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Dissection of *lin-11* enhancer regions in *Caenorhabditis elegans* and other nematodes

Sujatha Marri, Bhagwati P. Gupta *

Department of Biology, McMaster University, Hamilton, ON L8S 4K1, Canada

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ABSTRACT

The *Caenorhabditis elegans* LIM homeobox gene *lin-11* plays crucial roles in the morphogenesis of the reproductive system and differentiation of several neurons. The expression of *lin-11* in different tissues is regulated by enhancer regions located upstream as well as within *lin-11* introns. These regions are functionally separable suggesting that multiple regulatory inputs operate to control the spatiotemporal pattern of *lin-11* expression. To further dissect apart the nature of *lin-11* regulation we focused on three *Caenorhabditis* species *C. briggsae*, *C. remanei*, and *C. brenneri* that are substantially diverged from *C. elegans* but share almost identical vulval morphology. We show that, in these species, the 5′ region of *lin-11* possesses conserved sequences to activate *lin-11* expression in the reproductive system. Analysis of the *in vivo* role of these sequences in *C. elegans* has led to the identification of three functionally distinct enhancers for the vulva, VC neurons, and uterine π lineage cells. We found that the π enhancer is regulated by FOS homolog FOS-1 and LIN-12/Notch pathway effectors, LAG-1 (Su(H)/CBF1 family) and EGL-43 (EVI1 family). These results indicate that multiple factors cooperate to regulate π-specific expression of *lin-11* and together with other findings suggest that the mechanism of *lin-11* regulation by LIN-12/Notch signaling is evolutionarily conserved in *Caenorhabditis* species. Our work demonstrates that 4-way comparison is a powerful tool to study conserved mechanisms of gene regulation in *C. elegans* and other nematodes.

Introduction

The vulva in the nematode *C. elegans* serves as a paradigm to study mechanisms of gene interaction and function during organ formation (Inoue et al., 2005; Sternberg, 2005). The adult vulva is composed of 22 cells that are derived from 3 out of 6 equipotential vulval precursor cells (VPCs). The 3 VPCs are induced to adopt 1^0 or 2^0 cell fates by evolutionarily conserved signaling pathways mediated by Ras, Notch and Wnt (Sternberg, 2005). While much is known about the roles of various pathway components in specifying cell fates, little is understood how these pathways interact to regulate downstream genes in cell and tissue-specific manner. Genetic analysis of vulval development has identified some of the targets of signaling pathways including a LIM homeobox family member *lin-11* (Ferguson et al., 1987; Freyd et al., 1990). *lin-11* mutants are egg-laying defective due to the lack of a functional vulva, vulval-uterine connection, and associated neurons (Ferguson et al., 1987; Garriga et al., 1993a; Gupta et al., 2003; Newman et al., 1999). The analyses of mutant phenotype and reporter gene expression studies have revealed that *lin-11* is required for the specification of multiple cell types in the reproductive system (Gupta et al., 2003; Newman et al., 1999).

Genetic epistasis and reporter gene expression studies have shown that *lin-11* is regulated by the Ras, Notch, and Wnt signaling pathways (Ferguson et al., 1987; Gupta and Sternberg, 2002; Newman et al., 1999). The mechanism of how these pathways converge on the *lin-11* promoter is not fully understood. We had earlier dissected the genomic regions of *lin-11* and identified two enhancers for the vulva and uterine π lineage cells that respond to Wnt and Notch signaling, respectively (Gupta and Sternberg, 2002). Here we describe our findings on the evolutionary conservation of *lin-11* regulation in the reproductive system. We isolated *lin-11* 5′ sequences from *C. briggsae*, *C. remanei*, and *C. brenneri*, the three closest known relatives of *C. elegans* (Kiontke and Fitch, 2005), and examined their regulatory function. These species exhibit significant morphological similarity and conservation in some of the developmental processes, although their reproductive life styles are different (*C. elegans* and *C. briggsae*: hermaphroditic, *C. remanei* and *C. brenneri*: gonochoristic) (Barriere and Felix, 2006; Kiontke et al., 2007; Kiontke and Fitch, 2005). Phylogenetic studies have revealed that while *C. briggsae* and *C. remanei* are sister species, *C. brenneri* and *C. elegans* represent the two closest known outgroups (Fig. 1) (Kiontke and Fitch, 2005). Our *GFP* reporter expression and phenotypic rescue experiments illustrate that *lin-11* 5′ genomic regions in all four *Caenorhabditis* species share important functional elements. We identified conserved stretches of nucleotides and tested their *in vivo* role using deletion constructs. This led to the identification of three conserved tissue-specific enhancer elements for the vulva, ventral cord neurons (VCNs) and uterine π lineage cells. Further studies revealed that the π-specific enhancer possesses sites for transcription factors FOS-1 (FOS family), LAG-1 (Su(H)/CBF1 family) and EGL-43 (EVI1 family) and that these three factors are necessary for π-lineage expression of *C. elegans lin-11*. Since *lag-1*

* Corresponding author. Fax: +1 905 522 6066.
E-mail address: guptab@mcmaster.ca (B.P. Gupta).

