The Caenorhabditis elegans Homeobox Gene ceh-19 Is Required for MC Motorneuron Function

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Summary: Simplicity has made C. elegans pharyngeal development a particularly well-studied subject. Nevertheless, here we add the previously uncharacterized homeobox gene F20D12.6/ceh-19 to the set of transcription factor genes involved. GFP reporter assays revealed that ceh-19 is expressed in three pairs of neurons, the pharyngeal pace-maker neurons MC, the amphid neurons ADF and the phasmid neurons PHA. ceh-19(tm452) mutants are viable and fertile, but grow slightly slower, produce less progeny over a prolonged period, and live longer than the wild type. These phenotypes are likely due to the moderately reduced pharyngeal pumping speed arising from the impairment of MC activity. MC neurons are still born in the ceh-19 mutants but display various morphological defects. ceh-19 expression in MC is completely lost in progeny from animals subject to RNAi for pha-4, which encodes an organ-specifying forkhead transcription factor. CEH-19 is required for the activation in MCs of the excitatory FMRFamide-like neuropeptide-encoding gene flp-2. A regulatory pathway from pha-4 through ceh-19 to flp-2 is thereby defined. The resilience of MC identity in the absence of CEH-19 may reflect the buffering qualities of transcription factor regulatory networks.

Key words: Caenorhabditis elegans; transcription factors; homeodomain; nerve cells; pharynx

INTRODUCTION

The C. elegans pharynx is an anatomically self-contained structure dedicated to ingestion and transportation of bacteria into the intestine (Albertson and Thomson, 1976; Avery and Horvitz, 1989). The entire structure is surrounded by and isolated from the rest of the worm by a basement membrane (Sulston et al., 1983). The feeding behavior is accomplished by two distinct types of muscle contractions, namely pumping and peristalsis. Pumping ingests and concentrates bacteria in the anterior lumen and is followed by peristaltic contractions, which bring ingested bacteria through the isthmus (Avery and Shtonda, 2003). Feeding is regulated and modulated by the 20 pharyngeal neurons, almost completely independently of neuro-muscular activity elsewhere in the animal (Avery and Horvitz, 1989). Extensive laser ablation experiments have shown that the nervous system is not essential for pumping, but is important for normal feeding, growth rate, and fertility (Avery and Horvitz, 1989). Pharyngeal muscles pump even without the stimulation from neurons, but the pace of pumping is controlled by the motor neurons MC and M3, which initiate and terminate the muscle action potentials respectively (Raizen et al., 1995). The MC neuron is an excitatory cholinergic neuron. Its firing triggers a pharyngeal muscle action potential via the release of acetylcholine, which acts on a muscle nicotinic receptor. It is said to be necessary and probably sufficient for rapid pharyngeal pumping (Mukhopadhyay et al., 2007; Raizen et al., 1995).
third motor neuron, M4, is responsible for peristalsis of the isthmus; its inactivation has little effect on pumping but eliminates peristalsis causing animals to arrest with a "stuffed pharynx" (Avery and Horvitz, 1989).

Homeobox genes have been shown to be required for pharynx morphogenesis and cell differentiation. Notably, several NK-2 class homeodomain factors, including PHA-2, CEH-22, and CEH-24, are each required, independently of or in combination with other transcription factors, for target gene expression in and specification of one or a few of the 8 sets of pharyngeal muscles (pm1-pm8) (Harfe and Fire, 1998; Mango, 2009; Morck et al., 2004; Okkema and Fire, 1994; Okkema et al., 1997). NK-2 class homeodomain factors also function in the pharyngeal nervous system. For example, ceb-2 is expressed in the NSM and M3 neurons, and in a ceb-2 deletion mutant M3 was generated but its activity was substantially reduced, indicating ceb-2 acts in a late differentiation step for M3 development (Aspock et al., 2003). Similarly, CEH-28 is required for M4 function; ceb-28 inactivation results in irregularly spaced and sized M4 synapses in the isthmus, and frequent and prolonged peristalses (Ray et al., 2008).

Although MC has long been known to be the pacemaker neuron, regulatory factors critical for its specification and action, other than the FoxA transcription factor PHA-4 which is required broadly for pharyngeal cell fate specification, had not been identified. Here we report that a previously uncharacterized homeobox gene, F20D12.6/ceb-19 (Fig. 1), is specifically expressed in MCs from late embryogenesis through to adulthood. Well-fed, healthy animals bearing the ceb-19(tm452) or ceb-19(tm461) deletion mutations, that both remove most of the homeobox, displayed a moderate reduction of pharyngeal pumping speed. In ceb-19(tm452) mutants, the MC cells are generated, but with obvious axonal morphological defects, suggesting CEH-19 is required for proper specification of the MC neuronal type. Reporter fusions for ceb-19 also directed expression in two pairs of sensory neurons, the amphid and phasmid neurons ADF and PHA. We found that the ceb-19 expression level in ADF was up-regulated in wild type dauer animals, indicating a potential link of this homeoprotein to the C. elegans dauer and/or aging pathways.

RESULTS

F20D12.6/ceb-19 Expression Pattern

In (WormBase) (www.wormbase.org), ceb-19 is annotated to encode two transcripts, ceb-19a (F20D12.6a) and ceb-19b (F20D12.6b), based on the cDNA clones Z11795 and CN797884, and ORFeome sequence tags (OSTs). The extent of the homebox and both deletion alleles, tm452 and tm461, is indicated. ceb-19 is located between the genes csr-1 (F20D12.1) and F20D12.2, both transcribed in the opposite direction to ceb-19. The scale is in kb.

