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Transcription Factors GATA/ELT-2 and Forkhead/HNF-3/PHA-4 Regulate the Tropomyosin Gene Expression in the Pharynx and Intestine of Caenorhabditis elegans

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Transcription factors GATA/ELT-2 and forkhead/HNF-3/PHA-4 regulate the tropomyosin gene expression in the pharynx and intestine of *Caenorhabditis elegans*

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Abbreviations: TM, tropomyosin; RNAi, RNA interference; GST, glutathione-S-transferase; GFP, green fluorescence protein; ds, double-stranded

Running title: The tropomyosin gene regulation in *C. elegans*.

Summary

Gene regulation during development is an important biological activity that leads to synthesis of biomolecules at specific locations and specific times. The single tropomyosin gene of *Caenorhabditis elegans*, *tmy-1*/*lev-11* produces four isoforms of protein: two from the external promoter and two from the internal promoter. We investigated the internal promoter of tropomyosin to identify sequences that regulate expression of *tmy-1* in the pharynx and intestine. By promoter deletion of *tmy-1* reporters as well as by database analyses, a 100 bp fragment was identified that contained binding sequences for a GATA factor, for a chicken CdxA homolog and for a forkhead factor. Both the forkhead and CdxA binding sequences contributed to pharyngeal and intestinal expression. In addition, the GATA site also influenced intestinal expression of *tmy-1* reporter. We showed that ELT-2 and PHA-4 proteins interact directly with the GATA and forkhead binding sequences, respectively, in gel mobility shift assays. RNAi knockdown of *elt-2* diminished *tmy-1::gfp* expression in the intestine. In contrast to RNAi knockdown of *pha-4*, expression of *tmy-1::gfp* in *pha-4;smg-1* mutants was slightly weaker to that of the wild type. Ectopic expression of PHA-4 and ELT-2 by heat shock were sufficient to elicit widespread expression of *tmy-1::lacZ* reporter in embryos. We found no indication of a synergistic relation between ELT-2 and PHA-4. Based on our data, PHA-4 and CdxA function as general transcription factors for pharyngeal and intestinal regulation of *tmy-1*. We present models by which ELT-2, PHA-4 and CdxA orchestrate expression from the internal promoter of *tmy-1*.

Introduction

Tropomyosin (TM) is a conserved and ubiquitous protein. In vertebrates and some invertebrates, multiple genes encode TMs. For example, four genes encode mammalian TMs[1] and two genes encode *Drosophila* TMs.[2] The structure of TM genes suggests that all TM genes evolved from a single ancestral gene that possessed two alternative 5′ promoters and two alternatively spliced 3′ exons.[3] Combining the two promoters with alternative splicing and 3′ end processing, a large number of diversed TM isoforms are generated. For example, the *Tm1* gene of *Drosophila* encodes more than 5 isoforms[4] and the *Tmγ* gene of mouse has 11 non-muscle isoforms.[5]

Caenorhabditis elegans has a single TM gene, *tmy-1/lev-11*,[6] which shares structural characteristics with other TM genes of other species. *tmy-1* produces four isoforms: CeTMI and CeTMII are generated from an external promoter while CeTMIII and CeTMIV are generated from an internal promoter.[6,7] Expression of the isoforms transcribed from the external promoter does not overlap with expression of the isoforms transcribed from the internal promoter.[6,7] CeTMI and CeTMII are expressed in body wall, sex and anal muscles.[6] CeTMIII and CeTMIV are expressed in the intestine and muscle cells of the pharynx.[7] Knockdown of specific isoforms with RNA interference mostly leads to embryonic lethality and surviving worms are severely deformed.[7] The mechanisms that drive transcription and specification of these isoforms are unknown. We had previously identified several potential PHA-4 binding sites within the internal promoter of *tmy-1*[7] but did not experimentally analyze these sites.

PHA-4, the *C. elegans* ortholog of forkhead/HNF-3, a winged helix transcription factor,[8,9] specifies the pharyngeal primodium.[10] In *pha-4* mutants, derivatives of ABa

and MS cell lineages that produce pharynx[11] are converted into ectoderm.[8,10] PHA-4 expression is also detected at low levels in the intestine and may be under direct control of ELT-2,[9] a conserved GATA transcription factor.[12]

GATA factors are implicated in cell-fate specification, cell migration, cell fusion, and differentiation in both vertebrates and invertebrates.[13] *C. elegans* has eleven GATA factors that are expressed in EMS cell lineages,[14,15] vulval[16] and epidermal cells.[17,18] Out of the three GATA factors specifically expressed in both the embryonic and adult intestines, ELT-2 is the only factor that is essential for intestinal cell differentiation.[12,14] Homozygous deletion or inactivation of *elt-2* leads to embryos with malformed intestines that arrest at the L1 stage; lethality is likely due to starvation.[14] When expressed ectopically within the early embryo, ELT-2 is capable of re-programming non-E lineage cells into intestinal cells.[14,19] Recent evidence shows that the onset of ELT-2 expression initiates expression of most intestinal genes of the developing embryos.[12] Transcription factors for cell-fate specification and cell lineage of pharynx and intestine in *C. elegans* are summarized in Fig. 1A.[9,10,11,20]

In the present study, we investigated the mechanisms controlling the internal promoter of the *tmy-1* gene of *C. elegans*. Deletion analysis and site-directed mutagenesis of the promoter revealed a 100 bp fragment containing GATA, chicken CdxA homolog, and forkhead binding sequences. ELT-2 and PHA-4 can be shown to bind directly to the GATA and forkhead binding sequences, respectively, *in vitro*. We also present evidence that PHA-4 and CdxA are required for broad expression of TM in both pharynx and intestine, whilst ELT-2 directs intestinal expression only. Synergism between ELT-2 and PHA-4 was not detected. Based on our results together with other

published data, we propose that interaction between ELT-2 and PHA-4 occurs to coordinate activation of *tmy-1* in the intestine.