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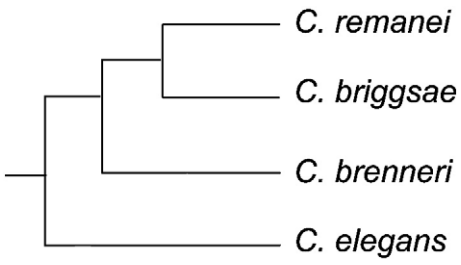

Fig. 1. The phylogenetic relationship among *Caenorhabditis* species used in this study.

and *egl-43* act downstream of the LIN-12/Notch pathway, these results suggest that, similar to *C. elegans*, LIN-12/Notch-mediated *lin-11* regulation may be involved in the formation of vulval-uterine connection in *C. briggsae*, *C. remanei* and *C. brenneri*.

Materials and methods

Strains and general methods

All strains were maintained at 20°C using standard culture methods (Brenner, 1974; Wood, 1988). Various strains used in this study are as follows. Wild type strains: N2 (*C. elegans*), AF16 (*C. briggsae*), SB146 (*C. remanei*), and PB2801 (*C. brenneri*). *C. elegans* strains: *lin-11*(*n389*) (Ferguson and Horvitz, 1985), *unc-119*(*ed4*) (Maduro and Pilgrim, 1995), *syIs80*[*pPGF11.13*(*Cel-lin-11p::GFP*)+ *unc-119*(+)] (Gupta et al., 2003). Transgenic *C. elegans* strains generated during the course of this work: *syIs103*[*pPGF11.13*(*Cel-lin-11p::GFP*)+ *unc-119*(+)], *syEx589* [*pPGF11.24*(*Cel-lin-11p::GFP*)+ *pha-1*(+)], *bhEx5*[*pCBlin11.45*(*Cbr-lin-11::GFP*)+ *unc-119*(+)], *bhEx20*[*pGLC3*(*Cre-lin-11::GFP*)+ *unc-119*(+)], *bhEx21*[*pGLC2*(*Cbn-lin-11::GFP*)+ *unc-119*(+)], *bhEx22*[*pGLC6*(*Cbr-lin-11::GFP*)+ *unc-119*(+)], *bhEx29*[*pPGF11.03*(*Cel-lin-11::GFP*)+ *unc-119*(+)], *bhEx32*[*GLF1*(*Cre-lin-11::GFP*)+ *unc-119*(+)], *bhEx33*[*GLF1*(*Cre-lin-11::GFP*)+ *unc-119*(+)], *bhEx34*[*GLF2*(*Cbn-lin-11::GFP*)+ *unc-119*(+)], *bhEx35* [*GLF2*(*Cbn-lin-11::GFP*)+ *unc-119*(+)], *bhEx39*[*pGLC18*(*Cel-lin-11::GFP*)+ *unc-119*(+)], *bhEx40*[*pGLC18*(*Cel-lin-11::GFP*)+ *unc-119*(+)], *bhEx41* [*pGLC17*(*Cel-lin-11::GFP*)+ *unc-119*(+)], *bhEx42*[*pPGF-LAG-1*(*Cel-lin-11::GFP*)+ *unc-119*(+)], *bhEx43*[*pPGF11.03-1*(*Cel-lin-11::GFP*)+ *unc-119*(+)], *bhEx44*[*pPGF11.03-1*(*Cel-lin-11::GFP*)+ *unc-119*(+)], *bhEx45*[*pGLC18* (*Cel-lin-11::GFP*)+ *unc-119*(+)], *bhEx46*[*pGLC16*(*Cel-lin-11::GFP*)+ *unc-119*(+)], *bhEx47*[*pGLC17*(*Cel-lin-11::GFP*)+ *unc-119*(+)], *bhEx60* [*pPGF11.23*(*Cel-lin-11::GFP*)+ *pha-1*(+)], *bhEx64*[*pGLC42*(*Cel-lin-11::GFP*+ *unc-119*(+)].

Transgenic worms were generated using *unc-119* (Maduro and Pilgrim, 1995) and *myo-2::GFP* (pPD118.33) (S. Q. Xu, B. Kelly, B. Harfe, M. Montgomery, J. Ahnn, S. Getz and A. Fire, personal communication) as rescue markers. The DNA microinjection technique was followed as previously described (Mello et al., 1991). While *GFP* transgene constructs were injected at 100 ng/μl, the *lin-11* phenotypic rescue constructs were injected at 25 ng/μl. To eliminate any array-specific bias in *GFP* expression or phenotypic rescue studies, more than one transgenic lines per construct were examined and the one with typical pattern was quantified and examined in detail.

Bacterial feeding RNAi experiments were carried out as previously described (Timmons et al., 2001). The *lin-11p::GFP* transgenic animals were grown on HT115 *E. coli* bacteria carrying RNAi constructs for *unc-22*, *sta-1*, *fos-1*, *lag-1*, and *egl-43*. Phenotypes were examined under Nomarski fluorescence microscopy.

