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A regulatory cascade of three transcription factors in a single specific neuron, DVC, in *Caenorhabditis elegans*

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ABSTRACT

Homeobox proteins are critical regulators of developmental gene transcription and cell specification. Many insights into transcriptional regulation have been gained from studies in the nematode *Caenorhabditis elegans*. We investigated the expression and regulation of the *C. elegans* homeobox gene *ceh-63*, which encodes a single-homeodomain transcription factor of 152 amino acids. *ceh-63* is expressed in the interneuron DVC in both sexes, from late embryogenesis through adulthood, and two pairs of uterine cells in reproductive hermaphrodites only. A reporter gene fusion, encoding GFP fused to the full-length CEH-63, also drove weak inconsistent expression in additional unidentified cells in the head and tail. A potential *ceh-63* null mutant had no obvious abnormalities, except for a possible increase in subtle defects of the DVC axon projection. No behavioural responses were observed upon either laser ablation of DVC or activation of DVC through light stimulation of channelrhodopsin-2 specifically expressed in this neuron. The function of DVC therefore remains enigmatic. A transcriptional regulatory cascade operating in DVC was defined from the LIM-homeodomain protein CEH-14 through CEH-63 to the helix–turn–helix transcription factor MBR-1. Both CEH-14 and CEH-63 individually bound the *mbr-1* promoter in a yeast one-hybrid assay. A model is proposed suggesting that CEH-14 activates *ceh-63* and then along with CEH-63 co-ordinately activates *mbr-1*.

1. Introduction

In most animals, the nervous system is by far the most complex tissue raising fundamental questions about how each nerve cell identity is specified, how the axon of each cell follows the appropriate trajectory and how each synaptic connection is correctly established. The neuroanatomy of the nematode *Caenorhabditis elegans*, however, is described completely, from electron microscope serial section reconstructions, down to the level of individual synaptic connections (Bargmann, 2006; Chalfie et al., 1985; Hall and Russell, 1991; White et al., 1986). The adult hermaphrodite has precisely 302 neurons in 118 distinct classes. These questions can therefore, in *C. elegans*, be addressed for specifically identified individual nerve cells.

Regulatory *trans*-acting factors, e. g. transcription factors and microRNAs, acting combinatorially, are known to be critical determinants of neuronal cell fate specification (Hobert, 2004; Johnston et al., 2005). Transcription factors often act hierarchically, in a network, to confer a progressive restriction in the developmental potential of a neuronal subtype until terminal differentiation is established (Altun-Gultekin et al., 2001). The homeodomain family of transcription factors controls neuronal identities in both spatial and temporal domains, and homeodomain-transcription factor networks directing the specification of various neuronal subtypes in *C. elegans* have been described (Altun-Gultekin et al., 2001; Hobert, 2005; Hobert et al., 1998; Sarafi-Reinach et al., 2001; Tsalik et al., 2003). There are approximately 100 homeodomain transcription factor genes in the entire *C. elegans* genome (Reece-Hoyes et al., 2005).

Anatomical gene expression patterns, as revealed by reporter gene fusions, allow visualization, *in vivo*, of dynamic differential gene expression through neuronal specification. We have previously investigated the expression patterns of promoter-GFP fusion genes for 366 of the approximately 940 *C. elegans* transcription factor genes (Reece-Hoyes et al., 2007). Transgenic lines were generated by microparticle bombardment transformation (Praitis et al., 2001) using reporter gene fusions created by high-throughput Gateway recombinational cloning (Hartley et al., 2000) based on the Promoterome resource (Dupuy

Abbreviations: AD, activation domain; ANOVA, analysis of variance; Bp, base pairs; ChR2, channelrhodopsin-2; DAPI, 4′,6-diamidino-2-phenylindole; DB, DNA binding domain; DIC, differential interference contrast; DNA, deoxyribonucleic acid; EST, expressed sequence tag; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; Hr, hour; IPTG, isopropyl β-D-1-thiogalactopyranoside; modENCODE, model organism encyclopaedia of DNA elements; NGM, nematode growth media; NTP, nucleotide triphosphate; ORF, open reading frame; PCR, polymerase chain reaction; RNA, ribonucleic acid; RNAi, RNA interference; TF, transcription factor; UTR, untranslated region; Y1H, yeast one hybrid; Y2H, yeast two hybrid; YFP, yellow fluorescent protein.

