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The *pha-4* gene is required to generate the pharyngeal primordium of *Caenorhabditis elegans*

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SUMMARY

In the 4-cell *Caenorhabditis elegans* embryo, two blastomeres are destined to generate pharyngeal cells, each by a distinct developmental strategy: one pathway is inductive, while the other is autonomous. Here, we identify the *pha-4* locus. In animals lacking *pha-4* activity, an early step in pharyngeal organogenesis is blocked: no pharyngeal primordium is formed and differentiated pharyngeal cells are absent. Most other tissues are generated normally in *pha-4* mutants, including cells related to pharyngeal cells by cell lineage and position. Thus, *pha-4* activity is required to form the pharyngeal primordium. We propose that *pha-4* marks a convergence of the inductive and autonomous pathways of pharyngeal development and suggest that establishment of pharyngeal organ identity is a crucial step for pharyngeal organogenesis.

Key words: *pha-4*, organogenesis, *Caenorhabditis elegans*, embryogenesis, pharynx

INTRODUCTION

How are cells of multiple cell types and distinct cell lineages specified and assembled into a functioning organ? Organ formation depends on complex patterns of morphogenesis and differentiation that often arise from interactions between adjacent tissues. Little is known, however, about how these different tissues are initially brought together to form a primordium. For some organs, such as the heart and salivary gland of *Drosophila*, the assignment of cells to a primordium appears to depend largely on sequential subdivisions of positional information (Panzer et al., 1992; Bodmer, 1993). For example, the dorsal and ventral boundaries of the salivary gland placode are set by the patterning genes *decapentaplegic* and the *dorsal*-group genes (Panzer et al., 1992). For other organs, such as the vertebrate tooth and gonad, the relationship between regional identity and primordium formation is less clear, given that these structures are composed of cells that arise from distant locations within the embryo. To begin to address how disparate cells are assembled into a primordium, we have focused on the development of the *C. elegans* pharynx, an organ whose cells arise from unrelated cell lineages and by distinct genetic pathways.

The pharynx is a neuromuscular organ that grinds up food and delivers it to the intestine. The mature pharynx is composed of eighty cells that can be grouped into five cell types: muscles, glands, neurons, epithelia and structural cells called marginal cells (Albertson and Thomson, 1976). These cells derive from precursors that migrate internally from the embryo's ventral surface and cluster into a primordium during mid-embryogenesis, essentially after the completion of cell division (Fig. 1; Sulston et al., 1983). Subsequently, the primordium undergoes morphogenesis to form a highly organized structure that consists of anterior and posterior lobes connected by an isthmus (Figs 1, 2; Albertson and Thomson, 1976).

The pharynx is generated polyclonally during embryogenesis. Normally, embryonic development begins with a series of asymmetric cell divisions to generate six founder cells that differ with respect to their molecular composition, their cleavage patterns and the ultimate fates of their descendants (Strome and Wood, 1982; Sulston et al., 1983; Bowerman et al., 1993; Evans et al., 1994). Two 4-cell-stage blastomeres, ABa and EMS, contribute descendants to the pharynx, whereas their sisters do not (Fig. 1). The production of pharyngeal cells by ABa and EMS depends on two distinct pathways. The ABa pathway is dependent on intercellular signalling between blastomeres and on maternally supplied *glp-1* product (Priess and Thomson 1987; Priess et al., 1987). In contrast, the EMS pathway appears independent of intercellular signalling and does not require *glp-1* function (Priess and Thomson, 1987; Priess et al., 1987; Bowerman et al., 1992). Rather, EMS depends on the activity of a second maternal gene, *skn-1*, to generate pharyngeal cells (Bowerman et al., 1992). Thus, the production of pharyngeal cells by ABa and EMS depends on two separate pathways that are genetically distinguishable.

