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pha-4 is *Ce-fkh-1*, a *fork head*/HNF-3α,β,γ homolog that functions in organogenesis of the *C. elegans* pharynx

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SUMMARY

The *C. elegans Ce-fkh-1* gene has been cloned on the basis of its sequence similarity to the winged-helix DNA binding domain of the *Drosophila fork head* and mammalian HNF-3α,β,γ genes, and mutations in the zygotically active *pha-4* gene have been shown to block formation of the pharynx (and rectum) at an early stage in embryogenesis. In the present paper, we show that *Ce-fkh-1* and *pha-4* are the same gene. We show that PHA-4 protein is present in nuclei of essentially all pharyngeal cells, of all five cell types. PHA-4 protein first appears close to the point at which a cell lineage will produce only pharyngeal cells, independently of cell type. We show that PHA-4 binds directly to a 'pan-pharyngeal enhancer element' previously identified in the promoter of the pharyngeal myosin *myo-2* gene; in transgenic embryos, ectopic PHA-4 activates ectopic *myo-2* expression. We also show that ectopic PHA-4 can activate ectopic expression of the *ceh-22* gene, a pharyngeal-specific NK-2-type homeodomain protein previously shown to bind a muscle-specific enhancer near the PHA-4 binding site in the *myo-2* promoter. We propose that it is the combination of *pha-4* and regulatory molecules such as *ceh-22* that produces the specific gene expression patterns during pharynx development. Overall, *pha-4* can be described as an 'organ identity factor', completely necessary for organ formation, present in all cells of the organ from the earliest stages, capable of integrating upstream developmental pathways (in this case, the two distinct pathways that produce the anterior and posterior pharynx) and participating directly in the transcriptional regulation of organ specific genes.

Finally, we note that the distribution of PHA-4 protein in *C. elegans* embryos is remarkably similar to the distribution of the fork head protein in *Drosophila* embryos: high levels in the foregut/pharynx and hindgut/rectum; low levels in the gut proper. Moreover, we show that *pha-4* expression in the *C. elegans* gut is regulated by *elt-2*, a *C. elegans* gut-specific GATA-factor and possible homolog of the *Drosophila* gene *serpent*, which influences *fork head* expression in the fly gut. Overall, our results provide evidence for a highly conserved pathway regulating formation of the digestive tract in all (triploblastic) metazoa.

Key words: *C. elegans*, Organogenesis, Pharynx, *pha-4*, *Ce-fkh-1*, Transcriptional regulation, Organ identity factor

INTRODUCTION

Organogenesis is a complex and critical feature of animal development, involving cell fate specifications, cell interactions, tissue assembly and intricate morphogenesis. During the past several years, key regulatory molecules have been identified in the formation of organs as diverse as insect eyes (Quiring et al., 1994) and mammalian livers, hearts and kidneys (Zaret, 1996; Fishman and Chien, 1997; Vainio and Muller, 1997). Despite this wealth of new information, it is far from clear whether different organs in different animals obey common developmental rules.

The *Caenorhabditis elegans* pharynx provides a relatively simple experimental system to study the molecular basis of organogenesis. The pharynx is a neuromuscular feeding apparatus, containing 80 nuclei within five distinct cell types: muscles, neurons, gland cells, epithelial cells and structural cells called marginal cells (Albertson and Thomson, 1976). Two distinct developmental pathways involving maternal-effect genes and precise cell-cell interactions act in the earliest stages of pharynx formation: an 'inductive' pathway, by which cells of the ABa lineage are induced to produce the anterior pharynx, and an 'autonomous' pathway, by which cells of the MS lineage produce the posterior pharynx (reviewed in Schnabel and Priess, 1997). What happens next is not clear: how do the apparently undifferentiated cells of the pharynx primordium adopt the five distinct cellular phenotypes and assemble into the mature functioning organ?

One could imagine two extreme models to explain pharyngeal organogenesis. In one model, the five distinct cell

types differentiate and assemble into the organ without expressing a common organ-specific regulator. In the opposite extreme, all cells that form the organ could express an 'organ identity factor' that coordinates development of the different cell types and directs their assembly into the final organ. The properties of such an organ identity factor should include the following: (1) in the absence of the factor, organ formation should be blocked at the earliest stages; (2) the factor should be present in all cells of the organ, beginning at the earliest stages of organ formation; (3) the factor should lie downstream of (and possibly integrate) pathways that are known to produce the organ primordium (in the case of the pharynx, the 'inductive' ABa and 'autonomous' MS pathways); and (4) ectopically expressed factor might induce ectopic organ formation, or at least ectopic expression of organ-specific genes.

Two candidates have already been proposed for zygotically produced pharynx-specific organ identity factors: PHA-1 and PHA-4. The important common feature of these two genes is that they influence formation of the pharynx as an organ, not as individual cell types, particular cell lineages or embryonic domains. In embryos mutant for the *pha-1* gene, the pharynx primordium forms but does not elongate correctly; some early pharynx differentiation can be detected but later differentiation is abolished (Schnabel and Schnabel, 1990; Granato et al., 1994). *pha-1* encodes a protein with similarity to bZIP transcription factors, suggesting it can act as a transcriptional regulator (Granato et al., 1994). Embryos mutant for the zygotically active *pha-4* gene arrest pharynx formation at an even earlier stage than do *pha-1* embryos; essentially no markers characteristic of the pharynx are expressed (Mango et al., 1994). Mango et al. (1994) showed that *pha-4* acts genetically upstream of *pha-1* but downstream of the maternal-effect genes that specify early blastomere fate.

In the present paper, we identify the *pha-4* gene as *Ce-fkh-1*, a gene that we have previously cloned (Azzaria et al., 1996) based on its high degree of similarity (approx. 75% amino acid identity) to the winged helix DNA-binding domains of the *Drosophila fork head* gene and the mammalian genes HNF-3α,β and γ. Mutants in *Drosophila fork head* have severe defects in digestive tract development, in particular the foregut and hindgut (Weigel et al., 1989a,b). HNF-3α,β and γ genes were originally identified as liver-specific transcription factors in adult rodents (Lai et al., 1990) and have subsequently been shown to be expressed in endoderm and axial structures of the early mouse embryo, as well as in the organizing centers necessary for axis formation (Ang et al., 1993; Monaghan et al., 1993; Ang and Rossant, 1994; Weinstein et al., 1994). The present study describes common features of expression patterns and mutant phenotypes for this set of *fork head*/HNF-3α,β,γ/*pha-4* genes and provides evidence for a highly conserved pathway of digestive tract formation in all (triploblastic) metazoa. However, our most important conclusion is that, during formation of the *C. elegans* pharynx, *pha-4* conforms to the criteria set out above for an 'organ identity factor.'