Results

A 470 bp fragment in the internal promoter of *tmy-1* is sufficient for pharyngeal and intestinal expression

To identify specific DNA sequences that contribute to expression of *tmy-1* from the internal promoter, we performed unidirectional deletion analysis of the *tmy-1* promoter fused to a GFP reporter. In worms transformed with a promoter length of 853 bp of *tmy-1*, embryonic expression of the reporter was first detected between the 300-cell and comma stages of development in regions that correspond to pharyngeal and intestinal cells.[7]

Post-embryonic expression of the reporter was observed in all pharyngeal muscle, pharyngo-intestinal valve, intestinal and rectal cells (Fig. 2A and B). The strongest pharyngeal muscle and intestinal expression of reporters containing 470 bp (Fig. 2B) and longer lengths of the 5′ upstream region were observed at the L2 stage and continued through to adulthood (Fig. 2A; data not shown). Reporter expression was more obvious in the pharynx than in the intestine. Amongst the intestinal cells, reporter expression was more intense in the posterior than in the anterior. This expression pattern did not changed after digestion of food at low background. In terms of timing and GFP intensity, the pharyngeal and intestinal expression of construct pTMGIV1570 (470 bp promoter fragment) replicated expression by longer 5′ regions such as pTMGIV1957 (853 bp) and pTMGIV4349 (3.2 kb).[7] In other words, the 470 bp of the upstream

flanking region appeared to be sufficient for wild type levels and patterns of *tmy-1* reporter expression (Fig. 2B).

Worms transformed with pTMGIV1538 (438 bp promoter fragment) expressed GFP in the pharynx but not in the intestine (Fig. 2C). Reporters containing 405 bp or fewer of the upstream region were not expressed in either the pharynx or intestine (Fig. 2D). Loss of intestinal expression but not pharyngeal expression observed with pTMGIV1538 (Fig. 2C) suggested that distinct and separable sequences regulate pharyngeal-specific and intestinal-specific expression of the *tmy-1* gene. The expression patterns observed with the various reporters have been summarized in Table 1.

GATA, CdxA and PHA-4 sites function in pharyngeal and intestinal expression of *tmy-1*

Analysis of the sequence between 350 and 450 bp upstream from the start codon (Fig. 3B) revealed a CTATCA, CACAAAT and TGTTTGT sequences, corresponding to GATA,[21] chicken CdxA homolog[22] and forkhead/HNF-3[23] binding sites, respectively (Fig. 3 C-E). The CTATCA is WGATAR consensus-binding site in the reverse orientation. In order to determine the functional contribution of the CTATCA (referred to only as GATA) to expression of *tmy-1*, we inactivated the core TATC sequence by site-directed mutagenesis to GCGA in the plasmid pTMmutGATA (Materials and Methods). Worms transformed with pTMmutGATA showed diminished GFP expression in the embryonic and post-embryonic intestines but not in the pharynx (Fig. 4B; data not shown). Pharyngeal expression of the pTMmutGATA reporter was similar in intensity and timing to the native pTMGIV1736.

When the CdxA homolog site was mutated in pTMmutCdxA, reporter expression was not observed in either the pharynx or the intestine throughout development (Fig. 4C). Following a similar regime, the TGTTTGT sequence was mutated to GTGGGTG resulting in pTMmutPHA-4. Transgenic worms containing the pTMmutPHA-4 failed to express the GFP in either pharynx or intestine (Fig. 4D). These results demonstrated that, together, the GATA, CdxA, and HNF-3 sequences conferred wild type expression of *tmy-1* in the intestine. CdxA and HNF-3 but not GATA binding sequences were required for pharyngeal expression. Table 1 summarizes the expression patterns of reporters.

ELT-2 and PHA-4 are required for *tmy-1* reporter expression

ELT-2, a GATA factor has been reported as a critical factor in expression of intestine-specific genes.[12] Because the GATA site within the minimal internal promoter is required for intestinal expression of the reporter (Fig. 2C and Fig. 4B), we tested if ELT-2 plays a role in activation of *tmy-1* in the intestine. *elt-2* function was reduced by RNA-interference (RNAi) in worms expressing the pTMGIV1957 reporter. All F1 progeny (*n*=48) showed gut-obstructed phenotype and arrested not later than the L1 stage. GFP expression was not observed in the intestine but pharyngeal expression of GFP was not affected (Fig. 5A(c)). The arrested L1 larvae died within two days. To eliminate the possibility that other intestine-specific GATA factors play a role in the reporter expression, *elt-7* (RNAi) was also performed in worms expressing the pTMGIV1957 reporter. No obvious phenotype was identified. The *elt-7* (RNAi) worms (*n*=63) maintained reporter expression in both pharynx and intestine (Fig. 5A(b)). As a

control, we also performed RNAi against *acn-1*, an angiotensin converting enzyme-like gene, which resulted in L1 arrest of F1 larvae. The arrested transgenic worms (*n*=51) expressed GFP reporter in the intestine and pharynx at intensities comparable to pTMGIV1957 transgenic worms (Fig. 5A(e)). Overall, our results indicate that *elt-2* is necessary for the *tmy-1* expression in the intestine but not in the pharynx.

We also tested RNAi knockdown of *pha-4* in pTMGIV1957 transgenic worms. Arrested F1 progeny (*n*=44) failed to form pharynges. The intestine did not express the reporter (Fig. 5A(d)). The posterior part of the intestine was detached from the deformed rectum (data no shown). The few worms that did not arrest, formed pharynges and reached adulthood. These worms likely hatched from eggs that were laid shortly after introduction of the double stranded RNA into their parents.

Expression of the pTMGIV1957 reporter was also characterized in *pha-4(zu225);smg-1(cc546ts)* mutants, in which the level of mRNA and character of PHA-4 are compromised.[24] The reporter was expressed in both pharynx and intestine. The level of GFP intensity was slightly weaker compared to that of the wild type worm (Fig. 5B(b)). Expression of pTMGIV1957 in *smg-1(cc546ts)* control worms was identical to the expression in the wild type worm (Fig. 5B(c)). Overall, we conclude that *pha-4* is necessary for *tmy-1* expression in both the pharynx and intestine.