Prior to the 15-cell stage, when *glp-1* and *skn-1* are active, most early blastomeres are pluripotent, contributing to multiple cell types. For example, ABa and EMS each produces both pharyngeal cells and non-pharyngeal cell types such as epidermis or body wall muscle (Sulston et al., 1983). In *glp-1* and *skn-1*

mutants, all of the cell types that normally derive from a given blastomere appear to be affected, not just pharyngeal cells. In these mutant embryos, ABa or EMS descendants follow the cell lineage and differentiation patterns characteristic of other early blastomeres (Bowerman et al., 1992; Hutter and Schnabel, 1994; Mello et al., 1994; Moskowitz, Gendreau and Rothman, submitted). This phenotype suggests that early blastomeres acquire unique identities that are specified by maternal genes like *glp-1* and *skn-1*. In contrast, later in development, cells that are destined to make the pharynx and that derive from different cell lineages, must coordinate their development to form an integrated, functioning structure. The processes that mediate the transition from the early stages of blastomere specification to later organogenesis are unknown.

Whereas ABa and EMS are pluripotent, their descendants become lineally restricted. Between the 50- and 350-cell stages, ABa and EMS descendants are born that will produce either all pharyngeal cells or no pharyngeal cells (Fig. 1). We define a pharyngeal precursor as a cell that is destined to produce only pharyngeal cells. The lineage restriction seen at this time applies to pharyngeal fate generally, not to individual cell types found within the pharynx. For example, one pharyngeal precursor born at the 200-cell stage (ABaraappaa; for a description of *C. elegans* nomenclature see Materials and Methods) divides twice to generate four pharyngeal cells: two muscle nuclei, one epithelial cell and one marginal cell (Sulston et al., 1983). This separation of pharyngeal and non-pharyngeal cell lineages raises the intriguing possibility that cells first acquire a pharyngeal organ identity, and only later become restricted to a specific cell type within the pharynx.

In this paper, we identify the *pha-4* locus and characterize its role in pharyngeal development. Development of the pharyngeal precursors is blocked in *pha-4* mutant embryos such that neither a pharyngeal primordium nor differentiated pharyngeal cells are produced. Non-pharyngeal cells that derive from ABa and EMS are made in *pha-4* mutants, arguing that *pha-4* does not specify the identity of ABa and EMS per se. Thus, *pha-4* defines the determination and assembly of the pharyngeal precursors as a genetically controlled developmental step. We suggest that cells acquire a pharyngeal organ identity and propose a model for how pharyngeal cells are specified during embryogenesis.

MATERIALS AND METHODS

Strains

Strains were maintained according to Brenner (1974).The wild-type strain was *C. elegans* var. Bristol strain N2. The following were used: LGI, *ced-1(e1735)*; LGII, *rol-1(e91), mex-1(zu121)*; LGIII, *lon-1(e185), par-3(it71), pha-1(e2123ts), pie-1(zu154), unc25(e156)*; *ced-4(n1162)*; LGIV, *dpy-13(e184sd), unc-5(e53), skn-1(zu67), unc-8(n491sd), ced-5(n1812), him-8(e1489)*; LGV, *unc-51(e369), unc-51(e1189), zen-3 (e2504), fog-2(q71), stu-3(q265), rol-9(sc148)*. In addition, the balancers *mnC1* on II and *qC1* on III and the deficiency *ozDf2* on V were used. The *mex-1* experiments were performed at 25°C since *mex-1* alleles are temperature sensitive (Mello et al., 1992). Unless indicated otherwise, the strain *fog-2 pha-4(q490)/stu-3 rol-9* was used.

Isolation of *pha-4* alleles

q400 and *n2498* (Chisolm and Horvitz, personal communication) were isolated in a screen for first stage larval (L1) lethals. Five additional alleles (from 12, 850 haploid genomes, frequency=3×10^{-4}) were isolated by screening for lethal mutations linked to either *fog-2* or *unc-51 rol-9*. Candidates were tested for complementation against *pha-4(q400)*, irrespective of their terminal phenotype. *pha-4(q506)* was isolated using 0.5 mM ethylnitrosourea; all other alleles were isolated with methanesulfonate (Brenner, 1974).