MATERIALS AND METHODS

Worm strains and transformation

The following *C. elegans* strains were used: wild type, Bristol N2; JK892, *unc-32(e189) glp-1(q231ts)* III; JJ1057, *pop-1(zu189) dpy-5(e61)/hT1* I; *him-5(e1490)/hT1* V; EU1, *skn-1(zu67)* IV/*nT1[unc-?(n754) let-?]* (IV;V); JJ529, *rol-1(e91) mex-1(zu121)/mnC1 [dpy-10(e128) unc-52(e444)]* II; JJ532, *pie-1(zu154) unc-25(e156)/qC1 [dpy-19(e1259) glp-1(q339)]* III; JK1521, *fog-2(q71) pha-4(q490)/stu-3(q265) rol-9(sc148)* V; GE42, *vab-7(e1562) pha-1(e2123ts)* III.

Embryos produced by *skn-1*, *mex-1*, *pie-1* and *pop-1* homozygous hermaphrodites were collected at room temperature. Homozygous *glp-1(q231ts)* and *pha-1(e2123ts)* worms were shifted from 16°C to 25°C as young adults and embryos collected at 25°C.

DNA constructs were transformed into N2 worms as described by Mello et al. (1991), using either *unc-22* antisense or *rol-6* as transformation markers (Fire et al., 1991; Mello et al., 1991). At least three transformed lines, usually more, were examined for each construct. When appropriate, transformed constructs were integrated into the genome by γ-irradiation to make 100% heritable lines (Egan et al., 1995).

Detecting the *pha-4(q490)* mutation in the *Ce-fkh-1* genomic sequence

To determine if *pha-4* and *fkh-1* are the same gene, single embryo PCR was performed on homozygous *pha-4(q490)* embryos, identified among the progeny of JK1521 by the Pha-4 phenotype; primers were designed to amplify portions of the known *Ce-fkh-1* genomic sequence (Azzaria et al., 1996). Both strands of the entire coding region were sequenced; only the single mutation described in the text was found.

PHA-4 antiserum production and immunofluorescence

A 175-base-pair *Bam*HI-*Hin*cII fragment from *pha-4* cDNA (*Ce-fkh-1* coordinates 1173-1347 in Azzaria et al., 1996), encoding 58 amino acids located towards the C terminus end, outside of the DNA binding domain, was cloned in-frame into the *Bam*HI-*Sma*I sites of pGEX-1 (Pharmacia Biotechnology) as well as into the *Sma*I site of pTRXfus (Invitrogen). A GST/PHA-4 fusion protein was induced and purified following manufacturer's instructions and used to immunize rabbits following standard injection protocols (Harlow and Lane, 1988). Thioredoxin/PHA-4 fusion protein was induced and purified by osmotic shock, following manufacturer's instructions, and used to affinity-purify PHA-4 antibodies from immunoblots (Harlow and Lane, 1988). Antiserum specificity is demonstrated by lack of staining in *pha-4* mutants and by extensive ectopic staining when a transgenic *pha-4* cDNA construct is activated by a heat-shock promoter (data not shown). Immunofluorescence analysis on embryos, larvae and adults was performed essentially as described in Miller and Shakes (1996). Primary antibodies were added for an overnight incubation either at 4°C or room temperature; slides were incubated for 2 hours at room temperature with a secondary antibody, either Cy3-labelled goat anti-rabbit IgG or FITC-labelled donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories). Pharyngeal muscle cells were identified by double staining *myo-2::lacZ* (*cc228*) larvae and adults (Okkema and Fire, 1994) with antibodies against PHA-4 and β-galactosidase (Promega). Rectal epithelial cells were identified by double-staining worms transformed with the enhancer trap construct pUL#38E12 (Hope, 1991), which expresses β-galactosidase in rectal epithelial cells (Mango et al., 1994). Other cell types were identified by position. Animals were stained for β-galactosidase activity as described in Fire (1992). PHA-4-staining cells were counted using deconvolution confocal microscopy.

Ectopic *pha-4* expression directed by heat shock

Full-length *pha-4* cDNA (corresponding to polypeptide I of Azzaria et al., 1996), was placed under the control of the *hsp16-2* promoter (Stringham et al., 1992) in the vector pPD49.78 (Mello and Fire, 1996). The construct was transformed into worms and integrated into the genome by γ-irradiation to produce the strain JM70. Males from

this strain were crossed to: (1) OK0039 (*cuIs2*), a transformed line containing integrated arrays of GFP under the control of eight copies of the C183 oligonucleotide (Okkema and Fire, 1994), and (2) PD8260 (*ccIs8260*), a line with integrated arrays of a *ceh-22::lacZ* fusion (Okkema and Fire, 1994). Since all transgenic arrays have *rol-6* as a marker, the presence of transgenes was determined either by single worm PCR using transgene specific primers, or by directly examining embryos for reporter gene expression and for lethality upon heat shock. Embryos from hermaphrodites transgenic for *hsp16-2::pha-4* and either reporter transgene were aged 30 to 60 minutes after being laid, incubated at 33°C for 1 hour and then allowed to recover at 16°C for approx. 7 hours before observing GFP expression or staining for β-galactosidase activity. This procedure results in developmentally arrested embryos with PHA-4 protein located in the majority of nuclei (data not shown).

As a control, the above crosses were repeated using strain JM53 (Fukushige et al., 1998), which contains an integrated transgenic array of a *hsp16-2::elt-1* cDNA fusion instead of *hsp16-2::pha-4*. ELT-1 is a GATA transcription factor (Spieth et al., 1991) involved in hypodermal development but not pharyngeal development (Page et al., 1997).

For experiments in which the monoclonal antibody 9.2.1 was used to detect ectopic pharyngeal myosin (Miller et al., 1986; Ardizzi and Epstein, 1987), 2- to 4-cell stage *hsp16-2::pha-4* embryos (JM70) were collected, aged 6 hours at 20°C, heat-shocked at 33°C for 2 hours, and allowed to recover at 20°C for 10 hours before staining. Control N2 embryos and embryos containing the *hsp16-2::elt-1* cDNA construct were treated identically.

pha-4 reporter gene expression driven by ectopic ELT-2 was examined as described in Fukushige et al. (1998) using embryos from worms carrying two independently integrated arrays: *hsp16-2::elt-2* (from JM57) and *pha-4::lacZ* from strain JM50 (containing pJM50; see below).

Electrophoretic mobility shift assays

PHA-4 protein was produced by a coupled transcription-translation system (Promega T_NT) using as template *pha-4* cDNA (encoding polypeptide I) cloned into pT7TAG (Pollock and Treisman, 1990). The electrophoretic mobility shift assay was performed essentially as described in Stroeher et al. (1994) using ^{32}P-end-labelled (double-stranded) C183 (Okkema and Fire, 1994), 100 ng poly(dIdC) non-specific competitor and a 40-fold excess of either specific (C183, double-stranded) or mutated (Cmut2, double-stranded) competitor.