ELT-2 and PHA-4 bind GATA and TGTTTGT sequences *in vitro,* respectively

To further explore the role of ELT-2 in the intestinal expression of *tmy-1*, we examined whether ELT-2 directly interacts with the GATA site of *tmy-1* using an electrophoretic mobility shift assay. Purified ELT-2 bound to a 38bp probe (Fig. 6A,

lane 2) identical to the native sequence of *tmy-1* containing the GATA sequence (Materials and Methods). Excess unlabeled wild type oligonucleotide effectively competed for ELT-2 (Fig. 6A, lanes 3 to 5). In assessing the ELT-2 binding specificity, the core GATA sequence of the competitor was mutated. Competition for ELT-2 binding by the mutant unlabeled oligonucleotide was ineffective even at 100-fold excess (Fig. 6A, lanes 6 to 8). These results showed that interaction of ELT-2 to the GATA sequence in the internal promoter of *tmy-1* is specific.

We also investigated PHA-4 direct interaction with the TGTTTGT sequence of *tmy-1* (Materials and Methods). PHA-4 interacted with the TGTTTGT sequence (Fig. 6B, lane 2). Molar excess of unlabeled wild type competed with the labeled probe for PHA-4 (Fig. 6B, lanes 3 to 5) whereas an unlabeled oligonucleotide containing a mutant PHA-4 site (GTGGGTG) was significantly less-effective in competing for PHA-4 (Fig. 6B, lane 6 to 8). These results indicate that PHA-4 interacts specifically with the TGTTTGT sequence of the *tmy-1* promoter.

ELT-2 and PHA-4 directly interact *in vitro*

To investigate whether ELT-2 and PHA-4 proteins directly interact, PHA-4 was overlaid on ELT-2 and detected by PHA-4 antibody following a protein-protein interaction procedure previously described.[25,26] In Fig. 7, PHA-4 antibody detected a single band corresponding to ELT-2-GST (lane 2) but not GST and total proteins from bacteria (lane 1) in which the ELT-2 was produced (Materials and Methods). These results demonstrated that PHA-4 directly binds to ELT-2. When worm protein extract was overlaid with PHA-4, the antibody detected two bands in the range of 47.5 and 62

kDa (Fig. 7, lane 4). These two bands had similar sizes as the predicted isoforms of PHA-4.[27]

Ectopic expression of ELT-2 and PHA-4 induce *tmy-1 in vivo*

To demonstrate whether ELT-2 and PHA-4 are sufficient for *tmy-1* expression, we separately placed *elt-2* and *pha-4* under control of the heat shock promoter. About 77.3% of transgenic embryos (*n*=53) containing both *hsp-16::elt-2* and pTMZIV1957 plasmids showed widespread activity of *lacZ* in almost all cells when heat-shocked (Fig. 8B). The level of *lacZ* staining was intense compared to control embryos that contained pTMZIV1957 alone. In addition, the double transgenic worms were arrested before any morphological development could occur. Control embryos showed *lacZ* activity only in regions corresponding to the developing pharynx and intestine (Fig. 8A). In contrast, when embryos containing both *hsp-16::pha-4* and pTMZIV1957 were heat shocked, 71.8% (*n*=78) of the embryos showed *lacZ* activity in most cells outside the intestine and pharynx (Fig. 8C). These results demonstrate that ELT-2 and PHA-4 are sufficient for *tmy-1* induction in embryos.

Induction of the pTMZIV1957 reporter expression in post-embryonic stages of the worm by ectopic ELT-2 and PHA-4 could not be observed because the affected embryos were arrested and did not hatch.

High levels of PHA-4 do not enhance activation of *tmy-1* by ELT-2

We investigated synergism between ELT-2 and PHA-4 in the regulation of *tmy-1*. High levels of PHA-4 were expressed under the control of intestine-specific *ges-1* promoter[28] (Materials and Methods) in pTMGIV1957 background worms. Transgenic embryos (*n*=78) and adult worms (*n*=31) containing both *ges-1* (promoter)::*pha-4* and the pTMGIV1957 expressed GFP at similar levels to those of worms expressing pTMGIV1957 reporter alone (data not shown). We did not see any evidence of synergism between ELT-2 and PHA-4 in the regulation of *tmy-1*. We conclude that low levels of PHA-4 detected in the intestine of wild type worms[9] are sufficient to cooperate with ELT-2 to activate intestinal expression of *tmy-1*. The low levels of endogenous PHA-4 may be adequate and further addition of PHA-4 via transgenes does not enhance synergism.

Discussion

This study focused on regulatory sequences within the internal promoter of the *C. elegans tmy-1* gene and their associated binding proteins that influence *tmy-1* expression in the pharynx and intestine. We showed that all the essential DNA sequences for pharyngeal and intestinal expression of *tmy-1*::GFP reporter are located within a 470 bp fragment of the internal promoter. Evidence provided in this study also shows that regulators of *tmy-1* expression are the GATA factor ELT-2, the forkhead winged helix factor PHA-4, as well as some unknown CdxA homolog.

Our previous study showed that the control region in the internal promoter of *tmy-1* is between 115 to 853 bp from the initiation codon.[7] Within the control region, C2, C3 and C4[7] are similar to PHA-4 binding sequence TRTTKRY.[23] Deletion analysis clearly demonstrates that C2, C3 and C4 are not essential but a TGTTTGT sequence, which corresponds to the TRTTKRY, at 363 bp from the initiation codon is essential for expression (Fig. 3B and E). Mutation of the TGTTTGT sequence greatly lowers *tmy-1* expression in intestine and we suggest that PHA-4 directs *tmy-1* expression through a specific interaction with this TGTTTGT sequence.

GFP expression in *pha-4* (RNAi) worms contrasts to that in the *pha-4(zu225);smg-1(cc546ts)* mutants (Fig. 5A(d) and Fig 5B(b)). Due to the severe phenotype of the *pha-4(RNAi)* worms, it is not possible to conclude that loss of reporter expression was a response to the absence of PHA-4, as opposed to simply the loss of the pharynx. The *pha-4(zu225);smg-1(cc546ts)* mutant at permissive temperature generates PHA-4 with a pre-mature stop codon after the DNA binding domain.[24] The truncated

PHA-4 did not affect *tmy-1* reporter expression (Fig. 5B(b)) suggesting that the mutant PHA-4 may have functioned in a manner similar to the wild type PHA-4.