The *pha-4* alleles map next to *rol-9* on the right end of chromo-

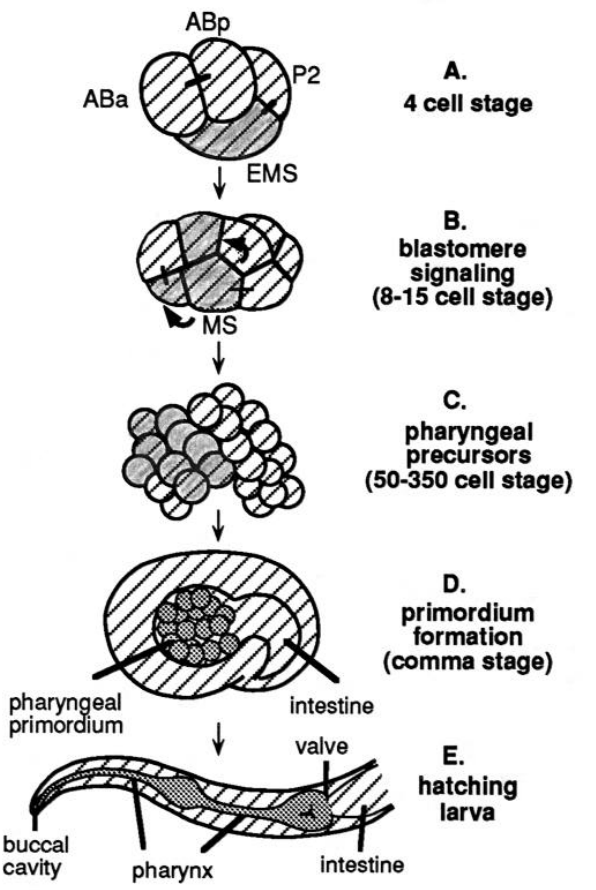

Fig. 1. A model for pharyngeal development in wild-type embryos. The developing pharynx is depicted from the 4-cell stage through hatching. Cells with the potential to produce pharyngeal cells are shaded. Cross hatching represents the potential of cells to produce non-pharyngeal cell types. (A) Two blastomeres, ABa and EMS, of the 4-cell embryo will generate the pharynx. These blastomeres will also contribute to other, non-pharyngeal cell types (cross hatching). EMS produces pharyngeal cells when isolated from other blastomeres (shading), whereas ABa does not (no shading; Priess and Thomson, 1987; Bowerman et al., 1992) (B) Between the 8- and 15-cell stages, an inductive signal (arrows) from MS to ABa descendants is required for these ABa descendants to generate pharyngeal cells. (C) Between the 50- and 350-cell stages, descendants of ABa and EMS are born that are lineally restricted such that they will either produce all pharyngeal cells (shading, no cross hatching) or no pharyngeal cells (cross hatching, no shading). Other cells have not yet become restricted (cross hatching and shading). (D) At the comma stage, when cell division is largely complete, pharyngeal precursor cells assemble into a ball that comprises the pharyngeal primordium. (E) A fully formed pharynx is shown, after the primordium has undergone morphogenesis and differentiation. For a complete description of the *C. elegans* embryonic cell lineage, see Sulston et al. (1983).

some V. Complementation and mapping indicate that *pha-4* is distinct from *rol-9*, *zen-3*, *sel-6* and *stu-3*.

Microscopy

25% of eggs laid by *pha-4*/+ mothers arrest as unelongated embryos or larvae. By Normarski microscopy, no pharyngeal-like cuticle, lumen or clumps of cells are seen in >99% of these arrested progeny (n>100 for each allele). Arrested embryos from heterozyogous mothers were scored for the ability to elongate by dissecting scope and Nomarski microscopy after overnight incubation.

Transmission electron microscopy was performed on decapitated L1 larva heads essentially as described in Priess et al. (1987). Longitudinal and transverse serial sections were examined for cells with a pharyngeal morphology. The contrast from the sections of the Pha+ control animal was computer-enhanced using Adobe Photoshop.