Full-length ELT-2 (Hawkins and McGhee, 1995) was also produced by in vitro transcription/translation and the electrophoretic mobility shift assay conducted as above, with the following modifications. The wild-type probe sequence was 5′-GTGTCAAACTCTC**TTATCA**A-TCAGTGCTCTTTTTCTCT; the mutated probe sequence was 5′-GT-GTCAAACTCTC**GGCGAC**ATCAGTGCTCTTTTTCTCT (where the GATA and mutated sites are depicted in bold); both wild type and mutant competitors (double-stranded) were present in an 80-fold excess over the probe (double-stranded).

pha-4 promoter analysis

Uni-directional deletions from the 5′ end of the 7 kb *pha-4* promoter were made as in Aamodt et al. (1991) beginning with plasmid pJM50 (a 9.0 kb *Bam*HI-*Eco*R1 *pha-4* fragment fused to *lacZ* at the second *pha-4* exon inserted into the vector pPD22.11 (Fire et al., 1990); pJM50 was referred to as Y4 in Azzaria et al. (1996)). Internal deletions were made by PCR overlap extension (Horton et al., 1993).

RESULTS

pha-4 is *Ce-fkh-1*

Ce-fkh-1 maps physically (Azzaria et al., 1996) and *pha-4* maps genetically (Mango et al., 1994) to the right side of *C. elegans* chromosome V. Because of the pharyngeal and rectal defects found in *pha-4* mutants (Mango et al., 1994) and because *Ce-fkh-1* is expressed throughout the embryonic digestive tract (Azzaria et al., 1996), it seemed possible that the two genes were one and the same. We sequenced the strong *pha-4* allele *q490* provided by S. Mango (University of Utah, USA); a single mutation (CAG→TAG; Q→Stop) was detected at amino acid 99 of the longest coding sequence (Azzaria et al., 1996). This mutation leads to truncation well upstream of the DNA binding domain of all three polypeptides predicted for this gene. Horner et al. have shown that other *pha-4* alleles also cause changes in the *Ce-fkh-1* coding sequence and that *Ce-fkh-1* RNA interference injections produce a *pha-4* phenocopy (M. A. Horner, S. Quintin, M. E. Domeier, J. Kimble, M. Labouesse and S. Mango, unpublished). We conclude that *pha-4* and *Ce-fkh-1* are the same gene, hereafter referred to as *pha-4*.

pha-4 is expressed in pharyngeal precursor cells

Fig. 1 summarizes the cell lineage of the *C. elegans* pharynx (Sulston et al., 1983). By the comma stage (approx. 560 total cells and 430 minutes post-fertilization at 20°C), the final cell divisions that produce the 80 nuclei of the pharynx have taken place and the pharyngeal precursor cells cluster together to form the undifferentiated pharynx primordium (Sulston et al., 1983; Schnabel and Schnabel, 1990; Mango et al., 1994; Schnabel et al., 1997). Fig. 2A shows a typical comma-stage wild-type embryo (430 minutes) stained with an affinity-purified rabbit polyclonal PHA-4 antibody; the DAPI image of the same embryo is shown in Fig. 2B. In six comma-stage embryos, we counted an average of 80.5 (± 2.5 s.d.) PHA-4-staining pharyngeal nuclei by confocal microscopy (maximum count = 85). Analysis of later stage animals (see below) shows clearly that all six cells of the pharyngeal-intestinal valve (Albertson and Thomson, 1976) contain PHA-4 protein; Mango et al. (1994) have previously noted valve defects in *pha-4* embryos. Thus the total number of potentially staining nuclei (pharynx + valve) should be equal to 86. We conclude that, within the error associated with counting closely apposed nuclei, all cells of the pharyngeal primordium (+ valve) contain the nuclear factor PHA-4. The same embryos also show nuclear PHA-4 in 6-8 rectal cells, including the two rectal valve cells and the three rectal epithelial cells. At the lima bean and comma stages of embryogenesis, a low but significant level of PHA-4 can be detected in the gut (Fig. 2C,D).

Do pharyngeal precursor cells express *pha-4* prior to the formation of the pharynx primordium? Fig. 2E,F shows an embryo that contains 177 DAPI-staining nuclei and hence corresponds to approx. 200 minutes of development (aligned as embryo 2E on the cell lineage in Fig. 1). At this stage, there are 28 cells whose lineages will ultimately give rise to pharyngeal cells (including the pharyngeal-intestinal valve cells) and 24 of these lineages are clonal, i.e. will give rise only to pharyngeal cells (Sulston et al., 1983). The embryo shown in Fig. 2E contains 21 nuclei with detectable levels of PHA-4 protein, as counted by confocal microscopy. In embryos one cell division earlier (approx. 100 total cells; 16 pharyngeal precursor cells, of which 11 give rise only to pharyngeal cells), we can detect PHA-4 in 3-10 nuclei but at very low levels (data

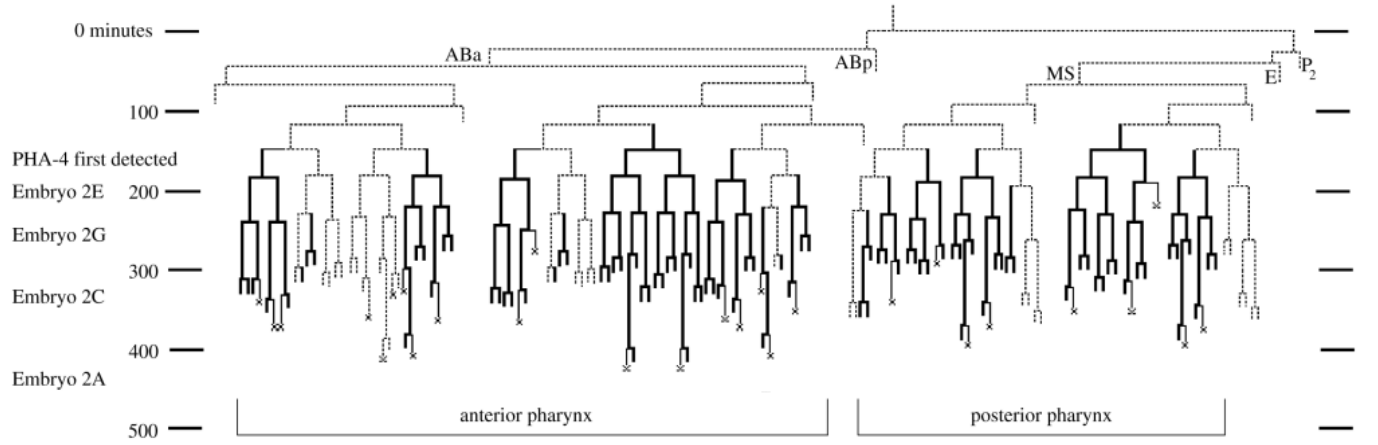

Fig. 1. The lineage of the sub-set of the ABa-derived and MS-derived cells that form the *C. elegans* pharynx (Sulston et al., 1983). A bold line represents lineages that will produce only pharyngeal cells (including pharyngeal-intestinal valve cells); cell death (X) is disregarded. The ages of individual embryos shown in Fig. 2 are aligned on the time scale on the left.