FIG. 1. The ceh-19 gene model. In WormBase, ceh-19 is annotated to encode two transcripts, ceh-19a (F20D12.6a) and ceh-19b (F20D12.6b), based on the cDNA clones Z11795 and CN797884, and ORFeome sequence tags (OSTs). The extent of the homebox and both deletion alleles, tm452 and tm461, is indicated. ceh-19 is located between the genes csr-1 (F20D12.1) and F20D12.2, both transcribed in the opposite direction to ceh-19. The scale is in kb.
neurons were identified unambiguously as MCL/R (Fig. 2a–c).

The other two GFP expressing cells in the head had cell bodies outside of the pharynx, just anterior to the posterior pharyngeal bulb, with dendrites extending to the head tip and therefore were identified as amphids. Specific identity was narrowed down by their dual ciliated sensory endings (Fig. 2g inset), a morphological feature of only ADFL/R and ADLL/R. Furthermore as the GFP expressing cells did not take up externally delivered DiI (Fig. 2d1), which stains ASI, ADL, ASK, AWB, ASH, and ASJ, the expressing cells were identified as ADFL/R. In addition, the GFP signal increased in this pair of amphids in the dauer stage (Fig. 2g) and ADFs control entry into the dauer stage (Bargmann and Horvitz, 1991b), suggesting a connection between ceh-19 regulation and dauer formation. The GFP signal was much higher in MCs than in ADFs during normal developmental stages (Fig. 2a, c, d2, f) whereas the strength of signal in the two neuron types was approximately the same in the dauer stage due to the considerable increase of signal within ADFs (Fig. 2g). In UL2701, which contains the transgene as an extrachromosomal array, the difference in intensity in non-dauer stages was not as apparent (Fig. 2b).

The two cells in the tail showing GFP expression have cell bodies located just behind the rectum and short processes in both anterior and posterior directions (Fig. 2b inset, h, i), a morphology suggestive of the phasmid neurons, PHAL/R or PHBL/R. DiI filling, which stains both of these phasmid neuron types revealing their relative positions, confirmed that the pair of neurons expressing GFP in the tail is the more anterior pair, PHAL/R (Fig. 2, E1/2). The extrachromosomal array in UL2701 directed stronger GFP expression in PHA than the integrated array in UL2702 and UL2703. In all three strains there was no apparent difference of signal in PHA between non-dauer and dauer animals (Fig. 2h, i). Although the tail neural anatomy is substantially modified in the male, no male specific expression was observed for the ceh-19bprom::gfp fusion in any of the three transgenic strains.

We further generated two gfp fusions for ceb-19b in a large genomic background by recombineering the
fosmid WRM0620bD04. This fosmid contains 18,586 bp upstream and 15,366 bp downstream of ceh-19 and would be expected to contain all ceh-19 regulatory elements. Strains UL3010, UL3011, and UL3012 independently transformed with the fosmid fUL#HF005.1, with the ceh-19b protein-coding region replaced by gfp, showed consistent GFP expression in MCL/R, ADFL/R and PHAL/R in UL3010 and UL3012 (a1–3). Expression was detectable in axons of MC and PHA (a1 inset, a2 arrow head). The recombineered fosmid fUL#HF004.1, with gfp inserted immediately before the stop codon of ceh-19b, also drove GFP expression in MCL/R, ADFL/R and PHAL/R, in UL3014 and UL3308 (b1–3), but to a low level and with nuclear-localization. Expression in ADFs increased in dauer animals such that the axon of ADF was visible in UL3010 (a3, arrow head) and ADF nuclei became much brighter in UL3308 (b3). Bars represent 25 μm in a1–3, b1–3 and inset, and 10 μm in a1 inset.

FIG. 3. GFP expression of fosmid-based reporter fusions for ceh-19b. The recombineered fosmid fUL#HF005.1, with the ceh-19b protein-coding region replaced by gfp, drove GFP expression in MCL/R, ADFL/R and PHAL/R in UL3010 and UL3012 (a1–3). Expression was detectable in axons of MC and PHA (a1 inset, a2 arrow head). The recombineered fosmid fUL#HF004.1, with gfp inserted immediately before the stop codon of ceh-19b, also drove GFP expression in MCL/R, ADFL/R and PHAL/R, in UL3014 and UL3308 (b1–3), but to a low level and with nuclear-localization. Expression in ADFs increased in dauer animals such that the axon of ADF was visible in UL3010 (a3, arrow head) and ADF nuclei became much brighter in UL3308 (b3). Bars represent 25 μm in a1–3, b1–3 and inset, and 10 μm in a1 inset.

A reporter fusion was constructed to assay the expression pattern of the annotated ceh-19a transcript, supported by the single EST Z11795 and SAGE tags for exon 1 (Naito et al., 1992). The gfp reporter was fused to the 2770 bp region upstream of the ceh-19a start codon,
including the start of transcript b and the entire intergenic region, by Gateway recombinational cloning, creating pUL#HF053. However, in two strains transformed with this plasmid no GFP expression was observed, throughout the normal life cycle or in the dauer, suggesting the ceh-19a transcript may not be functional or may only be expressed in circumstances not assayed. The absence of additional expressing cells for the C-terminal translational fusion is also consistent with ceh-19a not adding to the expression arising from ceh-19b.

Molecular Phylogeny of CEH-19 and Its Homologues

Within the C. elegans genome, ceb-30, ceb-31, tab-1, and ceb-1 have the highest similarity to ceb-19b by BLAST. Each of the five paralogues has one orthologue (best reciprocal BLAST match) in Caenorhabditis briggsae, C. remanei, D. melanogaster, and H. sapiens, in comparison with the more general consensus homodomain. The arrow points to position 49 of the homodomain, which is a characteristic tyrosine (Y) residue for the typical BarH homeoproteins rather than the more common phenylalanine (F). Alignment was performed using ClustalW software and edited with Jalview 2.5.1.