Strength of alleles

The *pha-4* alleles were ordered on the basis of ability to elongate and production of pharyngeal muscles, using 3NB12 staining. The seven *pha-4* alleles can be grouped into three classes (Table 1): severe (*q490, q506, n2498, and q496*) intermediate (*q487* and *q400*,) and weak (*q500).*

Cell staining

For antibody staining, the protocols of Albertson (1984) or Bowerman et al. (1993) were followed, with the following differences: microscope slides for mounting embryos were treated with a solution of 0.1% poly-L-lysine obtained from Sigma Chemical Co. Tween 20 was omitted from all buffers. Some embryos were fixed with 4% paraformaldehyde and were not incubated in paraformaldehyde, but frozen directly. Embryos were mounted for viewing in elvanol mounting media supplemented with 2.5% 1,4-diazobicyclo-[2.2.2]-octane (DABCO) and a pinch of p-phenylenediamine (S. Strome, unpublished data). Elongated larvae were stained in Figs 2, 3, 5A-D; developing embryos were used in Figs 4, 5E-H, 7, 8. Given the *pha-4* elongation frequency (Table 1), most of these embryos would not have elongated properly if allowed to develop to completion.

Cell type assignment

Pharyngeal muscles were stained with either 9.2.1 (Miller et al., 1983) or 3NB12 monoclonal antibodies. 3NB12 was identified in a search for tissue-specific antibodies (Okomoto and Thomson, 1985) and further characterized by Priess and Thomson (1987). 3NB12 stains a subset of pharyngeal muscles starting at approximately 6 hours of development as well as the two intestinal muscles and two small neuronal-looking cells located just posterior to the intestinal muscles. In embryos, we designated $3NB12^{+}$ cells as intestinal muscle cells if they were located in the posterior of the embryo, in pairs, often with the two posterior neuronal-like cells, and if they had a feathery appearance typical of the intestinal muscles.

Other antibodies used were anti-IFA (pharyngeal marginal cells Pruss et al., 1981; Priess and Thomson, 1987), J126 (pharyngeal glands, rectal valve cells, S. Strome, unpublished data), MH4 and MH5 (marginal cells, Francis and Waterston, 1991), 1CB4 (pharyngeal glands, intestine, Il2 neurons, Okamoto and Thomson, 1985). Amphid neurons in *pha-4* larvae were visualized with the vital dye DiOC18 (Molecular Probes, E. Hedgecock, unpublished). Body wall muscles were stained with the NE8/4C6.3 monoclonal, isolated in a search for tissue-specific antibodies to *C. elegans* (Okamoto and Thomson, 1985) and further characterized by A. Fire and J. R. Priess (personal communication). The rectal epithelial cells were visualized in larvae and embryos with the promoter-*lacZ* fusion construct pUL#38E12 (Hope, 1991; Young and Hope, 1993). These cells were designated rectal-epithelial cells because there were three cells and because these cells were located in the rectum, just behind the rectal-intestinal valve cells, as determined by double staining experiments with anti-β-galactosidase and J126 antibodies (S. E. M., unpublished observations). The protocol of Fire, 1992 was followed for enzymatic β-galactosidase staining. Epidermal cells were stained with fluorescein-conjugated soybean agglutinin (Vector Laboratories, Inc.) which recognizes epidermal cells (Mello et al., 1992).

In most staining experiments, *pha-4* animals were distinguished from their healthy Pha^{+} siblings by double staining with either 3NB12 or MH27. In the *skn-1; pha-4* double mutant experiments and in the (ABa+EMS) ablation experiments (for the rectal-intestinal valves), the genotype of individual embryos could not be assessed.

Determination of the fate of the 'pharyngeal cells' in *pha-4* mutant embryos

No extra cell deaths are observed in *pha-4* embryos: no general necrosis is seen in developing *pha-4* embryos, nor any ectopic programmed cell deaths in *ced-1; ced-5; pha-4* triple mutants. These two *ced* genes inhibit the engulfment of dying cells (Hedgecock et al., 1983; Ellis et al., 1991). In addition, the *pha-4* phenotype is not suppressed by *ced-4* mutations, in which programmed cell deaths do not occur (Ellis and Horvitz, 1986). Rather, the heads of arrested *pha-4* larvae are filled with neuronal-looking cells (Fig. 2B,D). Worms heads normally have many neurons. Therefore, to address the fate of the 'pharyngeal cells' in *pha-4* mutant embryos, we ablated all blastomeres except EMS at the four-cell stage (normally EMS produces six neurons and thirty-seven pharyngeal and intestinal valve cells (Sulston et al., 1983)). After development, the operated embryos were examined by Nomarski microscopy and by staining. Cells with a neuronal morphology were seen in the operated embryos. However, we failed to obtain unambiguous staining results with neuronal markers.