not shown). Fig. 2G,H shows an embryo at approx. 250 minutes of development, when there are 52 pharyngeal precursor cells of which 49 give rise to only pharyngeal cells; 40-50 strongly PHA-4-staining nuclei were counted in embryos at this stage. From these and similar observations, we conclude that the *pha-4* gene is expressed in pharyngeal precursor cells well before the formation of the pharynx primordium. Our data are consistent with a model in which PHA-4 first appears at the point when pharyngeal lineages become clonal and produce only pharyngeal precursor cells, independently of the particular pharyngeal cell type. However, considering the natural variability of normal embryogenesis (Schnabel et al., 1997), this assertion must be verified by direct lineage analysis of individual embryos.

pha-4 persists in all stages of the life cycle

PHA-4 can be detected immunologically in most but not all nuclei of the larval and adult pharynx. PHA-4 is detected in nuclei of all epithelial cells, muscle cells, marginal cells, gland cells and pharyngeal intestinal valve cells, but is detected only in about 8 of 19 neuronal nuclei (Fig. 2I,L). Based on reduced counts of total staining nuclei, we suggest that these neuronal cells had ceased *pha-4* expression at mid to late embryogenesis.

PHA-4 is also detected in one (comma stage) to several (pretzel stage and later) head cells outside of the pharynx (data not shown). As expected from previous transgenic analysis (Azzaria et al., 1996), PHA-4 can also be detected in nuclei of the developing somatic gonad, including the distal tip cell and ventral uterine cells (data not shown).

pha-4 expression lies downstream of maternal-effect genes that control the identity of pharynx-producing blastomeres

PHA-4 distribution was examined in embryos mutant for maternal-effect genes that establish the two pathways of pharynx production (Schnabel and Priess, 1997). In *glp-1* embryos, the induction of anterior pharynx is abolished (Austin and Kimble, 1987; Priess et al., 1987); as shown in Fig. 3A,B, PHA-4 is detected only in the posterior pharynges of *glp-1(q231ts)* embryos. The autonomously produced posterior pharynx is abolished in *pop-1* embryos (Lin et al., 1995); Fig. 3C,D show that PHA-4 is detected only in the anterior pharynges of *pop-1(zu189)* embryos, plus rectal cells. In *skn-1* embryos, no pharynx forms (Bowerman et al., 1992); as shown in Fig. 3E,F, no PHA-4 is detected in the pharynx of *skn-1(zu67)* embryos, but rectal staining persists as expected. *mex-1* or *pie-1* mutations cause the production of ectopic pharyngeal cells (Mello et al., 1992); excess PHA-4 staining is detected in *mex-1(zu121)* (Fig. 3G,H) and *pie-1(zu154)* embryos (data not shown). In summary, the *pha-4* expression patterns detected in all the above mutants suggest that *pha-4* must lie downstream of the convergence of the two distinct inductive and autonomous regulatory pathways determining the pharynx primordium. The distribution of PHA-4 molecules is thus completely consistent with genetic epistasis tests (Mango et al., 1994). Neither mutant nor wild-type embryos show obvious differences in the PHA-4 level or appearance times in the ABa-derived or MS-derived cells of the pharynx.

pha-4 and the zygotic gene *pha-1*

pha-4 expression was examined in embryos mutant for the zygotic gene *pha-1*, which is required for pharyngeal morphogenesis and expression of late pharyngeal markers (Schnabel and Schnabel, 1990; Granato et al., 1994). PHA-4 distribution in *pha-1(e2123ts)* embryos is essentially wild type if assayed 5-9 hours after embryos are laid (Fig. 3I,J). However, PHA-4 is not detected in *pha-1(e2123ts)* embryos when assayed later (data not shown), suggesting that *pha-1* is required for the maintenance of PHA-4 levels. *pha-1* is also required for the maintenance of *ceh-22* expression (Okkema et al., 1997). Thus the role of *pha-1* might be to increase the long-term stability of various pharyngeal transcription factors. It is interesting that the temperature-sensitive period and transgenic expression studies suggest that *pha-1* does not seem to be required later than the 1.5-fold stage (Schnabel and Schnabel, 1990; Granato et al., 1994).

PHA-4 interacts directly with a pan-pharyngeal enhancer element in the pharyngeal myosin *myo-2* gene

Of the 86 nuclei of the mature pharynx (including the six

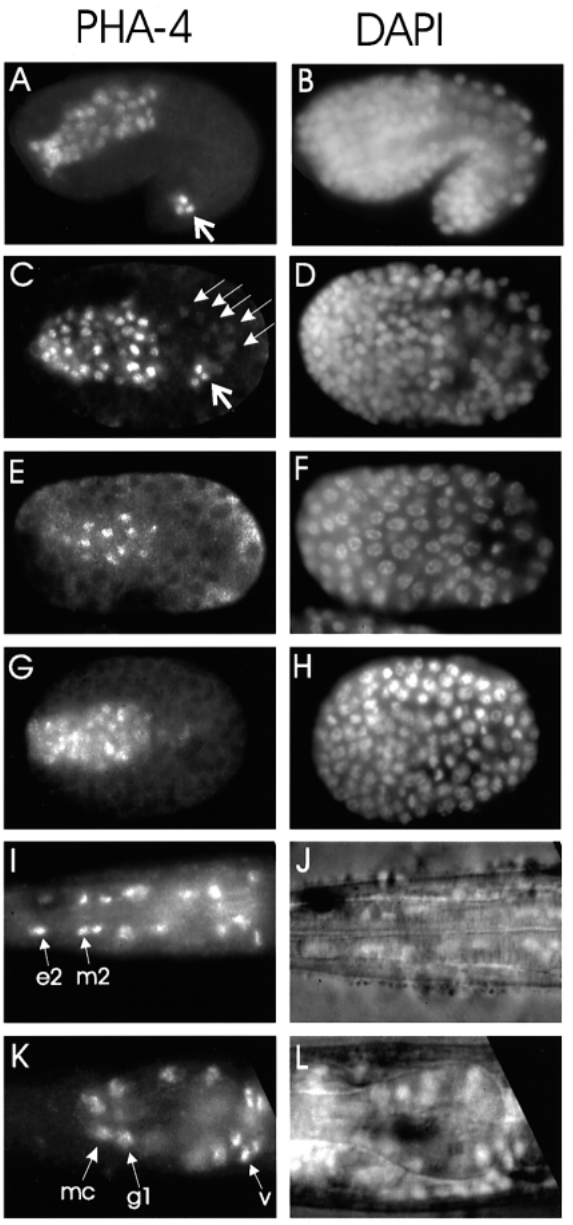

Fig. 2. (A,C,E,G) PHA-4 immunolocalization in wild-type embryos at the comma stage, the lima bean stage, and at approx. 200 minutes and 250 minutes post-fertilization, respectively. (B,D,F,H) show the corresponding DAPI-stained embryos. Large arrows point to rectal PHA-4 expression and small arrows to the low levels of PHA-4 gut expression. Ages of embryos are indicated on Fig. 1. (I,K) PHA-4 expression and (J,L) DAPI staining in a pharyngeal anterior bulb (I,J) and posterior bulb (K,L) of wild-type L2-L3 larvae; *pha-4* expressing cell types are labelled as follows: e2, epithelial, m2, muscle, mc, marginal cell, g1, gland and v, pharyngeal intestinal valve.