Four GATA factors MED-1, MED-2, END-1, and END-3 are transiently expressed during endoderm specification.[15,29,30] Three other GATA factors, ELT-2, ELT-4, and ELT-7 are endoderm-specific and are continuously expressed after specification of the endoderm in *C. elegans*.[20] ELT-4 has no transcriptional activity.[31] ELT-2 and ELT-7 are not redundant in the activation of *tmy-1* (Fig. 5A(b) and (c)). ELT-7 could not activate *tmy-1* reporter expression in *elt-2* (RNAi) worms, thus ELT-7 has no effect on *tmy-1* activation. Our conclusion agrees with the suggestion that the function of *elt-7* is dispensable.[12] Contrary to the *elt-7* knockdown, depletion of *elt-2* is detrimental to *tmy-1* activation in the intestine. The adverse effect of *elt-2* elimination is not limited to *tmy-1* activation but also applies to other intestine-specific genes.[28,32,33,34,35] Indeed, ELT-2 binds to the GATA sequence of *tmy-1* (Fig. 6A) and ELT-2 is sufficient for ectopic expression of *tmy-1* reporter (Fig. 8B). This is consistent with a recent suggestion that most intestinal genes are regulated by ELT-2.[12] We conclude that ELT-2 binds to the critical GATA sequences and activates *tmy-1* expression in the intestine.

Transcriptional coordination of intestinal expression

The simultaneous presence of both ELT-2 and PHA-4 are required in the intestine to activate *tmy-1* expression (Fig. 2B, Fig. 5A(c) and Fig. 5A(d)). This is consistent with the simultaneous presence of GATA-4 and HNF-3 in enhancing activation of mouse albumin genes in endoderm.[36] It is known that GATA-4 and HNF-3

occupy their binding sites *in vivo* on the promoter of albumin gene of mouse but remain inactive until additional required factors are present.[36] Additional factors such as Sp1 and Sp3 in the presence of GATA-4 and HNF-3 activate *Hex* of mouse.[37] ELT-2 and PHA-4 require additional factors to activate transcription of *tmy-1*. Our mutation analysis shows that a CdxA-like factor may also play a role as an additional factor (Fig. 4C). Cdx interacts with GATA as well as HNF-1α in activation of intestine specific sucrase isomaltase gene in mouse.[38] Based on the evidence in this study and other published data, we propose a model by which *tmy-1* is activated from the internal promoter in intestinal cells (Fig. 1C). Cooperation of ELT-2, PHA-4 and CdxA homolog initiates *tmy-1* activation. Thus, all the three factors are necessary for expression in the intestine although we have not experimentally determined any interaction between CdxA homolog and PHA-4.

PHA-4 and CdxA as general transcription factors of *tmy-1* gene

Because PHA-4 is expressed in all pharyngeal cells,[9,10,23] pharyngeal muscle specific expression of *tmy-1* requires cooperation of PHA-4 with other factors. CEH-22, a member of the NK-2 homeobox transcription factor differentiates pharyngeal muscles cells and activates gene expression.[39,40,41] Although we did not test for role of CEH-22, expression of *tmy-1*::GFP reporter specifically in pharyngeal muscle cells implicates CEH-22. Recent data analysis of sequences downstream of the PHA-4 site of the *tmy-1* internal promoter revealed three potential CEH-22 sequences (unpublished data). It is likely that expression of the *tmy-1* in pharyngeal muscle cells could involve CEH-22 activity (Fig. 1B).

PHA-4 had been widely implicated in activation of pharyngeal specific genes[9,10,23,41] but mutation of the PHA-4 binding site in *tmy-1* shows that at least, one gene expressed in the intestine is dependent on PHA-4. The involvement of PHA-4 in intestinal expression of *tmy-1* was unexpected but not surprising. Low levels of PHA-4 are detected in the intestine but the role PHA-4 plays in the intestine is unknown.[9] Although ELT-2 activates almost all intestinal genes,[12] this study (Fig. 2D and Fig. 4D) suggests that the function of PHA-4 in the intestine is to co-activate genes that are also expressed in the pharynx. An identical observation was seen when the CdxA binding site was mutated (Fig. 4C). Both PHA-4 and CdxA homolog are required at the same time for expression to occur in the pharynx and intestine. Thus, PHA-4 and CdxA are general factors for *tmy-1* expression.

Control mechanisms of tissue and isoform specificities of *tmy-1*

Isoforms CeTMIII and CeTMIV share the same internal promoter region for expression in the pharynx and intestine. Differences between these two isoforms are due to alternative splicing. The differences have profound effects on the morphology of the worm (Anyanful et al., 2001). Distinguishing specific functions of these two isoforms has been a difficult task to perform. Recently, Kuroyanagi *et al.* (2006)[48] succeeded in developing a technique that differentiates between expression of isoforms from the same gene and isolation of mutants defective in alternative splicing. This technique combined with how *tmy-1* is transcribed opens a new approach in solving mechanisms that lead to one gene-many isoforms phenomenon.

Materials and Methods

Maintenance and generation of transgenic nematodes

Nematodes were culture as described[44] except *pha-4;smg-1* and *smg-1* which were cultured at 24°C.[24] *C. elegans* strain, Bristol N2[45] was used as wild type. Plasmid reporter transformation was carried out by microinjection into the gonads of N2 hermaphrodite as described.[46] Processing of nematodes was as described.[7]

Construction of reporters for deletion and mutagenic analyses

Upstream deletions of *tmy-1* internal promoter were performed using the plasmid pTMGIV1957 as template by PCR. GFP primer served as an antisense primer. Sense primers are: TMDel-1736 (5′-CCAAAGCTAAAGCTGCAGCCTACCAGAACC), TMDel-1609 (5′-CCTCTCTCCATCTGCAGTCACTCTCCACG), TMDel-1538 (5′-CATCACTCGGCACCTGCAGTCTGCGGCTCC), TMDel-1505 (5′-CCACAAATCAGACTGCAGATACCGCCAACTCC) and TMDel-1404 (5′-GCATAAGACACTCGGCTGCAGGAGAGAAGG) for the reporter constructs pTMGIV1736, pTMGIV1609, pTMGIV1538, pTMGIV1505 and pTMGIV1404, respectively. We used ABI PRISM^{TM} 310 Genetic Analyzer (Applied Biosystems) for sequencing DNA. The DNA sequenced data was analyzed by GENETYX-MAC Version 11.2.3 software.