Cell lineage analysis

Embryos were lineaged either by direct observation using a Zeiss Axioskop microscope or with a 4D time-lapse videorecorder (Hird and White, 1993). To assess whether a Pha^{-} or Pha^{+} embryo had been lineaged, embryos were scored for the presence of a pharynx after overnight incubation. MSaaaa, MSaaap, MSaapa, MSaapp, MSpaaa and MSpaap and E descendants were followed. Small case letters denote the relative position of a daughter blastomere (e.g. MSa is the anterior daughter of MS). Several lineaged embryos failed to elongate normally, indicating that the abnormal morphogenesis seen in some *pha-4* animals is not due to an inability of EMS descendants to gastrulate properly.

Laser ablation

Laser ablation of embryonic blastomeres was performed according to Sulston and White (1980), Avery and Horvitz (1987) and Bowerman et al. (1992).

RESULTS

The *pha-4* locus

The *pha-4* gene is defined by seven recessive mutations that map to the right arm of chromosome V. Animals homozygous for a mutation in *pha-4* arrest as embryos or first stage larvae; heterozygous *pha-4*/+ animals appear normal (Fig. 2). As described below, *pha-4* mutants lack a pharynx. In addition, some animals are missing a subset of rectal cells and some fail to undergo proper embryonic elongation. The seven *pha-4* alleles can be ordered with respect to the severity of their phenotypes (Table 1). Alleles with the strongest phenotype, such as *pha-4*(*q490*), are likely to be null alleles by three criteria. First, the phenotype of *pha-4*(*q490*) resembles the phenotype of the deficiency *ozDf2*, which removes *pha-4*. Second, *pha-4*(*q490*) behaves like a deficiency when placed in *trans* to the

weakest allele, *pha-4*(*q500*). Third, the phenotype of *q490* is as severe when homozygous as when placed over a deficiency. Thus, *pha-4*(*q490*) is either a null allele or nearly null. It is therefore unlikely that there are defects associated with *pha-4* mutations other than those described below.

Differentiated pharyngeal cells are missing in *pha-4* animals

The most striking and consistent phenotype associated with *pha-4* mutations is the absence of a pharynx. By Nomarski DIC microscopy nothing that morphologically resembles a pharynx is visible in *pha-4* homozygotes (>99% arrested embryos from *pha-4*/+ mothers; Fig. 2). In addition, cells that resemble pharyngeal cells are not seen in the heads of arrested *pha-4* larvae, as scored by transmission electron microscopy (Fig. 2). To determine whether *pha-4* homozygotes make differentiated pharyngeal cells, animals carrying the strong allele *pha-4*(*q490*) were stained with antibodies that recognize three pharyngeal cell types. This analysis showed that most *pha-4* embryos and larvae lack any pharyngeal muscles (94%, *n*=245; Fig. 3A,B), glands (99%, *n*=77; Fig. 3C,D) and marginal cells (100%, *n*=63; Fig. 3E,F). Thus, the hallmarks of a mature pharynx are missing from most *pha-4*(*q490*) animals, including differentiated pharyngeal cells and structures that morphologically resemble a pharynx.

In addition to the pharynx itself, the pharyngeal-intestinal valve is absent in *pha-4* homozygotes (Fig. 3E,F). The valve, which links the pharynx to the intestine, is closely related to pharyngeal cells both spatially and by cell lineage. Because of this close lineal association and because the valve is missing in *pha-4* homozygotes, we propose that the valve is an integral part of the pharyngeal organ.

