-25 or the GABA transporter UNC-47 are expressed in these neurons and required for the execution of the cycle (Jin et al., 1999; Liu and Thomas, 1994; McIntire et al., 1993b, 1997; Thomas, 1990). The expression of \textit{lim-6} in these neurons suggests that the focus of \textit{lim-6} action in the defecation cycle is these neurons. The impact of \textit{lim-6} loss of function on the neuroanatomy of AVL and DVB is consistent with this model (see below).

The expression of \textit{lim-6} in RME\textsubscript{L}/R, two out of a set of four GABAergic head motoneurons (RMEL, RMER, RMED, RMEV) required for head foraging (Hart et al., 1995) indicates a possible \textit{lim-6} function in these neurons. Although we have not tested head foraging behavior in detail, a preliminary analysis suggests that \textit{lim-6} mutant animals do indeed display abnormal nose movements (S. Narrish, personal communication).

The GABAergic neurons that express LIM-6 are generated but their axons are defective in \textit{lim-6} mutant animals

The \textit{lim-6} mutant defects in GABAergic mediated behaviors suggests that the \textit{lim-6} transcription factor mediates a developmental step in the differentiation of these neurons. We attempted to define the cellular basis of these defects by examining the neural fate and the neuroanatomy of \textit{lim-6}-expressing neurons in the \textit{lim-6} mutant animals. As a first step to monitor the cell fate of the \textit{lim-6}-expressing neurons, we crossed the \textit{lim-6int3::GFP} and \textit{lim-6prom::GFP} reporter gene constructs (Table 1) into \textit{lim-6} mutant animals. The expression of these reporter genes as well as other reporter genes described below is largely unaffected in the \textit{lim-6} mutant, thus revealing that \textit{lim-6}-expressing neurons are generated and continue to survive in the mutant (data not shown).

The cell fate of \textit{lim-6}-expressing neurons was further analyzed using three differentiation markers of the neural types that express \textit{lim-6}. First, we examined the expression of the GABA vesicular transporter \textit{unc-47} (McIntire et al., 1997) in \textit{lim-6} mutant, thus finding it to be correctly expressed in AVL and DVB, as well as the other GABAergic neurons that express \textit{lim-6}, RMEL/R and RIS (Figs 4, 5). Thus, expression of the GABA transporter \textit{unc-47} is not dependent on \textit{lim-6} gene activity. Second, we examined the expression of the glutamate receptor \textit{glr-1}, which is a cell fate marker for the DVB and RME motoneurons (Hart et al., 1995); like \textit{lim-6}, \textit{glr-1} is only expressed in the right and left types of the otherwise fourfold symmetric RME motoneurons. \textit{lim-6} mutant animals

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**Fig. 3.** Enteric muscle contractions are defective in \textit{lim-6} mutant animals. (A) \textit{lim-6} mutant animals are constipated. Micrographs were taken using differential interference contrast (DIC) microscopy under Nomarski settings. The black arrows point to the intestinal lumen, which is bloated in \textit{lim-6(nr2073)} but not wild-type animals. (B) Analysis of the rhythmic defecation motor program. Each dot represents an animal tested. Each animals was assayed for 5-11 defecation cycles. The percentage EMC per animal indicates how often an intact EMC was observed per animal (e.g. if observed for 10 cycles, 9 intact EMCs will make a 90% value). 'Intact EMC' is defined as a EMC that occurred within 3-5 seconds following a pBoc. In \textit{lim-6} mutant animals those 75% EMC that do not count as intact, include two classes: entirely absent (59%) or delayed (16%), meaning that it occurs sometime between 6-15 seconds after the pBoc. Also note that the \textit{lim-6} mutant phenotype is not 100% penetrant. \textit{lim-6} mutant animals were rescued with the extrachromosomal, \textit{lim-6}-expressing array \textit{mgEx446}. Animals that showed partial rescue as manifested by an enteric muscle contraction that was visible but did not lead to the expulsion of significant amounts of gut contents were omitted from the count.

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containing a chromosomally integrated glr-1::GFP reporter gene, nuIs1, show unaltered expression of this neurotransmitter receptor in RMEL/R and DVB compared to wild-type animals (data not shown). Third, we observed FMRFamide expression in lim-6(nr2073) animals using anti-FMRFamide antibodies. FMRFamides are neuromodulatory peptides expressed in a subset of neurons in C. elegans, including the DVB, PVT and RIGL/R neurons (Schinkmann and Li, 1992); FMRFamide expression is correctly specified in DVB, PVT and RIGL/R in lim-6(nr2073) mutant animals (Fig. 4). In summary, this analysis demonstrates that lim-6 is not required for the lim-6-expressing neurons to be generated nor to assume certain aspects of their respective identities.