Site-directed mutation of the GATA site was performed in three steps. PCR I: TMDel-1736GATAmut (5′-CATCACTCGGCACGCGAAGTCTGCGGCTCC) and GFP primers amplified a 410bp fragment of the template pTMGIV1957. PCR II: M13 and GFP primers amplified a 632bp fragment of the template pTMGIV1957. PCR III: the GFP and M13 primers then joined products of PCRs I and II. The end product of PCR III contained the mutagenized GATA site. This was then ligated into the *PstI* site of pPD95.81 containing exons 3b, 4a and 5c of *tmy-1*.[7] Similar regime was employed for mutagenesis of PHA-4 and CdxA sites by the sense primers 5′-CCCCGCTCTCTAGTGGGTGAGAGAGGCTGG and 5′-CAGTCTGCGGCTCACACCCGCAGATGCTCC, respectively.

For high-level expression of PHA-4, a 2 kb *ges-1* promoter region was inserted upstream of *pha-4* cDNA, yk649e3 (from Dr Yuji Kohara). Concentrations of 100-550μg/ml with a fixed concentration of 100μg/ml of pTMGIV1957 and pRF4 marker were injected into N2 worms.

RNA interference

elt-2, *acn-1* and *elt-7* plasmids (from Dr James D. McGhee) were amplified by PCR and double stranded RNAs (dsRNAs) were synthesis *in vitro* using Ambion MAXIscript T7/T3 RNA polymerase transcription kit (Ambion Inc). DNA degradation, RNA extraction, and annealing were as described.[47] *pha-4* cDNA clone, yk649e3 (from Dr Yuji Kohara) was also used to synthesize dsRNA as described above.

Gel retardation assay

ELT-2 –GST (from Dr Julie D. Saba) was as described.[35] Transformation was done in *Escherichia coli* strain, BL21. ELT-2-GST was induced with isopropyl 1-thio-β-D-galactopyranoside. Glutathione Sepharose 4B kit (from GE Healthcare Bio-Sciences Corp.) was used to purify ELT-2 following the manufacturer's instructions. Labeling of probe (5′-CTGTCATCACTCGGCACTATCAGTCTGCGGCTCCACAA) containing the native GATA (underlined) and binding of the purified ELT-2 to the probe were performed using non-radioactive DIG Gel Shift Kit (from Roche Diagnostics GmbH, Mannheim, Germany) following manufacturer's instructions. For binding specificity experiment, the TATC of the oligonucleotide was mutated to GCGA. Competition experiments were performed utilizing unlabeled 38bp identical to the labeled probe.

pha-4 cDNA yk649e3 (from Dr Yuji Kohara) was cloned into the *HindIII* site of pET28 vector. PHA-4 was induced as above and purified using His-tag purification kit (Novagen) following manufacturer's instruction. Labeling of PHA-4 probe (5′-TCCCCCGCTCTCTAATGTTTGTAGAGAGGCTGGGCGC) was done as described above. In binding specificity assay, TGTTTGT was mutated to GTGGGTG. In both cases, electrophoresis was done using 6% non-denaturing acrylamide gel.

Ectopic expression

elt-2 plasmid (from Dr James D. McGhee) was amplified by 5′-CCGTGATATCATGGATAATAACTACAATGATAATGTC and 5′-

AGGCGATATCTTAAGAATCTCCGTCGACCGCTTCC primers. The PCR product was cloned into *EcoRV* site of pPD49.83 resulting in *hsp-16*::*elt-2*. *hsp-16::pha-4* was constructed by inserting the *pha-4* cDNA clone into *EcoRV* site of the pPD49.83. Transgenic worms were generated by separate co-injection of the *hsp-16*::*elt-2* and *hsp-16::pha-4* with pTMIVZ1957 and pRF4 marker plasmid at a concentration of 100µl/ml into young adult hermaphrodites. To induce ectopic expression, eggs removed from the transgenic worms were heat shocked for 1 hour at 34°C and incubated overnight at 20°C. Staining and detection of β-galactosidase activity was as described.[46]

Anti-serum production and protein overlay assay

Full-length *pha-4* cDNA yk649e3 (from Dr Yuji Kohara) was ligated into *HindIII* site of pET-28b(+) vector (Novagen) and transformed into *E. coli* strain BL21. Anti-serum preparation and SDS-PAGE were as described.[25,26] Worm extract was prepared by collecting mixed-stage worms and sonicated in PBS (pH=7.4). Equal volume of 2x Laemmli was added and boiled at 95°C for 2 minutes. The mixture was centrifuged at 4°C and supernatant used for SDS-PAGE as described above.

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Figure legends

Fig. 1. Cell lineage and models of pharyngeal and intestinal activation of *tmy-1* in *C. elegans*. (A) Embryonic fate map and transcription factors of *C. elegans* pharynx, intestine and rectum from the four-cell stage.[11] Horizontal lines represent cell divisions. Transcription factors that determine cell fate are indicated.[9,10,11,20] (B and C) Models depicting co-operation of ELT-2, CdxA homolog and PHA-4 in activation of *tmy-1* in pharynx and intestine, respectively. Details are in the text.

Fig. 2. Unidirectional deletion analysis of *tmy-1* internal promoter. The GATA, CdxA homolog and PHA-4 binding sites are indicated in the upstream region of the constructs. C2-C4 indicate *C*-elements of *myo-2*.[7] The corresponding GFP reporter expressions of pTMGIV deletion series are on the right panels (A-D). Filled boxes represent exons and numbers above the boxes represent the first-three exons of *tmy-1* isoform CeTMIV.[7] The +++ and – signs indicate relative level of expression intensities of GFP reporters on a scale of strong to undetectable, respectively. Summary of reporter expression patterns are in Table 1.