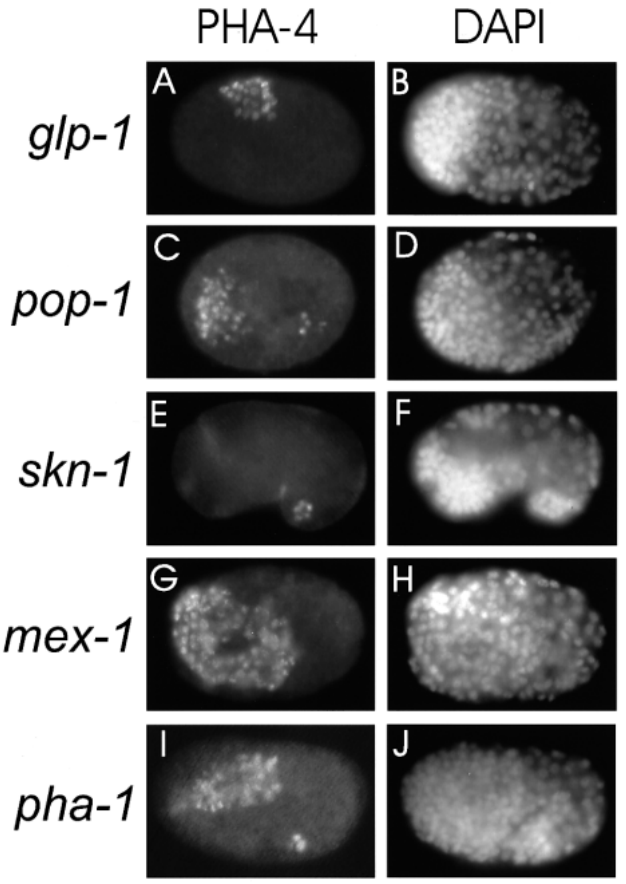

Fig. 3. *pha-4* expression and corresponding DAPI staining in mutations that alter pharynx formation (reviewed in Schnabel and Priess, 1997). (A,B) Embryo produced by *glp-1(q231ts)* hermaphrodite at 25°C. Anterior pharynx is not correctly formed and PHA-4 is found only in cells of the posterior pharynx. (C,D) Embryo from *pop-1(zu189)* hermaphrodite. The posterior pharynx is not correctly formed and PHA-4 is detected only in cells of the anterior pharynx and rectal cells. (E,F) Embryo from *skn-1(zu67)* hermaphrodite in which no pharynx is produced and in which pharyngeal PHA-4 is never detected. ABp-derived rectal cells stain as expected. (G,H) Embryo from *mex-1(zu121)* hermaphrodite in which extra pharyngeal PHA-4 is detected in the ectopic pharynx in the expected distribution. (I,J) *pha-1(e2123ts)* embryo raised at 25°C (Schnabel and Schnabel, 1990). In all cases, over 100 embryos were examined.

pharyngeal-intestinal valve cells), 37 are muscles and express the pharyngeal myosin *myo-2* gene (Dibb et al., 1989). Okkema and Fire (1994) have analyzed the *myo-2* promoter in transgenic nematodes and have identified two enhancer modules, denoted the *B* and *C* subelements. Dimers of the *B* subelement direct reporter gene expression in a subset of pharyngeal-muscle cells but not in non-muscle cells; sequences in the *B* subelement have been shown to bind the NK-2 class homeodomain protein CEH-22, a pharyngeal-muscle transcription factor (Okkema and Fire, 1994; Okkema et al., 1997). In contrast, dimers of the *C* subelement direct reporter gene expression into all cell types of the pharynx. Okkema and Fire (1994) suggested that the 'pan-pharyngeal' enhancer in the *C* subelement could be the direct target of a gene such as *pha-4*, because mutations in *pha-4* caused such widespread pharyngeal defects. We now show that this suggestion appears to be correct.

Fig. 4A shows the sequence of a short region of the *myo*-2 *C* subelement, oligonucleotide C183 in Okkema and Fire (1994). Multiples of C183 can direct pharynx-wide reporter gene expression in wild-type embryos; a mutation (Cmut2) in

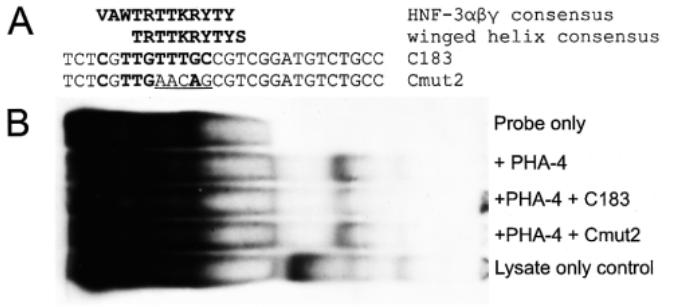

Fig. 4. (A) Sequence of oligonucleotide C183, representing a portion of the *C* subelement of the *myo-2* promoter; the sequence of the mutated element Cmut2 is shown with base-pair changes underlined (Okkema and Fire, 1994). Consensus binding sites for winged helix proteins (Kaufmann et al., 1995) and HNF-3α,β,γ factors (Costa, 1994) are shown above; base pairs in C183 and Cmut2 that match these consensus sequences are shown in bold. (B) Electrophoretic mobility shift assay of PHA-4 with ^{32}P-end-labelled (double-stranded) C183; complex formation is effectively competed with a 40-fold excess of double-stranded C183 but poorly competed with the same concentration of Cmut2.

this sequence abolishes this activity (Okkema and Fire, 1994). Multiples of C183 show no activity in *pha-4* embryos (data not shown). As shown in Fig. 4A, the Cmut2 mutation lies within a sequence that agrees well with consensus binding sites determined for HNF-3α,β,γ factors (Costa, 1994) and for winged-helix proteins in general (Kaufmann et al., 1995). This suggests a model in which PHA-4 binds directly to the C183 sequence. As a test of this model, PHA-4 protein was produced in vitro and shown to form a complex with (double-stranded) C183, as detected using an electrophoretic mobility shift assay (Fig. 4B). This binding is effectively self-competed but is poorly competed by a double-stranded oligonucleotide containing the Cmut2 mutation.

pha-4 acts through the C183 subelement to activate *myo-2* expression

Given that PHA-4 binds to C183 in vitro, we wished to demonstrate *pha-4*-dependent enhancer function in vivo. Worms were produced containing two independent transgenic arrays: the first array contained eight tandem copies of the C183 oligonucleotide fused to a *GFP* reporter; the second array contained the *pha-4* cDNA under the control of the *C. elegans hsp-16-2* promoter (Materials and methods). As shown in Fig. 5A, doubly transgenic embryos arrest after heat shock and express the reporter construct in most cells of the embryo. In heat-shocked control embryos that contain only the reporter construct array (i.e. do not contain *hsp16-2::pha-4*), reporter expression remains restricted to the presumptive pharynx and rectum (Fig. 5B). As an additional control, we examined embryos in which ectopic ELT-1, a GATA transcription factor essential for hypodermis development but uninvolved in pharyngeal development (Page et al., 1997), is produced throughout the embryo by heat shock. In heat-shocked *hsp-16-2::elt-1* embryos, C183 reporter gene expression remains restricted to the pharynx and rectum (Fig. 5C).