Close examination of the neuroanatomy of the DVB motorneuron, using the unc-47::GFP fusion gene reveals severe neuroanatomical defects in the lim-6 mutant animals (Table 3). 73% of the animals display additional small axons of varying lengths that either emanate directly from the DVB cell body or from the main axonal process in close vicinity of the cell body (Fig. 4). These extra axons often have additional branches. This axonal morphology is very rarely observed in wild-type animals (Table 3). Due to the proximity of AVL to

<table>
<thead>
<tr>
<th>Normal axon morphology</th>
<th>Additional axons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype</td>
<td></td>
</tr>
<tr>
<td>lim-6(nr2073)</td>
<td></td>
</tr>
<tr>
<td>unc-25(e156)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.** DVB and AVL motorneuron axon morphology

DVB and AVL axon morphologies were visualized using the GABAergic neural marker unc-47::GFP provided on the integrated array oxIs12 (McIntire et al., 1997).

*We observed 3 types of abnormal extensions in the VNC: main axon turns at its end or partly runs in an aberrant path outside the VNC (36%), the main axon terminates prematurely at less than half the way from the cell body to its normal termination position at the vulva (52%), the main axon extends beyond its normal termination position (12%).

†unc-30(e191) mutant animals were used to eliminate unc-47::GFP expression in the ventral cord D motorneurons, which would obscure the visualization of the AVL and DVB axons in the ventral cord.

‡We refer to the following cases as abnormal extension: No AVL axon visible in the ventral nerve cord (note that an AVL axon in the anterior third of the ventral nerve cord would be obscured by the RMEV motorneuron); prematurely terminated AVL axon in the ventral nerve cord; AVL axon in an aberrant path outside the ventral nerve cord. In lim-6(nr2073) the unc-47::GFP reporter gene is often less strongly expressed in AVL than in wildtype animals. We counted only those cases, in which the unc-47::GFP was roughly as strong in AVL as in DVB so that we would not miss the axon in the ventral nerve cord if it existed.
other neurons in the head ganglia, we were unable to observe whether AVL displays similar sprouting defects.

The main axonal trajectories of the DVB and AVL motorneurons are also affected by the lim-6 null mutant. Both of these neurons are monopolar and extend their single axonal process along the ventral nerve cord (VNC) (White et al., 1986). The visualization of these axons by unc-47::GFP is obscured by the processes of the GABAergic D-type motorneurons in the VNC. However, the expression of unc-47::GFP in the D-type neurons can be disrupted without affecting expression in the other GABAergic neurons by a mutation in the unc-30 homebox gene. In unc-30(e191) animals, the only VNC axons that contain unc-47::GFP are those of the RMEV, AVL and DVB motorneurons (Basson and Horvitz, 1996; Jin et al., 1994), and the axon projections of these neurons are normal (Fig. 4). We observed, however, that in more than half of the unc-30(e191); lim-6(nr2073) mutant animals, the main axonal process of the DVB motorneuron displays abnormalities along the VNC (Fig. 5; Table 3). Either the axon does not terminate at its normal termination position at the vulva, or it takes an abnormal path outside the VNC, or it turns at the vulva and projects aberrantly (Fig. 5). The main axon of the AVL motorneuron, which runs along the entire length of the VNC was more difficult to examine since it is obscured in the anterior quarter of the VNC by the RMEV motorneuron, which also expresses unc-47::GFP (but not lim-6). Nevertheless, in the majority of lim-6 mutant animals, the AVL motorneuron does not extend past the RMEV motorneuron (Table 3; Fig. 5). In summary, both the DVB and the AVL motorneurons require lim-6 either to acquire or maintain intact axon morphology.

The PVT neuron is a presumptive guidepost of the VNC and is possibly involved in directing axons of the ventral cord (Wadsworth et al., 1996). Since PVT expresses lim-6, we examined whether a presumptive PVT function in VNC organization requires lim-6. VNC organization was monitored...
using a cat-1::GFP reporter gene construct (S. Nurrish and J. Kaplan, personal communication) and a lin-11::GFP reporter gene construct (Hobert et al., 1998), each of which is expressed in axons of the right and left VNC. Transgenic lim-6 mutant animals expressing these reporter genes revealed no obvious defect in VNC organization.

We conclude that lim-6 is not necessary for neurons to be generated and to execute certain aspects of their differentiation program. However, their patterns of neurite outgrowth depend on lim-6 gene activity.

**lim-6 participates in the cell-type-specific regulation of glutamic acid decarboxylase**

Glutamic acid decarboxylase (GAD) is the rate limiting enzyme for synthesis of the neurotransmitter γ-aminobutyric acid (GABA) and is encoded by the *C. elegans* unc-25 gene (Jin et al., 1999). A translational fusion of the promoter and the first 13 codons of unc-25 to GFP is expressed in all GABAergic neurons (Jin et al., 1999). The expression of this fusion gene in *lim-6(nr2073)* mutant animals is significantly decreased in the GABAergic neurons DVB and RIS and to a lesser extent in AVL (Fig. 6; Table 4). unc-25::GFP expression is normal in the RMEL/R motorneurons, which normally express lim-6, and in the RMED/V motorneuron and the D-type motorneurons of the VNC, neither of which express lim-6. A translational GFP fusion to unc-25, that encompasses the full coding sequence of unc-25 (see Material and Methods) showed a similar pattern of expression in GABAergic neurons and was also affected by the absence of lim-6 gene activity; while 96% (*n* = 23) of wild-type animals show strong expression in DVB, only 28% (*n* = 25) of lim-6(nr2073) mutant animals show equally strong expression, 44% show weak expression and 28% show no expression (data not shown). We conclude that lim-6 contributes to the cell-type-specific regulation of unc-25 expression in the DVB, RIS and AVL neurons but because the lim-6 effects are quantitative, other factors also contribute to unc-25 expression.