![FIG. 4. Protein sequence alignment of the homodomains of C. elegans CEH-19 and its homologues from C. briggsae, C. remanei, D. melanogaster, and H. sapiens, in comparison with the more general consensus homodomain. The arrow points to position 49 of the homodomain, which is a characteristic tyrosine (Y) residue for the typical BarH homeoproteins rather than the more common phenylalanine (F). Alignment was performed using ClustalW software and edited with Jalview 2.5.1.](C. ELEGANS CEH-19 167)

cel-19 (tm452) Has Reduced Pharyngeal Pumping Speed

To investigate the function of ceb-19, two C. elegans ceb-19 mutant alleles were obtained and examined for a phenotype altered from the wild type. The


ceb-19\textsuperscript{tm452} and ceb-19\textsuperscript{tm461} alleles, generated by TMP/UV mutagenesis, have 995 bp and 701 bp deletions, respectively. Both alleles lack the two middle exons of ceb-19\textsuperscript{b} and the first three exons of ceb-19\textsuperscript{a} (Fig. 1). This was verified by PCR and sequencing. The mutations delete most of the ceb-19 homeobox and potential splicing together of the remaining two exons of transcript ceb-19\textsuperscript{b} would cause a shift to an incorrect translational reading frame for the last exon. The truncated peptide encoded by such a hypothetical transcript would retain the potential acidic transcriptional activation domain but none of the homeodomain. Therefore, \textit{tm452} and \textit{tm461} are likely to be null alleles. The \textit{tm452} allele was backcrossed to the N2 wild type seven times to generate UL3128, to remove other mutations that might be present in the original strain.

The original strains bearing the \textit{tm452} and \textit{tm461} alleles and UL3128 are viable and fertile and have no immediately obvious morphological, physiological, or locomotion defects. However, the specific expression of \textit{ceb-19::gfp} in the pharyngeal motor neuron MC, which is necessary for rapid pharyngeal pumping (Avery and Horvitz, 1989), suggested the \textit{ceb-19} mutants might be defective in pharynx function. Any such defect would need to be to a degree that is not strong enough to cause other more obvious defects, such as a starved body appearance. Closer examination of pumping behavior indicated that pharyngeal pumping rate of UL3128 was indeed reduced by more than 30% compared to N2 animals (Fig. 5a). In N2 individuals the pharynx pumped 239 ± 48.5 times per minute (mean ± s.d.) compared to 148 ± 30 times per minute for UL3128. The unbackcrossed \textit{tm461} mutant animals have an almost identical pumping speed to UL3128 (data not shown), consistent with the two \textit{ceb-19} alleles having essentially the same consequences. On an agar plate in the presence of abundant bacteria, pharynxes of N2 pump continuously at a near steady frequency. In contrast pharynxes of UL3128 pump in alternate cycles of 7-10 pumps at high and low frequencies, the slower-pumping phase responsible for reducing the average speed. This defect is not as severe as when both MC neurons were killed with a laser, which reduced the speed to 45 ± 6 pumps/minute (Raizen et al., 1995), indicating that MC function is not completely inactivated in the \textit{ceb-19} mutants. The \textit{ceb-19} mutant defect is not even as severe as that for \textit{eat-2\textsuperscript{(ad465)}} (Fig. 5a) which affects synapses between the pharyngeal muscles pm4 and pm5, post-synaptic to MC (Mckay et al., 2004). As for N2, pharyngeal pumping is absent in UL3128 animals on plates with no bacterial food, and when pumping is restored by supplementation with 10 mM serotonin UL3128 still pumps at a slower rate than N2. This suggests that the compromised pumping rate of UL3128 is due to a defect in the pharynx, and presumably MC itself, rather than in the ability to sense food. UL3128 was transformed with the fosmid fUL#HF004.1 bearing the full-length \textit{ceb-19::gfp} translational fusion to rescue the \textit{ceb-19} defect. The rescued strain, UL3548, had recovered pumping speed (241 ± 19.2 pumps/minute), very close to that of wild type worms, indicating that the pharynx defect in UL3128 is indeed due to the absence of \textit{ceb-19} (Fig. 5a).

### Consequences of ceb-19\textsuperscript{tm452} for Other Life-History Traits

Reduced food intake can influence other life-history parameters such as the development rate, and UL3128 animals showed slightly slower growth than N2, presumably as a result of the slower pharyngeal pumping. Growth rate was assessed in two ways. First, when two synchronized L1s were placed on each of six seeded agar plates, and incubated for 70 h at 20°C, 10 of 12 N2 worms had developed into mature adults, and there were scores of laid eggs with a few hatched L1 larvae on each plate. In contrast, when UL3128 L1s were used, each plate had only around ten eggs and no L1s had appeared. By the tenth day of incubation, the N2 populations had exhausted the bacteria but the UL3128 populations required another 20–24 h to use up the food source. In a second assessment, after 40 hr growth at 20°C, 3 of 145 synchronized N2 L1s had grown into young adults, 8 were late L3s/early L4s, and the remainder were mid/late L4s. For 161 UL3128 L1s, after 40 hr there were also 3 young adults, but the vast majority were late L3/early L4s and only 14 were mid/late L4s, in clear contrast with the N2s (Fig. 5b).