Microscopy

Worms were mounted as described (Wood, 1988) and examined under Nomarski optics using Zeiss AxioImager D1 microscope. For *GFP* reporter expressing strains, epifluorescence was visualized by using a Zeiss AxioImager D1 or a Nikon Eclipse 80i microscope equipped with the GFP filter HQ485LP (Chroma Technology).

Genomic sequence and bioinformatics

The *Cre-lin-11* genomic sequence was retrieved from the Washington University St. Louis Genome Sequencing Center web server (*http://genomeold.wustl.edu/blast/client.pl*). In the case of *Cbn-lin-11*, a fosmid clone containing *lin-11* 5′ region and part of the open reading frame was sequenced (John DeModena and Paul W. Sternberg, personal communication). The *C. elegans* and *C. briggsae lin-11* sequences were obtained from the Wormbase (*http://www.wormbase.org*).

The MultiPipMaker (web-based) (*http://pipmaker.bx.psu.edu/pipmaker/*) and Mussa (standalone version, stable release 1.0.0) (*http://woldlab.caltech.edu/cgi-bin/mussa*) alignments were carried out using 5.0 kb *Cel-lin-11*, 4.5 kb *Cbr-lin-11*, 4.9 kb *Cre-lin-11*, and 3.9 kb *Cbn-lin-11* 5′ regulatory sequences. For MultiPipMaker, *Cel-lin-11* was chosen as a reference sequence for alignments. Mussa requires two user-defined parameters, a sliding window length and the minimum desired conservation (within each window), to search for the conserved blocks in input sequences. Mussa alignments were carried out using 67% identity in the overlapping blocks of 30 nucleotides.

Molecular biology

Except for the *lin-11* rescue constructs, that involved using *Cel-lin-11* cDNA as a PCR template, all other amplifications were carried out using N2 (*C. elegans*), AF16 (*C. briggsae*), SB146 (*C. remanei*), and PB2801 (*C. brenneri*) genomic DNA. The sequences of various primers are given in Supplementary Table 1. The *GFP* reporter constructs were made by subcloning genomic DNA into the Fire lab vectors pPD95.69 and pPD107.94 (a gift of S. Q. Xu, B. Kelly, B. Harfe, M. Montgomery, J. Ahnn, S. Getz and A. Fire).

The *Cel-lin-11p::GFP* plasmids were made by subcloning PCR amplified fragments into pPD107.94. The primer pairs and the corresponding products are as follows: FBG19 and BBG13 (334 bp, pPGF11.03-1), FBG18 and LAG-1-D1 (323 bp, pPGF11.03), GL326 and GL327 (59 bp, pPGF-LAG-1), GL198 and GL197 (355 bp, pGLC16), FBG18 and GL197 (407 bp, pGLC17), GL199 and GL197 (92 bp, pGLC18), and FBG17 and GL197 (150 bp, pGLC42). The PCR products were digested with *Hind*III and *Sph*I (pPGF11.03-1, pPGF11.03, pGLC16, pGLC17, and pGLC18), *Sph*I and *Stu*I (pPGF-LAG-1) and *Sph*I alone (pGLC42). The pPGF11.15 plasmid was made by dropping out *Xba*I fragments from pPGF11.33 (Gupta and Sternberg, 2002) and ligating the vector backbone.

The *C. briggsae* plasmids pCBlin11.45 and pGLC6 were constructed as follows. For pCBlin11.45, a 4.5 kb genomic fragment was amplified using primers cb-lin-11-up2 and cb-lin-11-down2. The PCR product was digested with *Sph*I, *BamH*I and subcloned into pPD95.69. For pGLC6, primers GL52 and GL53 were used to amplify a 764 bp genomic fragment that was digested with *Sph*I, *Xba*I and subcloned into pPD95.69. The *C. remanei* plasmid pGLC3 was made by subcloning a 4.9 kb *Sph*I, *Pst*I digested *lin-11* genomic fragment (amplified using primers GL37 and GL38) into pPD95.69. To obtain the *C. brenneri* plasmid pGLC2, a 3.9 kb *lin-11* genomic fragment was amplified using primers GL33 and GL34. The PCR product was digested with *Pst*I, *Hind*III and subcloned into pPD95.69. The *GFP* fusion products GLF1 (*Cre-lin-11p::GFP*, 2.8 kb) and GLF2 (*Cbn-lin-11p::GFP*, 2.4 kb) were constructed by nested PCR technique (Boulin et al., 2006). First, a 1.9 kb *GFP* coding region was amplified from pPD95.79 using primers GL25 and GL26. In two separate reactions, 3.1 kb *Cre-lin-11* and 2.1 kb *Cbn-lin-11* genomic fragments were obtained using primer pairs GL124, GL125 and GL33, GL121, respectively. These were then combined with 1.9 kb *GFP* product in nested PCR experiments to obtain GLF1 (primer pair GL181, GL174) and GLF2 (primer pair GL166, GL174).