We tested whether the neuroanatomical defects of the DVB motorneuron in the lim-6 mutant might be related to defects in GABAergic signaling, perhaps due to LIM-6 regulation of unc-25 expression. For example, active DVB synaptic signaling may be required for normal axon morphology in a manner similar to other activity-dependent structural changes in the nervous system (Dahm and Landmesser, 1988, 1991). To observe DVB morphology in animals lacking GABA signaling, we observed DVB axon morphology using the unc-47::GFP reporter gene in GABA-deficient *unc-25* mutant. We found that even in the complete absence of GABA, the DVB motorneuron maintained its correct axon morphology (Table 3). An electron microscopical reconstruction of the synaptic connectivity of *unc-25* mutant animals has led to a similar conclusion (Jin et al., 1999). It is conceivable that lim-6 indirectly affects unc-25 expression through the effects of lim-6 on axonal pathfinding. However, this possibility is unlikely since several genes known to affect axonal pathfinding of GABAergic neurons, such as *unc-34, unc-71* or *unc-76*, do not affect GABA synthesis and thus unc-25 expression (McIntire et al., 1992). Thus, it is most likely that the effect of lim-6 on DVB axonal morphology represents a pathway that is parallel to its regulation of unc-25 expression.

### Table 4. unc-25::GFP (juIs8) expression in lim-6 expressing neurons

<table>
<thead>
<tr>
<th></th>
<th>DVB</th>
<th>RIS</th>
<th>AVL</th>
<th>RMER/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>(<em>n</em> = 30)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lim-6(nr2073)</td>
<td>10%</td>
<td>22%‡</td>
<td>61%§</td>
<td>100%</td>
</tr>
<tr>
<td>(<em>n</em> = 30)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Expression was not uniform; in 57% of the animals expression was strong, in the remainder the expression was weak but still significant.

‡In these 22% the expression was as strong as in wildtype, in the remaining 78% the expression ranged from very faint to entirely absent.

§These 61% include strong (22%) and weak (39%) expression, in the remainder the expression ranged from very faint to entirely absent.

**Dauer arrest is regulated by GABA and lim-6**

Sensory inputs to *C. elegans* not only elicit motor outputs. As in other animals, there are endocrine outputs of the nervous system. For example, sensory detection of a pheromone...
controls neuroendocrine outputs mediated by insulin-like and TGFβ-like signaling molecules that in turn regulate the metabolism of the whole animal and regulate whether development is arrested at the dauer diapause stage or continues to the reproductive adult stages (Riddle and Albert, 1997). A variety of sensory inputs are integrated by distinct neural circuits, for example food signals, crowding and temperature, to affect the neuroendocrine control of dauer stage entry (Riddle and Albert, 1997). We noted that unlike wild-type animals lim-6(nr2073) animals occasionally form dauers under conditions in which the animals are fairly crowded yet not completely starved; their pheromone responsiveness was unaffected, however (data not shown). As these dauer inducing conditions were difficult to reproduce, we investigated lim-6 regulation of dauer arrest by sensitizing the genetic background to make the animals more prone to enter the dauer stage. We used a strain mutant for the daf-7/TGFβ gene, a neuroendocrine signaling molecule that synergizes with an insulin-like signaling cascade to repress entry into the dauer stage (Kimura et al., 1997; Ogg et al., 1997; Ren et al., 1996). In the absence of daf-7/TGFβ signaling, the animals are sensitized to enter the dauer stage; e.g. they now become hypersensitive to specific environmental stimuli such as changes in their ambient temperature (Riddle and Albert, 1997). Consistent with the enhanced dauer arrest under weakly dauer inducing conditions of the lim-6 mutant alone, lim-6(nr2073); daf-7(e1372) double mutant animals are significantly enhanced for dauer arrest compared to daf-7(e1372) at 15˚C (Table 5).