The period of fecundity is prolonged in UL3128 animals. Although the average number of embryos produced by each UL3128 hermaphrodite (brood size = 242 ± 31, \(n=13\)) appeared slightly less than that of N2 (brood size = 260 ± 21, \(n=15\)) (Fig. 5c) this difference was not found to be statistically significant. However, the period of fecundity of UL3128 seemed prolonged (Fig. 5d). N2 hermaphrodites laid most of their eggs (83.2 ± 4.1%, \(n=8\)) within the first 3 days of the reproductive period with a peak on the second day (Fig. 5d). UL3128 animals laid a smaller proportion of eggs during the first 3 days (72.1 ± 9.4%, \(n=8\)) and laid more eggs on subsequent days, especially on Day 4. In addition, UL3128 showed considerably more variation in individual egg-laying curves than N2. When the number of eggs retained in the uterus was checked at 90 h after hatching (on the 2nd day of the reproductive period), N2 adults retained on average 13 ± 4.6 (\(n=10\), mean ± s.d.) eggs in the uterus and UL3128 had 8.7 ± 4.2 (\(n=10\), mean ± s.d.). At 96 h after hatching, N2 had 9.5 ± 1.5 eggs within the uterus of each hermaphrodite (\(n=14\), mean ± s.d.) and UL3128 had 8.2 ± 2.3 (\(n=13\), mean ± s.d.). This provided further evidence of the
FIG. 5. Effect of ceh-19 deletion on life-history traits. (a) Mean pumping speeds ± s.d. are presented for N2, UL3128 (the backcrossed ceh-19 mutant), UL3548 (the ceh-19 rescued strain) and eat-2 (add465) mutants. (b) The growth rates of N2 and UL3128 were compared under standard culturing conditions by recording the number of individuals that had become late L3/early L4s, mid/late L4s or young adults, 40 hrs after hatching. (c) UL3128 hermaphrodites appeared to produce slightly fewer progeny than N2 but the difference was not statistically significant (P = 0.082) according to a one-way ANOVA. (d) Daily egg-laying of 8 UL3128 hermaphrodites was scored (black) and compared with that of 8 N2 hermaphrodites (grey) over the six-day reproductive period. (e) The average mean life span (with standard deviation) of N2, UL3128 and UL3548 individuals is plotted in days. (f) The percentage of individuals surviving on each day was determined for N2, UL3128 and UL3548. * indicates a statistically significant difference by a one-way ANOVA test, P < 0.0001.
UL3128 has an extended life span. Reduced food intake lengthens life span in *C. elegans* as observed for many *eat* mutants with their defects in pharyngeal function (Lakowski and Hekimi, 1998). UL3128 is also essentially a weak *eat* mutant and the life span assay for this strain was performed twice, independently (Fig. 5e,f). N2 worms on average lived 18.9 ± 3.3 days (*n* = 156, mean ± s.d.), very close to the 19.5–21.6 days reported previously by Lakowski and Hekimi (1998) who also fed the worms on live OP50 bacteria. For UL3128 the lifespan was 23.8 ± 4.1 days (*n* = 175, mean ± s.d.), a lifespan extension similar to that of *eat-1(e2343)* (23.9 ± 0.9 days, mean ± SEM) and *eat-6(ad997)* (23.6 ± 0.7 days, mean ± SEM) mutants, when assayed with the same protocol (Lakowski and Hekimi, 1998). The pharyngeal pumping speed of *eat-1(e2343)* mutants is not published, but the pharyngeal pumping rate of *eat-6* (*ad997*) mutants (~150 pumps/minute) (Doi and Iwasaki, 2008) was very close to that of UL3128. These observations are consistent with the good correlation between life span extension and the severity of the eating defect, as has been observed previously (Lakowski and Hekimi, 1998).

Rescue of *ceh-19* in UL3548 shortened lifespan compared to that of the *ceh-19* mutant, towards that of the wild type (Fig. 5e,f). The rescued animals had a lifespan of 19.2 ± 3.3 days (*n* = 92, mean ± s.d.). Statistically, the UL3548 lifespan was significantly shorter than for UL3128 and was not significantly different from that for N2. Transformation with CEH-19 did appear to rescue the lifespan extension phenotype of the *ceh-19* mutant, as for the decreased pharyngeal pumping speed phenotype.

UL3128 animals enter into and exit from the dauer stage normally. Expression of *ceh-19* in ADF and PHA neurons might suggest a role connected with the dauer stage. Although UL3128 animals develop to adults at a slower rate, dauer formation was not observed in normal growth conditions; the *ceh-19* deletion does not confer a dauer formation constitutive (Daf-c) phenotype. Efficiency of dauer recovery for N2 and UL3128 was also compared and no difference was observed. All dauer animals (*n* ~ 200) resumed development within 24 hrs after food became available again. Possible further roles of *ceh-19* in chemosensation, suggested from expression in these sensory neurons, were not investigated.

Finally, defection did not appear affected in the *ceh-19* mutant. The period of the defection cycle of UL3128 remained at 40 ± 1 s, similar to, but apparently even more regular than, N2 at 42 ± 3.8 s (mean ± s.d., *n* = 5 worms each, 10 min of observation for each worm). This is consistent with previous observations that bacteria intake has only a minor effect on the period of the defection cycle (Thomas, 1990).

**CEH-19 Is a Novel Eat Gene**

Previous screens for pharyngeal pumping defects had yielded the *eat* mutants (Avery, 1993). Almost all *eat* mutations characterized so far result in severe impairment to the pharynx and most of them also affect tissues outside the pharynx (Avery, 1993; Shibata et al., 2000). The molecular nature of some of these genetically identified *eat* genes, including *eat-1*, *eat-8*, *eat-9*, *eat-10*, *eat-13*, *eat-14*, *eat-15*, and *eat-17*, remain to be determined. Among these, *eat-1* and *eat-10* have been mapped onto linkage group IV where *ceh-19* is found. *eat-10(ad606)* is on the left arm of LGIV (IV: −26.74 ± 0.306cM), far from *ceh-19* (IV: 3.51 ± 0.001cM). However, *eat-1* was mapped onto LGIV at 4.8 ± 6.395cM, and both its recessive alleles, *ad427* and *e2343*, have slow and irregular pharyngeal pumping, like *tm452*. The very large confidence interval of the *eat-1* location presumably reflects problems with the genetic mapping but does cover the locus of *ceh-19*. We found, however, that all three *eat-1* mutants have even slower pumping speed than the *ceh-19* deletion mutants: DA531[*eat-1(ad427)IV]* at 89.1 ± 21.7, CB4394 [*eat-1(e2343) unc-31(e928)IV]* at 87 ± 17.9, and DA449 [*eat-1(e2343) dpy-20(e1282)IV]* at 106 ± 17.4 times per minute, respectively (mean ± s.d, *n* = 10). And direct tests revealed *ceh-19* and *eat-1* are not allelic; PCR amplification of *ceh-19* from genomic DNA of *eat-1* mutants yielded products of wild type sizes and *tm452* complements genetically all three *eat-1* mutant alleles. Therefore, the molecular nature of *eat-1* remains to be determined and *ceh-19* is a newly identified *Eat* gene, a homeobox gene that is necessary for *C. elegans* pharynx development and proper pumping.