Fig. 3. Regulatory sequences of the *tmy-1* internal promoter. (A) Partial gene organization of *tmy-1*. Filled boxes represent exons and numbers above the boxes are exon numbers. Horizontal lines connecting the exons indict introns.[7] B1-B3 and C1-C4 indicate *B* and *C* regulatory subelements of *myo-2*, respectively. (B) Boxes indicate GATA/ELT-2, CdxA homolog and forkhead/HNF-3/PHA-4 binding sequences. Numbers below the enhancer sequence represent the position of nucleotides from the

ATG start codon. The 5′ ends of pTMGIV1538 and pTMGIV1505 used in deletion analysis are illustrated. (C-E) Sequence comparisons of (C) GATA/ELT-2 (D) CdxA homolog and (E) forkhead/HNF-3/HNF-3 binding sites. Nucleotides in lower cases represent incomplete match of the consensus sequence. Asterisks indicate binding sites that are oriented to conform to the consensus sequence. References: *ges-1*,[28] *pho-1*,[34] *spl-1*,[35] *albumin*,[36] *ADA*,[43] *SI*,[42] *ceh-22*,[41] *myo-2* [9] and *Hex*.[37]

Fig. 4. Mutation analysis of regulatory sequences. (A) Wild type. (B) Mutated GATA binding sequence. (C) Mutated CdxA binding sequence. (D) Mutated PHA-4 binding sequence. Mutated sequences are in the text. Filled boxes, numbers, +++ and – signs are as described in Fig. 2. X indicates mutation. Summary of reporter expression patterns are in Table 1.

Fig. 5. RNAi analysis and *tmy-1* expression in mutant background. (A) pTMGIV1957 reporter expression in (a) Wild type, (b) *elt-7* (RNAi), (c) *elt-2* (RNAi), (d) *pha-4* (RNAi) and (e) *acn-1* (RNAi) worms. (B) pTMGIV1957 reporter expression in (a) wild type worm, (b) *pha-4; smg-1* mutant worm and (c) *smg-1* mutant worms. The +++ and – signs indicating the relative level of GFP expression intensity are as described in Fig. 2.

Fig. 6. Gel mobility shift assay of ELT-2 and PHA-4. (A) Lane 1: Labeled wild type GATA oligonucleotide. Lane 2: ELT-2 and labeled wild type GATA oligonucleotide complex. Lanes 3-5: Competition for ELT-2 by labeled and increasing amounts of unlabeled wild type GATA oligonucleotides. Lanes 6-8: Competition for ELT-2 by labeled wild type and unlabeled increasing amounts of mutant oligonucleotides. (B)

Lane 1: Labeled wild type TGTTTGT oligonucleotide probe. Lane 2: PHA-4 and labeled wild type TGTTTGT oligonucleotide complex. Lanes 3-5: Competition for PHA-4 by labeled and increasing amounts of unlabeled wild type TGTTTGT oligonucleotides. Lanes 6-8: Competition for PHA-4 by labeled wild type TGTTTGT and increasing amounts of unlabeled mutant GTGGGTG oligonucleotides.

Fig. 7. Protein overlay analysis of ELT-2 and PHA-4 interaction. Lane 1: Total protein of bacteria containing empty vector. Lane 2: ELT-2. Lane 3: PHA-4. Lane 4: Worm protein extract. Arrow and arrowhead indicate 55.4 kDa and 48.1 kDa bands, respectively.

Fig. 8. Ectopic expression of ELT-2 and PHA-4 in embryos. pTMZIV1957 reporter expression were observed for 7 hours (left) and 11 hours (right) after heat treatment. (A) Wild type, (B) ELT-2 and (C) PHA-4 embryos.

Table 1. Summary of expression patterns of *tmy-1* reporters

Plasmids	Promoter length (bp)	Binding sites			Expression patterns	
		ELT-2 (TATC)	CdxA (CACAAAT)	PHA-4 (TGTTTGT)	Pharynx	Intestine
pTMGIV1957	853	+	+	+	+++	++
pTMGIV1736	632	+	+	+	+++	++
pTMGIV1570	470	+	+	+	+++	++
pTMGIV1538	438	—	+	+	+++	—
pTMGIV1505	405	—	—	+	—	—
pTMGIV1404	300	—	—	—	—	—
pTMmutGATA	632	m	+	+	+++	—
pTMmutCdxA	632	+	m	+	—	—
pTMmutPHA-4	632	+	+	m	—	—

The +++ and - signs are as described in Fig. 2. m indicates mutation.