In vertebrates, the neurotransmitter GABA is expressed in several neuroendocrine tissues and affects the release of insulin (Gu et al., 1993; Sorensen et al., 1991). Considering the expression of lim-6 in GABAergic neurons we examined whether defects in GABAergic signaling could phenocopy the effects of lim-6(nr2073) on dauer arrest: unc-25(e156) mutant animals, which are GABA deficient (McIntire et al., 1993a), are neither defective in dauer arrest, nor do they enter the dauer stage constitutively (Table 5). However, like lim-6(nr2073), the unc-25(e156) mutation strongly enhances daf-7(e1372) at 15˚C (Table 5). This effect of unc-25 is not due to a requirement for unc-25 in the D-type ventral cord motorneurons, since unc-30, a gene that abolishes GABAergic cell fate in the D-type motorneurons, but not in the other GABAergic neurons (Jin et al., 1994), does not enhance daf-7(e1372) (Table 5). The enhancement of daf-7(e1372) by unc-25(e156) is unlikely to be a non-specific secondary consequence of constipation, since neither aex-2(sa3) mutant animals, which are 100% EMC defective (Liu and Thomas, 1994) nor sem-4(n2654) mutant animals, which are 84% EMC defective (Basson and Horvitz, 1996) enhance daf-7 induced dauer formation to a similar degree as unc-25 (Table 5). We conclude that GABA signaling normally activates reproductive development or represses dauer arrest. Since the absence of functional D-type motorneurons in unc-30 does not affect dauer formation, GABA regulation of dauer arrest is most likely mediated through either the RME, AVL, RIS or DVB neurons. As each of these neurons expresses lim-6 and as the absence of lim-6 gene activity phenocopies the absence of GABAergic function we consider it most likely that lim-6 activity in these neurons is required for their functional specification to in turn affect GABA control of dauer arrest. For example, neurons postsynaptic to these GABA neurons may secrete DAF-7 or one of the dozens of insulin-like genes that are likely to regulate dauer arrest (S. Pierce, L. Liu and G. R., unpublished).

**lim-6 affects asymmetric sensory receptor expression**

Many neurons in the nervous system of *C. elegans* come in bilaterally symmetric pairs (White et al., 1986), yet lim-6::GFP reporter gene constructs are only expressed in the left neuron (ASEL) of the ASER and ASEL chemosensory pair (Fig. 2, Table 1; Bargmann and Horvitz, 1991). Using the ASEL-expressed lim-6prom::GFP reporter gene construct, we found that ASEL is generated and pathfinds normally in lim-6 mutant animals (data not shown). We examined the expression of the two ASE differentiation markers gcy-6 and gcy-7, two putative guanylyl cyclase-type sensory receptors that like lim-6 are exclusively expressed in ASEL, but not in ASER (Yu et al., 1997). We found that gcy-6 and gcy-7 GFP reporter gene constructs are correctly expressed in lim-6 mutant animals (Fig. 7), suggesting that lim-6 gene activity is not necessary for these aspects of ASE neural maturation. However, lim-6 activity is required for the correct expression of another putative guanylyl cyclase-type sensory receptor, gcy-5. A gcy-5 reporter gene construct is normally expressed in the right neuron, ASER, but not in ASEL (Yu et al., 1997). In lim-6(nr2073) mutant animals, the gcy-5 reporter gene is ectopically expressed in ASEL (Fig. 7). Thus, lim-6 is normally required for repression of gcy-5 expression in ASEL. The LIM-6 transcription factor could either activate a repressor that directly acts on the gcy-5 promoter or LIM-6 could directly bind and repress the gcy-5 promoter. It is not known to what sensory inputs the gcy-5 receptor responds. Consequently, the physiological significance of disregulated gcy-5 receptor expression in lim-6 mutant animals is hard to assess at the moment. We did, however, examine lim-6(nr2073) animals for their responsiveness to various water-soluble chemicals that are sensed by ASEL and ASER, but could not detect any defects (data not shown).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Dauer arrest at 15°C (n)</th>
<th>Dauer arrest at 25°C (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0% (278)</td>
<td>0% (228)</td>
</tr>
<tr>
<td>lim-6(nr2073)</td>
<td>0% (179)</td>
<td>0% (204)</td>
</tr>
<tr>
<td>unc-25(e156)</td>
<td>0% (156)</td>
<td>0% (116)</td>
</tr>
<tr>
<td>unc-30(e191)</td>
<td>0% (378)</td>
<td>0% (79)</td>
</tr>
<tr>
<td>daf-7(e1372)</td>
<td>0% (379)</td>
<td>97% (64)</td>
</tr>
<tr>
<td>daf-7(e1372); lim-6(nr2073)</td>
<td>88% (189)</td>
<td>100% (&gt;100)</td>
</tr>
<tr>
<td>daf-7(e1372); unc-25(e156)</td>
<td>73% (127)</td>
<td>93% (244)</td>
</tr>
<tr>
<td>daf-7(e1372); unc-30(e191)</td>
<td>5% (552)</td>
<td>100% (61)</td>
</tr>
<tr>
<td>daf-7(e1372); aex-2(sa3)</td>
<td>26% (123)</td>
<td>100% (75)</td>
</tr>
<tr>
<td>daf-7(e1372); sem-4(n2654)</td>
<td>10% (380)</td>
<td>100% (&gt;100)</td>
</tr>
</tbody>
</table>
The expression of the vertebrate \textit{lim-6} homolog \textit{Lmx-1} is regulated by a vertebrate wingless homolog (Riddle et al., 1995; Vogel et al., 1995); wingless signaling in \textit{C. elegans} is involved in determining asymmetric features of cell division (Sawa et al., 1996). We tested whether \textit{lin-17}/wingless mutants affect the asymmetry of \textit{lim-6} expression, but found no effects (data not shown). Other wingless like genes that are present within the \textit{C. elegans} genome (Ruvkun and Hobert, 1998) might be involved in regulating asymmetric features of \textit{lim-6} expression.