**UL3128 Animals Have Morphological Defects in MC**

To assess whether the specification and development of MC, ADF and PHA were affected by the *ceh-19* deletion and also whether CEH-19 is required for its own expression in these neurons, their morphology was examined in the *ceh-19(tm452)* deletion background using *ceh-19*;F27 rescue strains. The chromosomally integrated transgenic array *leIs2703[ceh-19bprom::gfp]*, *unc-119(+)*, was crossed from strain UL2703 into the *ceh-19(tm452)* mutant background of UL3128 to give strain UL3413. The extrachromosomal array *leEx3011* and *leEx3012* both carrying the recombineered fosmid fUL#HF005.1 with the *ceh-19* protein coding region replaced with *gfp*, in the strains UL3011 and UL3012, respectively, were also crossed into the *ceh-19(tm452)* mutant background of UL3128 to give strains UL3013, and UL3019 and UL3022.

In each strain with the *ceh-19(tm452)* deletion background, GFP expression in all three pairs of neurons remained at a similar level to that in the wild type
background, suggesting MC, ADF, and PHA are still born normally in the absence of CEH-19, and ceh-19 expression is not self-regulated. The ceh-19bprom::gfp expression was also examined in dauer animals in the ceh-19 mutant background as the change in strength of expression in ADF in this stage might have depended on functional CEH-19, but again no difference from wild type levels was observed (data not shown). Although apparently lacking a role in the fundamental generation of these nerve cells, CEH-19 could still be functional in their specific terminal differentiation. Indeed, in 90% (n>100) of mutant animals, there were axonal defects in the MC neurons to varying degrees. MCs in wild type animals have symmetrically located cell bodies and well-organized, consistent axonal projections, as revealed under epifluorescence (Fig. 6a, b) and confocal microscopy (Fig. 6c, d). Although usually both MC cell bodies were still present in the ceh-19 mutant background, they were often different sizes and shapes (Fig. 6h-i). Moreover, severe abnormalities were observed for MC axons, by both epifluorescence and confocal microscopy (Fig. 6e-m), which presumably would result in abnormal inter-neuronal and neuro-muscular connections. Occasionally, in the mutant background, GFP was only apparent in one MC (Fig. 6m). These defects are consistent with the moderate pharyngeal pumping defect observed for the ceb-19 mutant animals.

No axonal defects were observed by epifluorescence microscopy for ADF and PHA neurons in the ceh-19 deletion background (data not shown). These amphid and phasmid neurons might have minor structural and/or synaptic defects beyond detection from observations of the GFP expression but which could be revealed by electron microscopy.

**Regulatory Context of ceh-19 in MC**

PHA-4 is the pharyngeal master regulator required for development of all cells of the pharynx (Mango, 2009) and the expression of ceb-19 in MC is dependent on this transcription factor. We assayed the dependence of ceh-19 expression on PHA-4 by applyingpha-4 RNAi to gravid UL2703, with the chromosomally integrated ceh-19bprom::gfp fusion, and examining GFP in the progeny. As expected, knocking down PHA-4 resulted in embryonic lethality and/or larval arrest for UL2703. Animals that did manage to hatch out appeared to lack a pharynx with no GFP corresponding to MC, although GFP in ADF and PHA remained (Fig. 7a1/2). Presumably no cell with an MC fate is generated with PHA-4 absent.

CEH-19 is required for flp-2 expression in MC. flp-2 and flp-21 both encode FMRFamide-like peptide
FIG. 7. Regulation upstream and downstream of ceh-19. Larva hatched from UL2703 animals transgenic for ceh-19bprom::gfp and subject to RNAi for pha-4 are pharynexitis (a1), and although expression in ADF and PHA was maintained, expression in MC was lost (a2). In a wild type background, flp-2prom::gfp is expressed in MC (b2 and b2 inset) and other neurons (b1/2). In the ceh-19(tm452) background, flp-2prom::gfp expression was not detected in MCs but was still present in other neurons at the same level as in wild type (c1/2). flp-2prom::gfp expression was observed in ventral cord motor neurons in the dauer stage only (d1/2) including the axon (d2 inset), here in the wild type. Bars represent 10 μm in a–c, b2 inset and d2 inset, and 25 μm in d. Corresponding images were captured by DIC (a–d1) or epifluorescence (a–d2) microscopy.
neurotransmitters and have been reported to be expressed in MC, in addition to multiple other nerve cells (Kim and Li, 2004). To assay the genetic dependence of these genes on CEH-19, we generated strains UL3795 and UL3793, transgenic for flp-2prom::gfp and flp-21prom::gfp fusions respectively, and indeed detected GFP expression in MC neurons in the wild type background. The reporter fusions were then crossed into UL3128 with the ceb-19 deletion background, resulting in strains UL3881 [ceb-19(tm452); flp-2prom::gfp] and UL3891 [ceb-19(tm452); flp-21prom::gfp], to assess their regulation by CEH-19. While there was no difference in MC expression of flp-21prom::gfp between the wild type and ceb-19 mutant (data not shown), flp-2 expression in MC appeared dependent on CEH-19. In the wild type background, flp-2prom::gfp gave a clear consistent GFP signal in MCs, in UL3795 and 3 other independent transgenic strains, although expression was normally weaker than in other cells (Fig. 7b1/2). In contrast, in the ceb-19 mutant, flp-2prom::gfp expression in MC was not detectable (Fig. 7c1/2), suggesting CEH-19 is required for flp-2 expression in MC. This requirement was specific to MC, the only cells in which ceb-19 and flp-2 expression overlaps, with flp-2 expression in other cells not affected in the more than 50 individuals where expression was carefully and specifically examined in this regard. MC expression of flp-2prom::gfp in dauer animals in both the wild type and ceb-19 mutant backgrounds was also compared. Although ceb-19bprom::gfp was expressed strongly in MC during the dauer stage, MC expression of flp-2prom::gfp in dauers with wild type background was weaker than in non-dauers or even absent. Nevertheless flp-2prom::gfp MC expression was never observed in the ceb-19 mutant dauers, confirming the requirement of CEH-19 for flp-2 expression, even in the dauer. Interestingly, while examining flp-2prom::gfp MC expression, a strong GFP signal was observed in ventral cord motor neurons of dauers in both wild type and ceb-19 mutant backgrounds (Fig. 7d1/2). Such expression for flp-2 was not reported previously and was not seen in non-dauers, indicating a potential involvement of flp-2 in dauer physiology.