Fig. 1

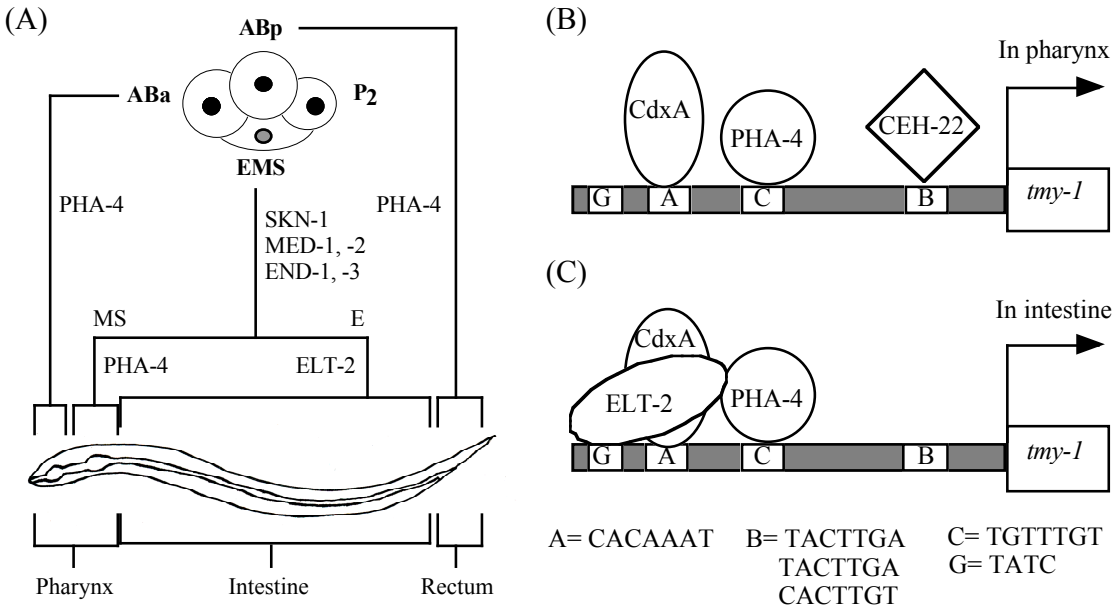


Fig. 2

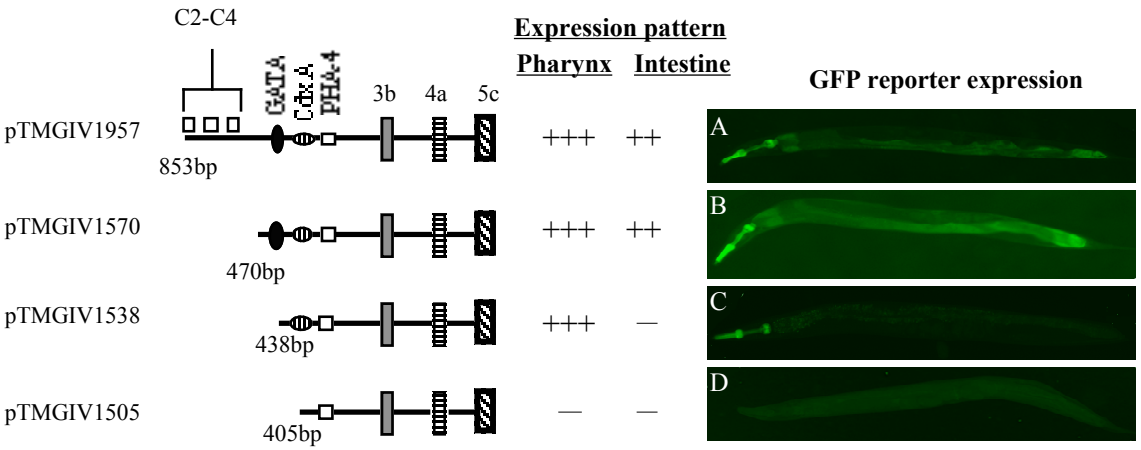
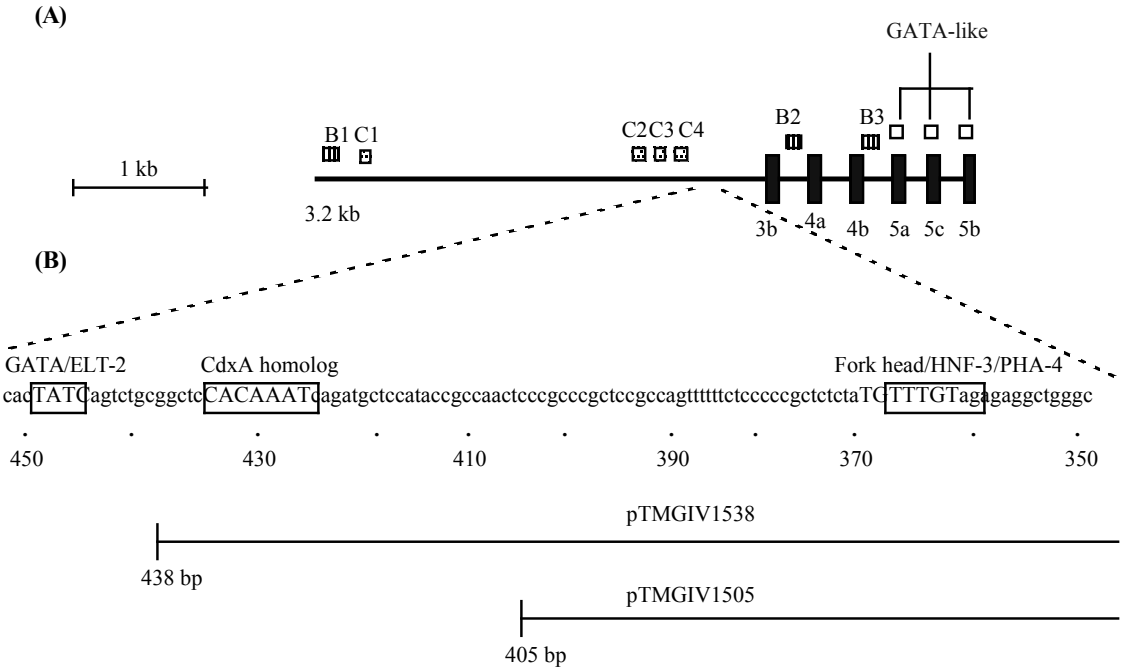

C2-C4
GATA
Cdx4
PHox4
3b
4a
5c
Expression pattern
Pharynx
Intestine
GFP reporter expression
pTMGIV1957
853bp
+++
++
A
pTMGIV1570
470bp
+++
++
B
pTMGIV1538
438bp
+++
—
C
pTMGIV1505
405bp
—
—
D

Fig. 3



(C)

	(A/T)GATA(A/G)
tmy-1	***TGATAG**
ges-1	TGATAA
pho-1	TGATAA
spl-1	TGATAA
serum albumin	TGATAc

(D)

	(A/C)TTTAT(A/G)
tmy-1	***ATTTgTG**
ADA	*ATTTATG
SI	CTTTATG

(E)

	T(A/G)TT(T/G)(A/G)(T/C)
tmy-1	**TGTTTGT**
ceh-22	TGTTTGT
	TGTcTAT
myo-2	TGTTTGC
Hex	TGTTTGT