\textbf{\textit{lim-6} and excretory gland cell function}

The excretory gland cell displays a neuron-like appearance; it contains specific types of secretory vesicles of unknown function and extends thin processes towards the nerve ring, where it receives synaptic input (Nelson et al., 1983). To examine the effects of the \textit{lim-6(nr2073)} mutation on excretory gland cell morphology and function, we attempted to observe the cell with the \textit{lim-6prom::GFP} reporter gene, which labels the entire gland cell in wild-type animals. Interestingly, the \textit{lim-6} reporter gene construct is not expressed in the excretory gland cell of \textit{lim-6} mutant animals, whereas its expression in the other neurons and epithelial cells is unaffected (data not shown). This suggests that \textit{LIM-6} may autoregulate its own expression in the excretory gland cell or that there are severe differentiation defects of the excretory gland cell. Because laser ablation of the excretory gland cell has no significant impact on growth, moulting, osmoregulation, fertility, longevity, and dauer larva formation (Nelson and Riddle, 1984), we did not assess the functional consequences of the absence of \textit{lim-6} activity in the excretory gland cell. Vertebrate \textit{Lmx-1} is required for the specification of basement membrane structures in the kidney. An ultrastructural analysis of \textit{lim-6} mutant animals will be required to examine whether \textit{lim-6} might have a similar function in \textit{C. elegans}.

We found that the extension of excretory gland cell processes uses guidance mechanisms similar to those used by neurons, since several neural pathfinding genes, such as \textit{unc-33}, \textit{unc-73} and \textit{unc-76} (Hedgecock et al., 1987), affect excretory gland cell morphology as monitored by crossing \textit{lim-6prom::GFP} into these mutants (data not shown). Since \textit{lim-6} is required to specify the correct neuroanatomy of several neurons, as described above, it is conceivable that by regulating a similar set of target genes \textit{lim-6} also affects the process morphology of the excretory gland cell.

\textbf{\textit{lim-6} function in uterine morphogenesis}

\textit{lim-6} mutant animals have a significantly reduced brood size (Table 6). Moreover, most of the progeny of \textit{lim-6} mutant animals hatch within the parent animal. The uterine expression of \textit{lim-6} prompted us to examine the morphology of the uterus in the \textit{lim-6(nr2073)} mutant. The main tissue types of the uterus are the epithelial uterine toroid (ut) cells, which line the uterine lumen and which express \textit{lim-6} (see above), and specialized utve and uv cells, which constitute the connection between the uterus and the vulva (Newman et al., 1996). Using DIC microscopy we found that the uterine lumen is not formed correctly in 78\% of \textit{lim-6} mutant animals and appears to be clogged (Fig. 8; Table 6). The extent of clogging varies from animal to animal and can range from a simple ‘bridge’ as shown in Fig. 8 to a complete closure of the uterine lumen. This tissue that clogs the uterine lumen expresses \textit{lim-6} and could represent ut cells that have not separated correctly (Fig. 8). The \textit{lim-6(nr2073)} egg laying defects could be explained by the animal’s inability to accommodate eggs in the uterus. A hindered passage through the uterus would congest the gonad and inhibit further fertilizations, thus explaining the low brood size of the animals (which is even smaller than those of vulvless animals, such as \textit{lin-11(n389)}; Freyd, 1991). However, the uterine closure defect is not 100\% penetrant, while the low brood size clearly is. This argues for additional defects in the reproductive system. The expression of \textit{lim-6} in the junction cells of the spermatheca described above could indicate that eggs in \textit{lim-6(nr2073)} mutants might have problems in passing through the spermatheca thus reducing fertility. In summary, we have shown that \textit{lim-6} is required for uterine morphogenesis.
DISCUSSION

lim-6 is one of seven C. elegans LIM homeobox genes revealed by the genome sequence. Most of these LIM homeobox genes detect mammalian orthologs (Fig. 1). The determination of the function of these C. elegans LIM homeobox genes with precise cellular identifications and correlations with neuron type and function should broadly indicate the function of these genes across phylogeny. lim-6 is an interesting case because while the function of the mammalian ortholog Lmx-1b was much better understood than lim-6 before this study, our genetic and cellular analysis suggests new functions to search for in mammals.