Evidence for direct interaction between PHA-4 and ceb-19 and between C. elegans and flp-2 were sought using the yeast one hybrid (Y1H) approach. The strategy could also have yielded additional targets of CEH-19 and regulators of ceb-19. A previously described C. elegans transcription factor yeast array (Reece-Hoyes et al., 2005) was used in a yeast-one-hybrid screen with the ceb-19b promoter as bait. Out of the 755 transcription factors in the array, three, TBX-8, TBX-9, and MLS-2, appeared to bind the ceb-19b promoter. Although genetically required for ceb-19 expression in MC, PHA-4 did not appear to bind the ceb-19 promoter in these Y1H TF array screens. This was also specifically tested with ceb-19bprom::lacZ bait yeasts directly transformed with the Gal4AD-pha-4 and Gal4AD-only prey plasmids. Both showed the same level of lacZ expression, yielding no support for specific binding of PHA-4 to the ceb-19b promoter. Twenty genes reported to be expressed in MC, ADF and/or PHA, including flp-2 and two transcription factor genes, lin-11 and lin-4, were selected as potential CEH-19 targets (Supplementary Table 2). Promoters for these genes, either retrieved from the Promoterome or cloned ab initio, were fused with the Y1H lacZ and His3 reporters for screening as baits versus the C. elegans TF yeast array. CEH-19 did not appear to bind to any of these baits, although various other transcription factors did (data not shown).

**DISCUSSION**

The *C. elegans* genome encodes ~100 homeodomain transcription factors, 24 of which lack any other highly conserved motif (Reece-Hoyes et al., 2005). Currently these “homeodomain-only” transcription factors, including ceb-19, are less well characterized although over half are essential, with various developmental and behavioral roles. Reporter gene fusions have been assayed for most and typically revealed expression in a range of cell types, e.g., (Reece-Hoyes et al., 2007). The restricted distribution of reporter expression for ceb-19, however, led to its study here.

The lack of reporter expression with a ceb-19a specific fusion, and lack of additional components for the fusion targeting both transcripts as compared to fusions targeting ceb-19b specifically, suggests ceb-19a is at best poorly expressed under laboratory conditions. The sequence of the donor splicing sites of ceb-19b intron 1 (AGgtaatcat) matches more closely to the well-conserved motif (Reece-Hoyes et al., 2005). Currently these “homeodomain-only” transcription factors, including ceb-19, are less well characterized although over half are essential, with various developmental and behavioral roles. Reporter gene fusions have been assayed for most and typically revealed expression in a range of cell types, e.g., (Reece-Hoyes et al., 2007). The restricted distribution of reporter expression for ceb-19, however, led to its study here.

Although the defect in pharyngeal pumping in the ceb-19 mutant is likely to relate purely to expression in MC, the clear expression of the ceb-19::gfp fusion in ADF and PHA suggests ceb-19 contributes to the sensory roles of these neurons. Defects relating to ADF and PHA sensory function may, however, be subtle due to redundancy with other cells or genes, or may not have been apparent because of ecological specificity. Nevertheless, the striking up-regulation in the ADF of dauers could be significant in this regard. *C. elegans* dauer entry and exit involve integration and transformation of environmental cues (dauer pheromone, nutrients, and temperature) into endocrine signals by amphid neurons. Amphids ADF, ASI and perhaps ASG act redundantly to prevent dauer formation in favourable
conditions, and amphid ASJ is critical to exit from the dauer stage with some minor contributions from ADF or ASI, or ASG (Bargmann and Horvitz, 1991a; Bargmann and Horvitz, 1991b). This redundancy amongst the amphids means the lack of an effect of the ceb-19 deletion on dauer entry or exit is not surprising. The up-regulation suggests increased levels of CEH-19 are important in preparing ADF for a role in the dauer stage, presumably for sensing of conditions for dauer exit or perhaps contributing to the resilience to adverse conditions characteristic of the dauer stage, in this case specifically of ADF. Such roles may only be revealed under conditions characteristic of the dauer stage, in this case specifically of ADF. Such roles may only be revealed with ASI, ASG and ASJ inactivated. The increased ceb-19 expression in ADF would itself be a consequence of endocrine signals acting to direct dauer entry. This, along with the flp-2::gfp expression in ventral cord neurons of only the dauer stage, and the expression of this reporter fusion in MC being ceb-19 dependent seems unlikely to be coincidental but the biological significance is not yet apparent. ceb-19 has not been identified previously in global expression analyses seeking genes expressed specifically or with altered levels in the dauer stage (Holt, 2006; Jones et al., 2001; Wang and Kim, 2003). This may be because the problem of low expression, as seen with other transcription factor genes, is further exacerbated by expression being restricted to just a few small cells. Reporter gene fusions provide the localized sensitivity needed to observe such subtle effects.