We next wanted to determine if ectopic *pha-4* could activate expression of the endogenous *myo-2* gene. Heat shock of embryos transformed with *hsp16-2::pha-4* results in the expression of pharyngeal myosin (detected by the monoclonal antibody 9.2.1) not only in the pharynx as expected, but also in dorsal and ventral regions of the embryo (probably body wall muscles) in approximately 20% of the embryos (Fig. 5D). When wild-type embryos or *hsp16-2::elt-1* transformed embryos are heat shocked under the same conditions and stained with 9.2.1, pharyngeal myosin is detected only in the pharynx (Fig. 5E and F, respectively).

In summary, we suggest that PHA-4 binds to the *C* subelement of the *myo-2* promoter and thereby participates directly in the activation of *myo-2* expression in vivo.

pha-4 also activates *ceh-22*, a pharyngeal-muscle-specific-homeodomain gene

ceh-22 encodes a NK-2 homeodomain protein that is found in a subset of pharyngeal muscle cells. CEH-22 binds to the *B* subelement of the *myo-2* promoter and can drive expression of *myo-2* when produced ectopically (Okkema and Fire, 1994; Okkema et al., 1997). Okkema and Fire (1994) have suggested that during normal pharyngeal development, it is the combination of *pha-4* and *ceh-22* that restricts *myo-2*

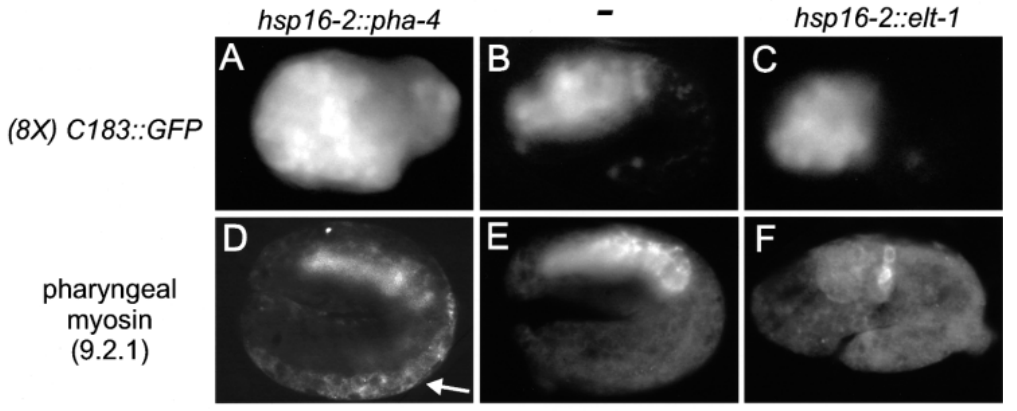

Fig. 5. (A) Heat-shocked embryos containing both the *hsp16-2::pha-4* cDNA transgene and the *(8×) C183::GFP* reporter gene express GFP throughout the embryo; (B) Heat-shocked control embryos containing only the reporter gene express GFP only in the pharynx and rectum, as do heat-shocked control embryos transformed with both the *(8X) C183::GFP* reporter gene and a *hsp16-2::elt-1* gene (C). *hsp16-2::elt-1* embryos arrest at a slightly earlier stage than do heat-shocked *hsp16-2::pha-4* embryos, whereas heat-shocked wild-type embryos usually do not arrest at all and hatch. (D) Heat-shocked *hsp16-2::pha-4* embryo stained with the monoclonal antibody 9.2.1. The arrow points to ectopic pharyngeal myosin staining. No ectopic pharyngeal myosin is detected in heat-shocked (E) wild-type N2 embryos or (F) *hsp16-2::elt-1* embryos. The *hsp16-2::elt-1* embryo in F does not express pharyngeal myosin in the anterior pharynx; this phenotype is seen in some *hsp16-2::elt-1* embryos when they are heat-shocked at the stage used in this experiment.

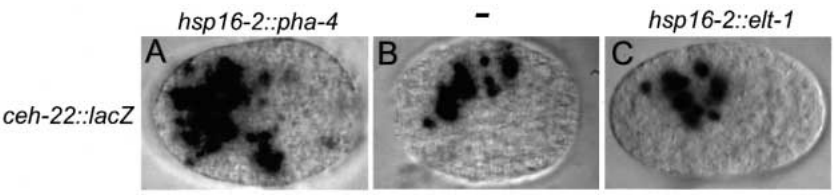

Fig. 6 (A) Heat-shocked embryos containing the *hsp16-2::pha-4* transgene and the *ceh-22::lacZ* reporter gene express β-galactosidase in more than twice the number of cells observed in (B) heat-shocked control embryos containing only the reporter transgene or (C) heat-shocked control embryos transformed with both the reporter gene and *hsp16-2::elt-1*.

expression to muscle cells. CEH-22 is first detected in a subset of pharyngeal-muscle cells at the lima bean stage of embryogenesis, 330 minutes after fertilization. As demonstrated above, PHA-4 is already present in pharyngeal precursor cells at the time that CEH-22 first appears in pharyngeal muscle (Okkema and Fire, 1994); furthermore, CEH-22 is not detected in *pha-4* mutants (Mango et al., 1994). This raises the possibility that *pha-4* might also activate *ceh-22*.

Fig. 6A shows that ectopically produced PHA-4 can indeed induce significant levels of ectopic expression of a *ceh-22*::*lacZ* reporter gene outside of the presumptive pharynx. In heat-shocked embryos transformed either with the *ceh-22::lacZ* construct alone, or doubly transformed with *ceh-22::lacZ* and *hsp16-2::elt-1*, reporter expression remains confined to the pharyngeal muscle cells (Fig. 6B,C). Cell counts confirm the increase in *ceh-22::lacZ*-expressing cells in embryos with ectopic PHA-4. Heat-shocked embryos containing only the reporter gene show an average of 17.1 (± 1.5 s.d.; $n = 25$) total staining nuclei. Heat-shocked embryos containing the reporter gene and the *hsp16-2::elt-1* control construct show an average of 13.3 (± 2.7 s.d.; $n = 26$) total staining nuclei. In contrast, heat-shocked embryos containing both the reporter gene construct and *hsp16-2::pha-4* construct show an average of 41.6 (± 8.2 s.d.; n=15) total nuclei staining. In these latter embryos, a maximum of 54 staining nuclei was observed: a number greater than 21, the total number of nuclei in which CEH-22 is found in the mature pharynx (Okkema and Fire, 1994), and 37, the total number of all nuclei in pharyngeal muscles.

pha-4 expression in the gut

PHA-4 protein can be detected at low, but significant, levels in the gut of lima bean- and comma-stage embryos (Fig. 2C,D), and *pha-4* mutant embryos show a mild gut phenotype. Staining with the monoclonal antibody MH-33 (Francis and Waterston, 1985) reveals the presumptive gut lumen to appear wider and more diffuse in *pha-4* embryos than in wild-type controls (Fig. 7A,B respectively). This is a subtle defect compared to defects found in the pharynx and rectum but is nonetheless reproducible. It is not clear whether the phenotype is a direct result of the absence of PHA-4 in the gut or an indirect effect caused by the failure of *pha-4* mutants to elongate.