Our results show that lim-6 functions mainly in GABAergic neurons that regulate enteric muscles and endocrine outputs, and uterine development. It is not yet clear which of the C. elegans lim-6 functions are generalizable to its mammalian orthologs Lmx-1a and Lmx-1b. It is possible that some of the lim-6 functions are served by one ortholog and others by the other. Consistent with such a model, the regulation of neuroendocrine signals by both C. elegans lim-6 (dauer regulation) and mammalian Lmx-1a (insulin regulation) suggests common, ancestrally related functions. Similarly, the requirement for Lmx-1b in mammalian kidney function and the lim-6 expression in the C. elegans excretory gland cell suggests a common function in excretory system differentiation for this LIM homeobox gene. Our data also suggests that expression of mammalian Lmx-1a and Lmx-1b in the central and peripheral nervous system should be correlated with GABAergic neural markers. Lmx-1b knock-out mice exist (Chen et al., 1998) but have not been analyzed for of GABAergic neuroendocrine or enteric muscle control defects. The C. elegans function of this class of LIM homeobox genes suggests that GABAergic function (for example sensitivity to GABA-related drugs such as valium) in the Lmx-1a or Lmx-1b knockout mice should be assayed.

Table 6. lim-6 is required for the development of the reproductive system

<table>
<thead>
<tr>
<th>Brood size</th>
<th>Wild type</th>
<th>lim-6(nr2073)</th>
<th>lim-6(nr2073); Ex[lim-6] or Ex[lim-6::VP16]</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of progeny at 22°C</td>
<td>207±42 (n=10)</td>
<td>15±10 (n=49)</td>
<td>125±26* (n=8)</td>
</tr>
<tr>
<td>Morphology of the uterine lumen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>both lumenal sides open</td>
<td>96%</td>
<td>22%</td>
<td>100%‡</td>
</tr>
<tr>
<td>one lumenal side closed</td>
<td>4%</td>
<td>41%</td>
<td>0%‡</td>
</tr>
<tr>
<td>both lumenal sides closed</td>
<td>0%</td>
<td>37%</td>
<td>0%‡</td>
</tr>
</tbody>
</table>

*The array line mgEx446 was tested for rescue. 8 out of 12 animals tested showed at least partial rescue of the brood size.
‡Due to the partial rescue of the lim-6 expression construct we increased the activity of the VP16 transcriptional activation domain. We had previously shown that adding the VP16 domain to the LIM homeobox gene ttx-3 significantly increases its ability to complement a ttx-3 null mutant (O. H., I. Mori, G. R., unpublished data). The lim-6::VP16 construct, expressed from the extrachromosomal arrays mgEx408 and mgEx409, is also very potent in complementation of the other lim-6 defects (see Materials and Methods).

Our data also suggests that uterine development should be analyzed. The expression of vertebrate Lmx-1 in the uterus has not been reported. However, Wnt-7a, which induces Lmx-1b expression in the developing limb (Riddle et al., 1995; Vogel et al., 1995), has recently been shown to act in uterine development (Miller and Sassoon, 1998). As in limb development the expression of Wnt-7a in the uterus might similarly be coupled to the induction of Lmx-1b expression in the uterus. However, it is also possible that some of the lim-6 functions in C. elegans may not be primitive and universal.

lim-6 function in the GABAergic nervous system

The most prominent site of expression of lim-6 in the nervous system are GABAergic neurons; five of the nine lim-6-expressing neurons are GABAergic. The complete GABAergic nervous system of C. elegans consists of 26 neurons (McIntire et al., 1999). They can be functionally subdivided into the D-motorneurons of the ventral nerve cord, which are required

![Fig. 8. Uterine defects in lim-6(nr2073) mutant animals. (A) Wild-type animal showing the continuous opening of the uterine lumen. (B,C) Two representative photomicrographs (using DIC microscopy) of uterine closure defects in lim-6(nr2073). The upper two arrows point to the uterine closure. Occasionally the whole uterine lumen is closed. (D) lim-6prom::GFP in lim-6(nr2073). The animal is identical to that in C.](image-url)
for locomotion, the AVL/DVB motoneurons required for enteric muscle contraction, the RME motoneurons required for foraging and the RIS interneuron with an unknown function. The unc-30 homeobox gene is exclusively expressed in and required by one specific subset of the GABAergic neurons, the D-type motoneurons, for the acquisition of the GABAergic neural cell fate (Jin et al., 1994). Interestingly, the expression pattern and function of lim-6 is almost entirely complementary to the the expression pattern and function of unc-30 in the GABAergic nervous system; lim-6 is not expressed in the D-type motoneurons, but is expressed in all other GABAergic neurons (with the exception of two dorsal and ventral types of the fourfold symmetric RME motoneurons). However, lim-6 does not appear to be the functional counterpart of unc-30 in these neurons, since unlike unc-30 the lim-6 gene is not as tightly required for the expression of all GABAergic-specific cell specializations. For example, the expression of the unc-47 GABA transporter in the non-D-type GABAergic neurons is largely unaffected and the expression of the unc-25 /GAD gene is only partially affected by the lim-6 null mutation. Presumably other transcription factors can also regulate unc-25 and unc-47 expression in the non D-type motoneurons, suggesting that the genes that define GABAergic neural identity are regulated by different regulatory mechanisms in different subtypes of GABAergic cells.