The minor nature of the ceb-19 mutant’s defects, compared with those upon MC ablation, suggests MC function is only partially disrupted. As MC cell processes are abnormal in ceb-19(tm452) mutants, a simple hypothesis would be that receipt or transmission of neural signaling is impaired due to the improperly specified synaptic connections between MC and muscles and/or other neurons. As tm452 and tm461 are likely to be null alleles, ceb-19 may only be required for partial or non-essential aspects of MC specification, with only limited types of terminal differentiation genes being controlled by CEH-19. Alternatively, the robustness of transcription factor regulatory networks may mean that the level of expression of genes expressed for MC terminal differentiation are only moderately perturbed by the complete absence of CEH-19 activity. CEH-19 would contribute to the level of expression of many genes needed for MC fate, but other transcription factors are required for MC differentiation, with roles that remain to be identified, and buffer against ceb-19 loss.

The gene flp-2, the identified target of CEH-19 regulation in MC, encodes two FMRFamide-like neuropeptides (FLPs), FLP-2A and FLP-2B. Different C. elegans pharyngeal neurons express different sets of modulatory FLP neuropeptides (Husson et al., 2007; Kim and Li, 2004). While eleven flp genes encode inhibitors of pharyngeal activity, eight encode excitatory peptides (Papaioannou et al., 2005). FLP-2A, expressed in pharyngeal neurons M4, MC and 15, enhances pharyngeal activity. In contrast, FLP-21, also expressed in MC, suppresses pumping rate (Papaioannou et al., 2005). Hence, in a single neuron, MC, at least two FLPs are expressed with opposing activity, acting in response to different environmental stimuli to fine-tune pumping rate. Presumably, complex transcription factor regulatory networks integrate the subtly distinct nerve cell fates with the different environmental situations encountered so as to achieve the expression of each member of the range of flp genes precisely where, when, and to the levels required. CEH-19 is essential for flp-2 expression in MC. However, interruption of flp-2 by RNAi caused embryonic lethality, slow growth and larval arrest (Simmer et al., 2003), a more severe phenotype than the slow pumping when only inactivated in MC and in accordance with its broad expression and a wider role. Clearly other transcription factors must input into flp-2 such that expression of flp-2 doesn’t occur wherever CEH-19 is expressed and can occur in cells beyond where CEH-19 is expressed. As the MC fate is only partially perturbed in ceb-19 mutants, other transcription factors must control other MC terminal differentiation genes, such as flp-21. Furthermore, transcriptional control of flp-2 is not the only role for CEH-19 in MC. The MC morphological defects in ceb-19 mutants indicate transcription of other genes is needed for proper MC differentiation.

Despite the genetic evidence of regulatory dependency, CEH-19 did not bind the flp-2 promoter and PHA-4 did not bind the ceb-19 promoter, in Y1H assays. These regulatory interactions could be indirect in vivo with other intermediate transcription factors, or direct but with co-factors required. A direct interaction of PHA-4 to the ceb-19b promoter might have been expected as PHA-4 directly activates many genes expressed in the different cell types in the pharynx (Gaudet and Mango, 2002). Furthermore, modENCODE ChIP experiments identified PHA-4 binding to the ceb-19 promoter, within the region assayed in the Y1H experiments, but only in L1s and not other stages (Gerstein et al., 2010). Given the lack of any other supporting data, the significance of our finding of TBox-8, TBox-9, and MLS-2 binding to the ceb-19 promoter in Y1H assays, for regulation of ceb-19 expression in vivo is unclear. Although our targeted Y1H screen for potential CEH-19 targets, using 20 candidate gene promoters, was unsuccessful, a larger-scale screen has since revealed Y1H binding of CEH-19 to promoters of cog-1, vba-15, blb-15 and lbp-8 (Reece-Hoyes et al., 2011). The biological relevance of these interactions for ceb-19 function in vivo is also as yet unclear. Genome-wide screens for direct protein::DNA interactions involving transcription factor combinations would be a huge
undertaking even with an assay as easy to apply as the Y1H. Other data, such as from expression pattern determinations or ChIP, may be needed to reduce the number of transcription factors that need to be tested, combinatorially, in such assays before the details of transcription factor regulatory networks can be revealed.

METHODS

C. elegans Strains and Mutant Alleles

All strains were maintained at 20°C on 5 cm NGM agar plates seeded with E. coli OP50 as food source (Sulston and Hodgkin, 1988). C. elegans N2 (Bristol) was used as wild type. Transgenic strains are UL2701 [unc-119(9ed3)III; leEx2701(ceb-19bprom::gfp, unc-119(+))], UL2702 [unc-119(9ed3)III; lels2702 (ceb-19bprom::gfp, unc-119(+))], UL2703 [unc-119(9ed3)III; lels2703(ceb-19bprom::gfp, unc-119(+))], UL2704 [unc-119(9ed3)III; lels2704(ceb-19bprom::gfp, unc-119(+))], UL2705 [unc-119(9ed3)III; lels2705(ceb-19bprom::gfp, unc-119(+))] (Reece-Hoyes et al., 2007). C. elegans strains with ceb-19(tm452) and ceb-19(tm461) alleles were from the Mitani Laboratory (http://www.shigen.nig.ac.jp). The ceb-19(tm452) allele was backcrossed into N2 seven times to generate the strain UL3128, which was used in most phenotypic assays.