Although endogenous PHA-4 protein (above) and *pha-4* transcripts (Azzaria et al., 1996) are present at low levels in the gut, we previously showed that 7 kb of *pha-4* promoter drives reporter gene expression strongly in gut cells (Azzaria et al., 1996). This discrepancy is likely to be caused by defects in transcriptional control and not by stable reporter protein, because in situ hybridization with transgenic embryos shows that reporter gene transcripts are present at high levels in the gut throughout embryogenesis (data not shown). We interpret the gut expression of the *pha-4* transgene to reflect missing transcriptional gut repressor

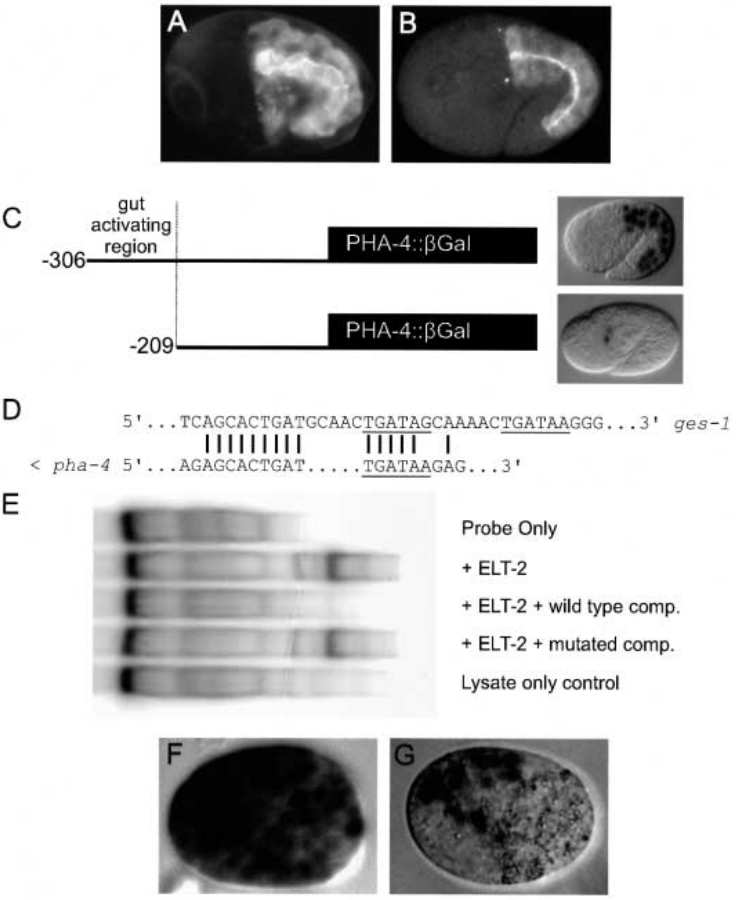

Fig. 7. (A,B) Embryos stained with the monoclonal antibody MH-33, which appears to stain a component of the intestinal brush border/terminal web. The presumptive gut lumen appears wider in *pha*-4 embryos (A) than in wild-type embryos (B). (C) Two of the constructs produced as uni-directional deletions of the 7 kb *pha-4* promoter; a β-galactosidase-stained embryo transformed with each construct is also shown. (D) Comparison of control sequence from the *ges-1* promoter with a sequence within the –306 to –209 base pair region of the *pha-4* promoter. GATA elements (WGATAR) are underlined and identical base pairs are indicated by bold vertical lines. (E) Electrophoretic mobility shift demonstrates a complex formed between the *pha-4* gut activating region and in vitro-produced ELT-2 protein. The binding is competed by an 80-fold excess of cold wild-type competitor, but not by the same concentration of mutated competitor. (F) Heat-shocked embryos containing both a *pha-4::lacZ* reporter gene and *hsp16-2::elt-2* transgene express β-galactosidase throughout the embryo in contrast to heat-shocked embryos containing only the reporter gene (G).

elements located outside of the 7 kb *pha-4* promoter region used for the reporter constructs.

An intriguing parallel can be drawn between the expression of the *pha-4* reporter gene and the expression of the gut esterase gene *ges-1*. *ges-1* is normally expressed exclusively in the gut (Edgar and McGhee, 1986). However, deletion of a 36-base-pair region containing a tandem pair of GATA elements from the *ges-1* promoter abolishes *ges-1* expression in the gut and activates expression in cells of the pharynx and rectum (Egan et al., 1995; Fukushige et al., 1996). The *pha-4* reporter constructs seem to show an inverse behavior: a dramatic increase in gut expression of the normally pharynx/rectum-specific gene. Overall, such observations suggest that each digestive tract module may contain factors to repress genes expressed in other modules of the digestive tract.

To investigate what controls *pha-4* expression in the gut, a series of uni-directional 5′-deletions of the *pha-4* promoter fused to a *lacZ* reporter gene was introduced into worms to produce stably transformed lines, whose embryos were then examined for β-Galactosidase activity. As shown in Fig. 7C, deletion of the *pha-4* promoter to only 306 base pairs before the first PHA-4 ATG still allows reporter expression in the gut; however, deletion to 209 base pairs before the first ATG abolishes gut expression. Within this 97-base-pair gut-activating region, there is a sequence with significant similarity to the promoter region that controls the gut to pharynx/rectum switch in *ges-1* (Fig. 7D) (Egan et al., 1995). The sequence in the *pha-4* gut-activating region has a single GATA element along with an additional 9/9 match to a sequence adjacent to the GATA elements, both in reverse orientation. The gut-activating ability of this 97-base-pair *pha-4* promoter region is redundant: when this precise region is deleted in the context of the entire 7 kb *pha-4* promoter, the reporter gene is still expressed in the gut (data not shown).

The sequence matches between the *pha-4* and *ges-1* gut-activating regions suggest that the same factor might be involved in their control. The ELT-2 GATA factor is an excellent candidate to control *ges-1* gut expression by binding to the GATA elements found in the *ges-1* promoter (Hawkins and McGhee, 1995; Fukushige et al., 1998). As shown in Fig. 7E, in vitro-produced ELT-2 does indeed bind to the gut-activating region in the *pha-4* promoter, as assayed by electrophoretic mobility shift. Furthermore, embryos that express ectopic ELT-2 under heat-shock control also express a *pha-4::lacZ* reporter construct throughout the embryos (Fig. 7F), compared to control embryos (Fig. 7G). Both of these observations suggest that *elt-2* might activate gut expression of *pha-4* in vivo.