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et al., 2004). A large proportion of these transcription factor gene promoters drove neuronal expression, reflecting the complexity of the *C. elegans* nervous system and the major role of transcription factors in the development of this tissue. Most expression patterns involving the nervous system were complex with GFP seen in many nerve cells and other tissues. However, the expression pattern driven by the promoter of one homeobox gene, then known as *C02F12.5* and now known as *C02F12.10* or *ceh-63* was strikingly simple consisting of a single nerve cell in the tail plus some weaker expression in the wall of the uterus (Reece-Hoyes et al., 2007).

With the characterization of *C02F12.5* ESTs, the gene initially annotated as *C02F12.5* in WormBase was subsequently annotated as two separate genes, *C02F12.10* and *C02F12.5* (Fig. 1). It is *C02F12.10* that contains the homeobox and for which the expression driven by the promoter had been assayed with the promoter::*gfp* fusion (Reece-Hoyes et al., 2007). *C02F12.10* has now been given the genetic gene name *ceh-63*. The absence of experimental data for the *ceh-63* gene model meant that *ceh-63* transcripts had to be characterized first before defining the *ceh-63* expression more precisely. We determined the identity of the nerve cell in which this gene is primarily expressed, investigated the function both of this nerve cell and of *ceh-63*, and defined a cascade of transcription factors working through CEH-63.

2. Materials and methods

2.1. C. elegans strains

All strains were maintained at 20°C on 5 cm NGM agar plates seeded with *Escherichia coli* OP50 as food source (Sulston and Hodgkin, 1988). *C. elegans* N2 (Bristol) was used as wild type. Transgenic *C. elegans* strains utilised were: TB513{*dpy-20*(*e2017*)*IV;chIs513* [*pHK107*(*ceh-14*(*1*st*exon*)*-gfp-w/o-NLS*),*dpy-20*(+)]*V*} (Cassata et al., 2000), PY2016{*OyIs32*[*lin-11::gfp*]} (Sarafi-Reinach et al., 2001), UL2650/2651/2652{*unc-119*(*ed3*)*III; pUL#JRH10H1*[*CO2F12.10*^{prom}*:: gfp, unc-119*(+)] (Reece-Hoyes et al., 2007) and an unnamed strain with the integrated transgene *Is*[*mbr-1*^{prom}*::gfp*] (Kage et al., 2005). Mutant *C. elegans* strains included UTK2 {*mbr-1*(*qa5901*)} (Kage et al., 2005), TB528 [*ceh-14*(*ch3*) *X*] (Cassata et al., 2000) and an unnamed strain with *ceh-63*(*tm541*) (http://www.shigen.nig.ac.jp). The *qa5901* deletion removes the first half of the protein-coding region plus the promoter region and is likely to be a null allele of *mbr-1*. The *ch3* allele causes a translational frameshift upstream of the homeodomain and is a null allele of *ceh-14*, as confirmed with genetic data (Cassata et al., 2000).

To examine *ceh-63::gfp* expression in the absence of *ceh-63* function, the transgenic extrachromosomal array *leEx2650*[*ceh-63*^{prom}*::gfp, unc-119*(+)] was crossed from strain UL2650 into the *ceh-63(tm541)* mutant background, both of the original strain, giving strain UL3105, and of the backcrossed strain, UL3122, giving strain UL3551. In addition, the extrachromosomal array *leEx3025*, carrying the recombineered fosmid with the *ceh-63* protein coding region replaced with *gfp*, in the strain UL3025 was also crossed into the *ceh-63(tm541)* mutant background of UL3122, giving strain UL3161. To examine *ceh-63::gfp* expression in the absence of *ceh-14* function, the *leEx3025* transgene was also crossed into the *ceh-14(ch3)* mutant background.