Fig. 4

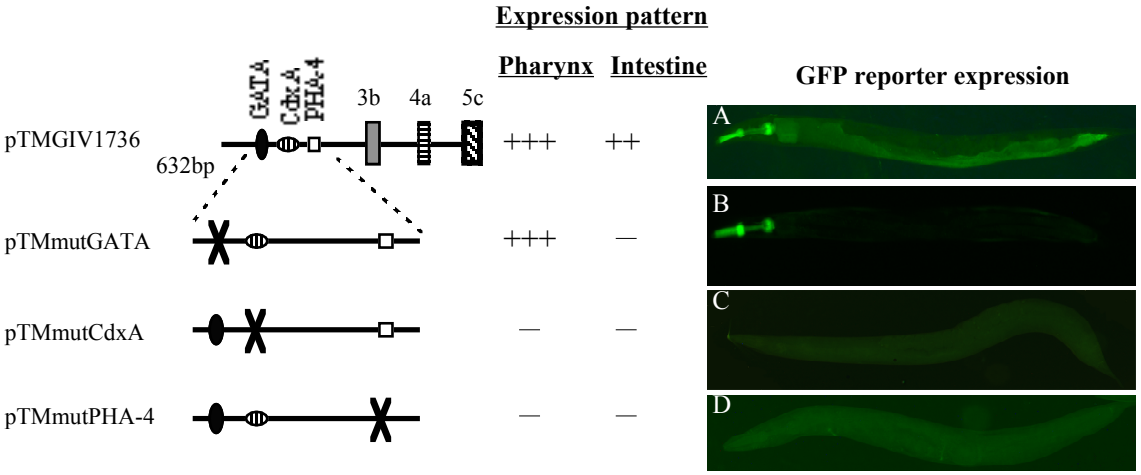

Expression pattern
Pharynx
Intestine
GFP reporter expression
GATA
CdxA
PHA-4
3b
4a
5c
pTMGIV1736
632bp
+++
++
pTMmutGATA
+++
—
pTMmutCdxA
—
—
pTMmutPHA-4
—
—
A
B
C
D

Fig. 5

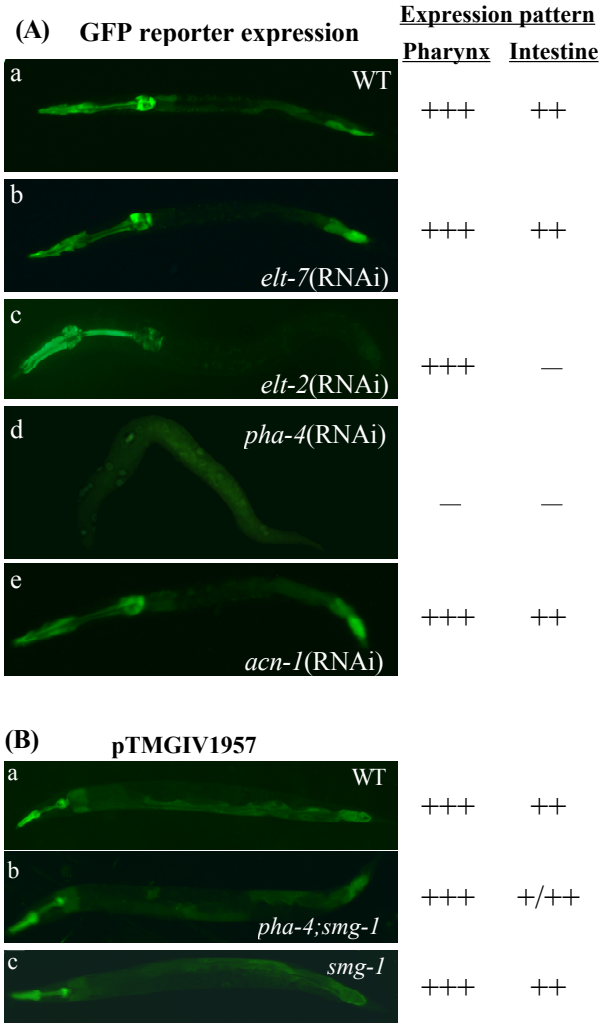

(A) GFP reporter expression
Expression pattern
Pharynx
Intestine
a
WT
+++
++
b
elt-7(RNAi)
+++
++
c
elt-2(RNAi)
+++
—
d
pha-4(RNAi)
—
—
e
acn-1(RNAi)
+++
++
(B)
pTMGIV1957
a
WT
+++
++
b
pha-4;smg-1
+++
+/++
c
smg-1
+++
++

Fig. 6

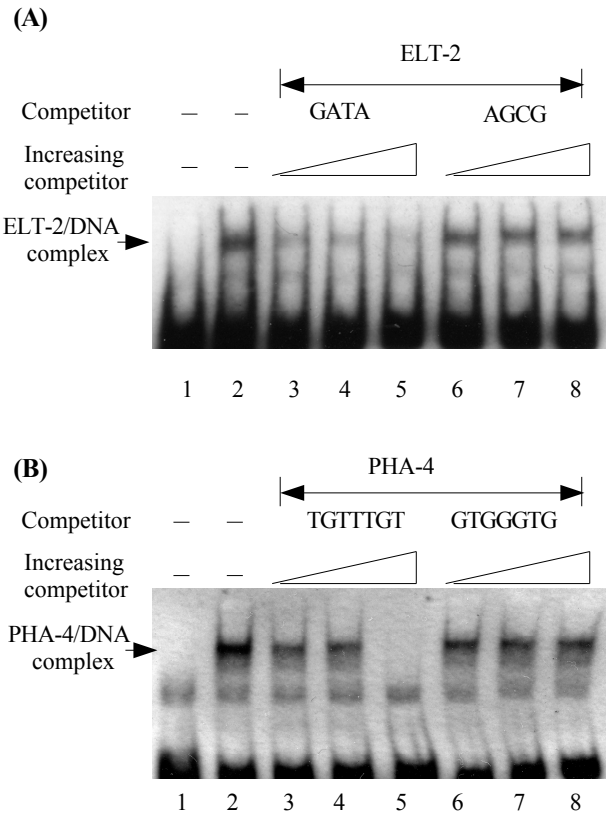

(A)
ELT-2
Competitor
– –
GATA
AGCG
Increasing competitor
– –
ELT-2/DNA complex
1 2 3 4 5 6 7 8
(B)
PHA-4
Competitor
– –
TGTTTGT
GTGGGTG
Increasing competitor
– –
PHA-4/DNA complex
1 2 3 4 5 6 7 8

Fig. 7

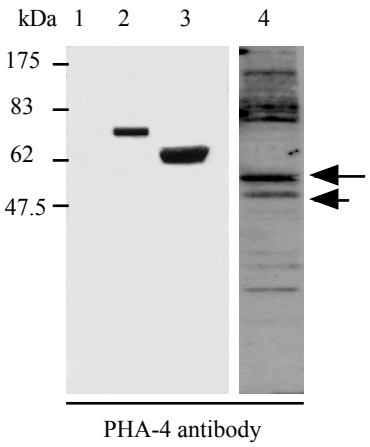

kDa 1 2 3 4
175
83
62
47.5
PHA-4 antibody

Fig. 8