Formation of the pharyngeal primordium is blocked in *pha-4* mutant embryos

To address when pharyngeal development is affected by *pha-4* mutations, we examined whether the pharyngeal primordium is formed in *pha-4* mutant embryos. In the wild type, the pharyngeal precursors assemble into a primordium midway through embryogenesis, after most cell divisions are complete (the comma stage, see Fig. 1). Formation of the primordium marks the first time the pharynx is visible as a discrete morphological entity, bounded by a basement membrane that separates the pharynx from the rest of the animal. In *pha-4* mutant embryos, no cluster of cells that resembles a pharyngeal primordium is visible by Nomarski DIC microscopy (Fig. 4A,B). In addition, no ectopic dying cells are seen in developing *pha-4* embryos (data not shown; see Materials and Methods).

Normally, three antibodies stain pharyngeal precursors in the developing pharyngeal primordium. Two antibodies,

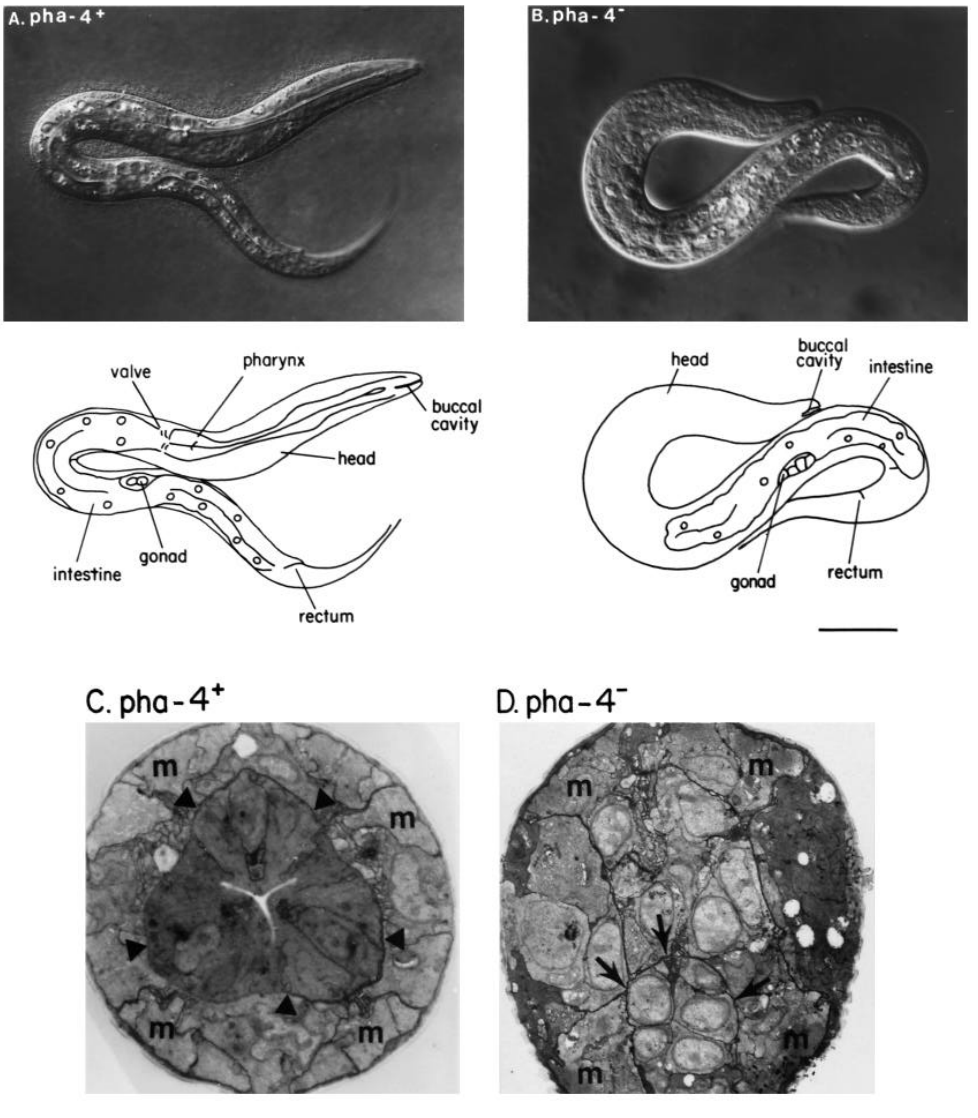

Fig. 2. *pha-4*$^{+}$ and *pha-4*$^{-}$ larvae. (A,B) *pha-4*$^{+}$ (A) and *pha-4*$^{-}$ (B) larvae are shown with Nomarski optics. The *pha-4*$^{-}$ larva has elongated normally and has a buccal cavity, intestine and gonad. In the head, nothing that morphologically resembles a pharynx can be seen. Posterior, the rectum is not connected to the intestine. The allele *pha-4(q400)* was used. Bar, ~20 μm. (C,D) Electron micrographs from serial sections through the heads of *pha-4*$^{+}$ (C) and *pha-4*$^{-}$ (D) larvae. The outer boundary of the pharynx is indicated by arrowheads in C. This section passes through the procorpus of the pharynx. In C, nothing that resembles a pharynx is visible. For reference, body wall muscles are labelled m in C and D. In D, the ventral ganglion is indicated by arrows. This section passes through the head, close to where the terminal bulb is located in wild-type larvae. Magnification ~×5000.

Table 1.

(A) Penetrance of the different *pha-4* alleles

pha-4 allele*	% embryos with some pharyngeal cells† (*n*)	% elongated embryos‡ (*n*)
q490	6 (245)§	10 (259)
q506	8 (84)	8 (116)
n2498	9 (80)	10 (225)
q496	7 (107)	8 (265)
q487	19 (100)	12 (129)
q400	20 (101)	16 (221)
q500	53 (194)	41 (269)
ozDf2	9 (109)	N.D.