2.2. PCR amplification of ceh-63 cDNA

Sequences of PCR primers, for amplification of *ceh-63* cDNA fragments from a mixed-stage *C. elegans* cDNA library (Walhout et al., 2000), were based on the predicted gene model and flanking regions of the vector (pPC86) or directed at the poly-A tail (Supplementary Table 1). PCR was performed in 1× BioTaq buffer, 2.5 mM $MgCl_2$, 0.5 mM dNTPs, 0.5 μM of each primer, 0.25 units / μl of BioRed Taq enzyme, and 0.5 ng / μl purified cDNA library DNA. The PCR program consisted of 94°C 2 min, 30 cycles of 94°C 30 sec, 55°C 45 sec, and 72°C 2 min, and a final incubation at 72°C for 10 min. PCR products were either separated by agarose gel electrophoresis with distinct bands purified for sequencing or cloned into the pGEM-T-Easy cloning vector (Promega, USA) with screening by PCR before sequencing.

2.3. Recombineering of reporter gene fusions

Recombineering of *C. elegans* fosmid clones was carried out according to Bamps and Hope (2008). Primer sequences are provided in Supplementary Table 1.

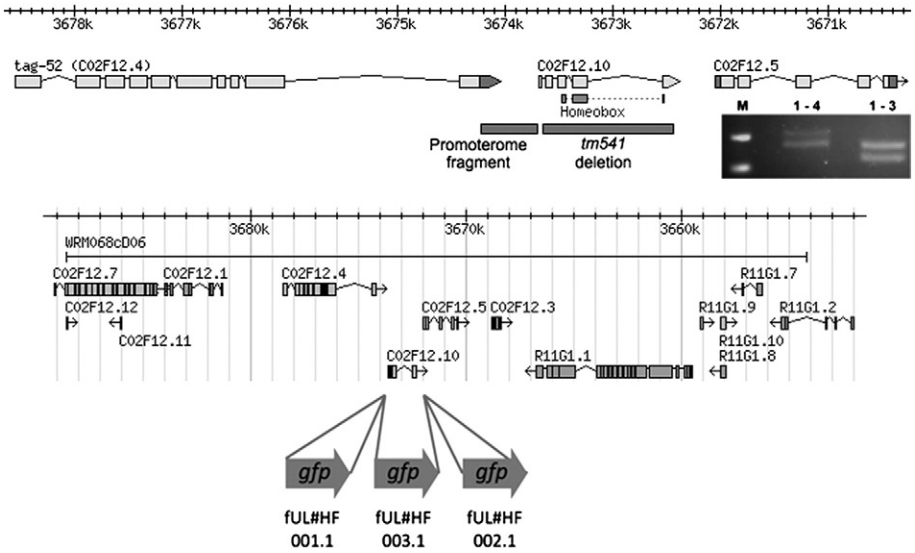

Fig. 1. *ceh-63* / *C02F12.10* gene structure with flanking genes. The intron / exon structures of the gene models as presented in WormBase are indicated. The untranslated regions, as previously defined by experiment, are in dark grey. The part of the *ceh-63* coding region encoding the homeodomain and deleted in the *tm541* allele are also indicated as is the region cloned in the Promoterome for this gene. The scales in kilobase pairs refer to position along the X chromosome. Inset: *ceh-63* cDNA fragments derived by PCR amplification of templates, in the *C. elegans* cDNA library, either containing (top band) or lacking (bottom band) intron 3. Products generated using primers For1 and Rev4 (1–4) or For1 and Rev3 (1–3) were resolved by agarose gel electrophoresis. (Primer sequences are provided in Supplementary Table 1.) DNA size markers (M) are 300 bp (top) and 150 bp (bottom). In the lower part of the figure, the genomic region contained in the fosmid WRM068cD06 is presented. The *gfp* reporter was inserted precisely at the start (fUL#HF001.1) or end (fUL#HF002.1) or to replace (fUL#003.1) the *ceh-63* protein-coding region by recombineering.