The functional and neuroanatomical defects of the AVL and DVB neurons in the lim-6 null mutant strongly suggest that the function of these neurons is defective. The neuroanatomical defects which manifest as sprouting defects or the inability of the main axonal projection to follow correct paths could either represent an inability of the neuron to correctly engage its axonal outgrowth machinery, or alternatively, could represent a secondary consequence of neural activity defects. Neural activity defects have been shown before to cause axonal misrouting in both C. elegans and vertebrates (Coburn and Bargmann, 1996; Coburn et al., 1998; Dahm and Landmesser, 1988, 1991; Shatz and Stryker, 1988). lim-6 could be required for neural activity of GABAergic neurons by regulating the expression of genes directly involved in generating electric activity or regulating genes required to generate the synaptic connectivity required to receive and transmit electrical signals.

The defects in non-D-type GABAergic function of the lim-6 mutant can account for the defects in defecation cycle that the animals show. Defecation behavior requires the activation of the defecation motor program. This program represents a rhythmic behavior composed of three stereotyped muscle contractions; one of these rhythmic contractions, the enteric muscle contraction is controlled by two GABAergic motoneurons, AVL and DVB (Liu and Thomas, 1994; McIntire et al., 1993b). Mutations in GABAergic signaling, for example in the unc-47 GABA transporter or the unc-25 GABA synthesizing enzyme, cause very similar enteric muscle contraction defects (McIntire et al., 1993a). Similarly, GABAergic neurons are also part of the neural circuit that controls gut function in vertebrates (Jessen et al., 1986) and enteric GABA has been shown to regulate the peristaltic effects in the vertebrate enteric system (Grider and Makhlouf, 1992). Moreover, muscle contractions of the enteric system are also rhythmically active in vertebrates. This rhythmicity is regulated by the electric pacemaker activity of the interstitial cells of Cajal, which function as an intermediary between enteric nerves and smooth muscle cells (Sanders, 1996). It will be interesting to see if human patients with Nail Patella syndrome, a haploinsufficiency of Lmx-1b, or mouse carrying Lmx-1a or Lmx-1b knockout mutations, also show defects in enteric motor function, perhaps due to a common GABAergic input to enteric muscles.

Our data favors an output from the GABAergic neurons to the neuroendocrine regulation of dauer formation which depends on lim-6 activity. We find that lim-6 loss of function strongly enhances the neuroendocrine signaling defects caused by a daf-7/TGFβ mutation. This neuroendocrine function of lim-6 is likely to be due to its function in GABAergic neurons, since unc-25 mutant animals with defects in GABA synthesis show the same phenotype. Because animals with defective D-type motor neurons do not have these neuroendocrine defects, these data argue that it is the lim-6-regulated GABAergic neurons that regulate C. elegans endocrine signaling. Although we can not exclude that lim-6 affects dauer arrest through other, as yet unidentified sites of expression, the simplest model is that lim-6 specifies functional aspects of the non-D-type GABAergic neurons that in turn regulate dauer arrest via GABAergic signaling.

The expression of lim-6 in the GABAergic neuroendocrine system also reveals an interesting similarity to the expression of its vertebrate homolog Lmx-1a. Vertebrate Lmx-1a participates in the control of insulin gene expression in the β cells of the pancreas (German et al., 1992) which also contain high levels of the neurotransmitter GABA. GABA regulates insulin secretion from the β cells via an autocrine loop (Gu et al., 1993; Sorensen et al., 1991). We find that both lim-6 and non D-type GABAergic neurons in C. elegans regulate dauer arrest, which is regulated by an insulin-like neuroendocrine pathway. It is possible that lim-6 regulates the development of the particular GABAergic neurons that in turn regulate insulin expression and secretion in C. elegans. For example, these GABAergic neurons may couple temperature or food or other sensory inputs to the endocrine output of dauer regulatory proteins such as the many insulin-like proteins that may converge on the single insulin-like receptor that clearly regulates dauer arrest. The synergizing effects of mutations in lim-6 and GABA signaling on the daf-7/TGFβ control of dauer formation indeed mirror the synergism between insulin signaling and daf-7/TGFβ signaling (Ogg et al., 1997). However, the complexity of the insulin family in C. elegans (Duret et al., 1998) complicates the matter significantly.

**Asymmetry in the C. elegans nervous system**

The major head ganglia of C. elegans display an obvious overall bilateral symmetry. The majority of neural cell types have both a left and right representative that share a comparable lineage history and morphology and make similar synaptic connections (Sulston, 1983; White et al., 1986). In those cases tested, both left and right neurons are required for a full functional response of, for example, a given pair a chemosensory neurons (Bargmann and Horvitz, 1991). The asymmetric expression of lim-6 in ASEL, but not in ASER thus represents a surprising observation. It may suggest a potential diversification of apparently symmetric sensory neurons possibly leading to a diversification in sensory function. This notion is supported by the asymmetric expression of several receptor-type guanylyl
cycrases in the ASER and ASEI neurons (Yu et al., 1997), one of which we have shown to be under control of lim-6 activity. As the function of these receptor-type guanylyl cyclases is as yet unknown, we could not assess the physiological consequences of ectopic activation of one of these receptors. Gross defects in ASE sensory functions were not obvious in lim-6 mutant animals (data not shown).