DISCUSSION

The role of *pha-4* in pharyngeal development as a model for organogenesis

The *pha-4* gene (which we had previously cloned as *Ce-fkh-1*) meets most of the criteria proposed above for an ‘organ identity factor’: (1) in the absence of *pha-4*, the pharynx does not form (Mango et al., 1994); (2) PHA-4 is present in essentially all cells of the pharynx primordium and appears early in the pharyngeal precursor cell lineages; (3) *pha-4* lies genetically (Mango et al., 1994) and molecularly downstream of the point at which the ABa-derived and MS-derived pathways of pharynx formation converge; and (4) although we were not able to show that ectopic PHA-4 causes formation of entire ectopic pharynges, ectopic expression of PHA-4 does lead to ectopic expression of at least two pharyngeal muscle-specific markers.

Both the persistence of PHA-4 protein into adults and the identification of *ceh-22* and *myo-2* as direct targets of *pha-4* regulation suggest that PHA-4 participates at multiple steps of organ formation, from the earliest activation of subsidiary regulatory factors to the final activation of structural genes. Fig. 8 contains a simple schematic model illustrating how PHA-4 might function during pharynx organogenesis, more specifically in muscle lineages. We suggest that integration of the (induced) ABa-derived and (autonomous) MS-derived pharyngeal pathways occurs at the level of the *pha-4* promoter. We further propose that PHA-4 is involved in succeeding waves of gene transcription throughout pharynx formation and that regulatory factors activated by PHA-4 at one stage of development (e.g. *ceh-22*) participate with PHA-4 in activating transcription of later genes (e.g. *myo-2*). Such progressive accumulation of transcription factors, which then cooperate in subsequent gene activation, might be a common feature in organogenesis.

It is clear that the regulatory scheme shown in Fig. 8 can reflect only a small fraction of the complexity inherent in building a pharynx. Our analysis of genes controlled by *pha-4* applies only to (a subset of) pharyngeal muscles, but we suggest that similar schemes involving *pha-4* may apply to other pharyngeal cell types (as well as to cells of the rectum). A major question is how the five different cell types are established within the *pha-4*-expressing cells of the pharyngeal primordium. For example, since *ceh-22* expression is restricted to a subset of muscle cells, there must be at least one gene (depicted as X on Fig. 8) lying between the initial activation of *pha-4* and activation of *ceh-22*. An additional complication is whether the three distinct PHA-4 polypeptides produced by alternative splicing (Azzaria et al., 1996) have different expression patterns or different functions.

Conservation of metazoan digestive tract development

Cloned *C. elegans* genes encoding proteins with the *fork head*/HNF-3α,β,γ DNA binding domain include *lin-31*, *pes-1* and *daf-16* (Miller et al., 1993; Hope, 1994; Ogg et al., 1997; Lin et al., 1997). Searches of the *C. elegans* genomic sequence reveal at least a dozen predicted genes encoding proteins with

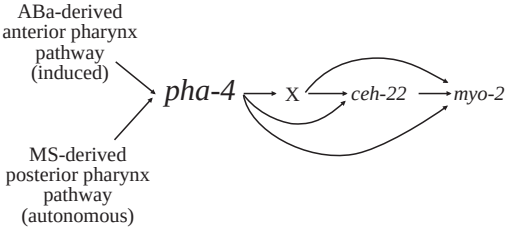

Fig. 8. A suggested pathway for *pha-4* action, as described in the text. *ceh-22* expression is restricted to muscle cells, suggesting that at least one gene (depicted here as X) must lie between the initial activation of *pha-4* and *ceh-22*.

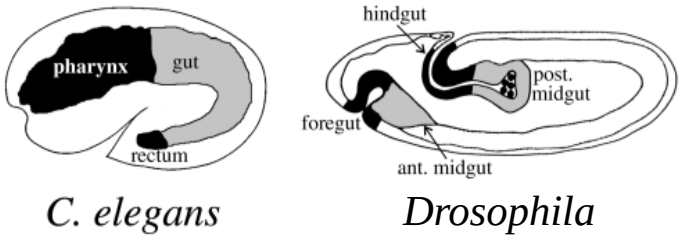

Fig. 9. Diagram comparing embryonic *pha-4* expression patterns in *C. elegans* to *fork head* expression in *Drosophila* (Weigel et al., 1989a,b). In addition to having similar mutant phenotypes, both genes are expressed at high levels at the ends of the digestive tract (pharynx and rectum in worms and foregut and hindgut in flies) and transiently at lower levels in the gut proper (anterior and posterior midgut in flies and intestine in worms).

winged-helix DNA binding domains. However, PHA-4 remains by far the most similar protein to *Drosophila fork head* (Azzaria et al., 1996).

C. elegans pha-4 and *Drosophila fork head* share obvious common features of their mutant phenotypes, in particular severe derangement of the pharynx/foregut and rectum/hindgut (Weigel et al., 1989a,b; Mango et al., 1994). Furthermore, as illustrated in Fig. 9, the expression pattern of *pha-4* in the *C. elegans* embryo bears a remarkable resemblance to the expression pattern of *fork head* in the digestive tract of the early *Drosophila* embryo (Weigel et al., 1989a,b). There are high levels of expression in domains at the digestive tract termini (pharynx/foregut and rectum/hindgut) and reduced expression in the gut proper.

Although comparison of expression patterns between *pha-4* and the mammalian genes HNF-3α,β,γ is necessarily more subjective, it might be pertinent that two of the early points of HNF-3α,β,γ expression in the definitive endoderm of the mouse embryo are in the invaginating foregut and hindgut (Ang et al., 1993; Monaghan et al., 1993); in addition, foregut morphogenesis is severely affected in HNF-3β (–/–) mouse embryos (Ang and Rossant, 1994; Weinstein et al., 1994).

There may be additional commonalities between digestive tract development in *C. elegans* and in *Drosophila*. In *C. elegans*, we have shown that *elt-2* may be involved in the gut expression of *pha-4*. ELT-2 is a gut-specific GATA factor (Hawkins and McGhee, 1995; Fukushige et al., 1998), highly similar to the *Drosophila* GATA factor *serpent* (Rehorn et al., 1996). *serpent* is required for *Drosophila* midgut development and acts genetically upstream of *fork head* (Reuter, 1994). This raises the possibility that HNF-3α,β,γ in vertebrate digestive tracts may be regulated by gut-associated GATA factors, such as GATA-4,5,6 (Laverriere et al., 1994).

In conclusion, we suggest that the *pha-4*-centered model proposed here for *C. elegans* pharynx formation may also apply to organogenesis regulated by *fork head*/HNF-3α,β,γ genes in the digestive tract of all (triploblastic) metazoa.

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