Transgenic strains generated in this work include: UL3010 [leEx3010(fUL#HF005.1, rol-6(su1006))], UL3011 [leEx3011(fUL#HF005.1, rol-6(su1006))], and UL3012 [leEx3012(fUL#HF005.1, rol-6(su1006))], each carrying the recombineered fosmid fusion fUL#HF005.1 in an extrachromosomal array; UL3014 [leEx3014(fUL#HF004.1, rol-6(su1006))] and UL3015 [leEx3015(fUL#HF004.1, rol-6(su1006))], each carrying the recombineered fosmid fusion fUL#HF004.1 in an extrachromosomal array; UL3413 [ceb-19(tm452)IV; lels2703(ceb-19bprom::gfp, unc-119(+))], created by crossing lels2703 from UL2703 into UL3128; UL3013 [ceb-19(tm452)IV; leEx3011(fUL#HF005.1, rol-6(su1006))], created by crossing leEx3011 from UL3011 into UL3128; UL3010 [ceb-19(tm452)IV; leEx3012(fUL#HF005.1, rol-6(su1006))], created by crossing leEx3012 from UL3128; UL3548 [ceb-19(tm452)IV; leEx3014(fUL#HF004.1, rol-6(su1006))], created by crossing leEx3014 from UL3014 into UL3128; UL3795 [leEx3795(flp-2prom::gfp, rol-6(su1006))], UL3793 [leEx3793(flp-2prom::gfp, rol-6(su1006))], and UL3891 [ceb-19(tm452)IV; leEx3891(flp-2prom::gfp, rol-6(su1006))], created by microinjection of the wild type, N2, or the ceb-19 mutant, UL3128, with the plasmid containing the flp-2prom::gfp or flp-2prom::gfp fusions constructed by Gateway recombination; UL3881 [ceb-19(tm452)IV; leEx3795(flp-2prom::gfp, rol-6(su1006))], created by crossing leEx3795 from UL3795 into UL3128.
reproductive period and then examined every day until their death, as determined by lack of movement even in response to physical stimulation. Each day, dead individuals were removed from the plates and the deaths were recorded.

Statistics analyses of the behavioral assays were performed using one-way ANOVA in OriginPro7.5 (Origin Lab Corporation).

**RNAi by Feeding**

RNAi by feeding was carried out as described by (Kamath and Ahringer, 2003). Standard NGM plates were supplemented with ampicillin (50 μg/ml), tetracycline (10 μg/ml), and IPTG (isopropyl β-D-1-thiogalactopyranoside) (1 mM), and seeded with bacteria, from the RNai library from Geneservice (Kamath and Ahringer, 2003), verified by restriction enzyme digestion of purified plasmids. A few L3-L4 hermaphrodites of strains transgenic for a gfp reporter fusion, were transferred from an area off of the bacterial lawn of an OP50 seeded NGM plate, first to an unseeded NGM plate for a few minutes, and then to the RNai plates. The RNAi plates were maintained at 20°C for 3 days before observing the progeny. The negative control was HT115 bacteria containing pL4440, as for clones in the RNAi library, but without an insert between the T7 promoters. An equivalent bacterial strain with a pL4440 insert for unc-22 was used as a positive control.

**Yeast One-Hybrid Screens**

Yeast one-hybrid (Y1H) screens and assays were performed as described previously (Deplancke et al., 2004; Reece-Hoyes et al., 2011; Vermeirssen et al., 2007; Walhout, 2006). Promoter entry clones were either retrieved from the C. elegans Promoterome library or generated ab initio by Gateway cloning. (Primers are listed in Supplementary Table 1.) Promoter::reporter (HIS3/lacZ) bait fusions were generated by Gateway LR recombination reactions between the promoter entry clones and the pMW2-HIS3 and pMW2-lacZ yeast expression vectors. Promoter::reporter bait fusions were linearized and used in transformation of the YM4271 (MATa) yeast host strain with integration into the genome by homologous recombination. Twelve clones from each integration were assayed for self-activation of HIS3 and lacZ expression and the clone with the lowest level for each bait was used for subsequent screens.

Screens for transcription factors that interact with a promoter (Y1H) bait were performed using the enhanced Y1H (eY1H) approach (Reece-Hoyes et al., 2011). eY1H screens are more efficient than traditional library screens because the transcription factors are presented to the baits as an array of yeast prey strains (Yα1867, MATα), each transformed with a different Gal4AD-TF fusion. 755 C. elegans transcription factors were included in the array, with each TF represented four times and thus retested inherently. A bench top robot (RoToR, Singer Instrument, Somerset, UK) was used to precisely transfer the (up to) 1536 yeast colonies present on each media plate. To prepare for screens, bait yeast cells were propagated as lawn cultures in standard RoToR dishes containing YAPD media. The transcription factor array was maintained on Sc-Trp media in sets of three 1536-colony plates each containing four copies of up to 384 AD-TF prey yeast clones. To set up a mating, each bait lawn and the transcription factor array were sequentially copied to YAPD plates. The yeasts were grown at 30°C for 3 days before being copied to Sc-His-Ura-Trp plates to select for successfully mated diploids. Diploids were grown for two days before being copied to Sc-His-Ura-Trp plus 5 mM 3-AT (3-amino-1,2,4-triazole) and 80 mg/ml X-gal (5-bromo-4-chloro-3-indolylβ-D-galactoside) plates and incubated at 30°C. Plates were monitored over the next 5–7 days for the expression level of the reporters. Colonies that can grow in the absence of histidine, overcome the inhibitory effects of 3-AT, and turn X-gal into a blue compound are expressing the reporters, indicating a transcription factor-promoter (Y1H) interaction. Positives, with at least two of the four colonies containing a particular transcription factor showing reporter expression, were identified according to their array coordinates. The transcription factor ORFs of positive colonies were PCR amplified using primers corresponding to the vector and sequenced to verify the identity of the transcription factors.

To directly test individual transcription factor - promoter (Y1H) interactions, plasmids encoding Gal4AD-TF prey fusions were transformed into haploid promoter::reporter bait strains and activation of reporters in transformants was assessed with a β-galactosidase assay on overlay filter membranes and from growth on selective plates plus 20, 40, or 60 mM 3-AT (Vermeirssen et al., 2007).

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