It will be interesting to determine how the asymmetry of lim-6 expression is established. In vertebrates, Lmx-1b expression is under control of wingless signaling (Riddle et al., 1995; Vogel et al., 1995); wingless signaling in C. elegans is involved in determining the polarity of certain cell divisions (Sawa et al., 1996). Although we have not found an impact of the wingless-like lin-17 gene on lim-6 expression (data not shown), it is possible that any other of the five wingless-like genes present in the C. elegans genome (Ruvkun and Hobert, 1998) are involved in regulating lim-6 expression and possibly its asymmetry. The asymmetrically expressed lim-6 reporter gene construct is a tool for the genetic identification of mutants involved in establishing asymmetry in the nervous system.

LIM homeobox gene function in C. elegans: a common theme?

Homeobox genes have been shown to act at a variety of different stages of neurogenesis, including such early steps as the determination of neural identity and later steps such as neural differentiation (Manak and Scott, 1994). The analysis of 4 of the 7 LIM homeobox genes in C. elegans demonstrates that they are required for the terminal differentiation of the neurons that express them (Hobert et al., 1998, 1997; Way and Chalfie, 1988, 1989; this study). In many respects the neural defects caused by mutations in these genes are similar. Mutations in lim-6, like mutations in the ttx-3 and lin-11 LIM homeobox genes do not affect the generation of the neurons that express them (Hobert et al., 1998, 1997) nor do they affect several aspects of neurotransmitter choice (this study and our unpublished data). However, the lim-6-, ttx-3- and lin-11-expressing neurons are functionally as well as structurally defective (Hobert et al., 1998, 1997; this report). Similarly, mec-3 is required for the structural integrity of mechanosensory neurons (Way and Chalfie, 1998). These similarities in LIM homeobox gene function suggest a common theme in the action of this class of transcriptional regulators. We speculate that each gene is required in its respective neuron to make a specific target choice and that in the absence of intact signaling partners, retrograde signaling events induce the neuron to find a signaling partner by sprouting additional processes or by inducing abnormal turns of the main axonal process. Alternatively, each gene might be required within its given neuron to directly regulate its axon outgrowth machinery. Our finding that defects in the GABA neurotransmitter synthesis do not cause axonal sprouting and the report of Jin et al. that GABAergic neurons and their postsynaptic partners show no defects in synaptic connectivity in unc-25 mutants (Jin et al., 1999) indicate that defects in synaptic outputs are unlikely to feedback on neurite outgrowth functions. However, defects in other synaptic outputs, such as signaling by neuropeptides might be feeding back into axonal sprouting or, alternatively, defects in synaptic inputs to the GABAergic neurons, caused for example by connectivity defects, could induce axonal sprouting.

Another common feature of all C. elegans LIM homeobox genes studied to date is that their onset of expression in postmitotic neurons and the maintenance of their expression throughout adulthood (Hobert et al., 1998, 1997; Way and Chalfie, 1989). These observations are unlikely to be a reporter gene artefact, since the expression in other tissues, such as lim-6 in the uterus or lin-11 in the vulva is dynamic and transient. Maintained expression of a regulatory gene throughout the life of a given cell suggests, but does not prove an involvement of the particular gene in maintenance of the differentiated features of the given cell. In the case of LIM homeobox genes it is possible that they are required for the maintenance of such neural features as synaptic connectivity.

Lastly, all of the C. elegans LIM homeobox genes described so far are expressed in a largely non-overlapping subsets of neurons (Hobert et al., 1998, 1997; Way and Chalfie, 1989; this study; D. H. and G. R., unpublished). The most prominent non-neural tissue that expresses C. elegans LIM homeobox genes is the epithelium of the somatic gonad and the vulva. Intriguingly, the LIM homeobox genes are also expressed in a complementary, non-overlapping pattern in this epithelium. lin-11 is expressed in the vulva and cells that connect the uterus to the vulva (Freyd, 1991; Hobert et al., 1998), lim-6 is expressed in uterine toroid cells and in spermathecal junction cells (this study), ceh-14 is expressed in the epithelial cells of the spermatheca (T. Bürglin, personal communication) and we found another LIM homeobox gene, lim-7, to be expressed in the gonadal sheath cells (O. H. and G. R., unpublished). The unifying feature of all these cells is their highly polarized morphology and their engagement in complex morphogenetic events for which specific cell-cell contacts are required (Newman et al., 1996). These morphological features are shared by neurons as well. Moreover, epithelial cells and neurons utilize similar sorting and targeting mechanisms for specific cell surface molecules (Bredt, 1998; Rongo et al., 1998). It is conceivable that by regulating the expression of specific cell surface proteins, LIM homeodomain proteins in C. elegans are generally involved in determining the specificity of cell-cell recognition and attachment to neighboring cells both in the nervous system and in epithelial cells. The structural defects of uterine cells in lim-6 mutants indeed point to a role at least of lim-6 in cell recognition and adhesion.

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