wild-type	100 (105)	N.D.

(B) *pha-4(q490)* behaves like a null allele

pha-4 genotype	% elongated embryos (*n*)
q500/ q500	41 (269)
q500/ ozDf2	30 (426)
q500/ q490	26 (170)
q490/q490	11 (332)
q490/ozDf2	9 (105)

A. *Markers linked to a given *pha-4* allele are: *fog-2 pha-4(q500) rol-9, pha-4(q400), fog-2 pha-4(q496), fog-2 pha-4(q487), pha-4(n2498), fog-2 pha-4(q490).*

†Embryos from *pha-4/+* mothers stained with the monoclonal antibody 3NB12 which recognizes a subset of pharyngeal muscles (Priess and Thomson, 1987).

‡Embryos from *pha-4/+* mothers were allowed to develop overnight before scoring for the ability to elongate to the three-fold stage.

§In addition, the strong allele *pha-4(q490)* was stained with the monoclonal antibody 9.2.1, which recognizes pharyngeal myosin (Miller et al., 1983). This staining also gave 6% positive embryos (*n*=147). The 9.2.1 antibody is completely specific for pharyngeal cells, whereas 3NB12 is not. N.D. not determined.

B. The strong allele *pha-4(q490)* behaves like a deficiency when placed in *trans* to the weak allele *pha-4(q500)* (top three lines). In addition, the phenotype of *pha-4(q490)* does not become more severe when reduced from two copies to one copy (bottom two lines). Strains are as in section A.

3NB12 (Priess and Thomson, 1987) and anti-*ceh-22* (P. Okkema and A. Fire, unpublished data), recognize cells that are destined to become pharyngeal muscle. Neither 3NB12 (Fig. 3A,B above, Table 1) nor anti-*ceh-22* (0/67; data not shown) recognizes any pharyngeal cells in most *pha-4*(*q490*) embryos. The antibody MH27 stains adherens junctions, which are found in polarized epithelial cells in both pharyngeal and non-pharyngeal tissues (Francis and Waterston, 1991). In *pha-4*(*q490*) mutant embryos, some MH27 staining is seen in the region of the head where the pharynx normally develops, but this staining is dramatically reduced compared to wild type (Fig. 4C,D). In contrast, non-pharyngeal epithelial cells from *pha-4* mutant embryos and larvae are stained normally by MH27 (for example, Fig. 5G,H). These data show that *pha-4* activity is required to form the pharyngeal primordium. Other primordia (for example, the gonad and the intestine) are formed normally in *pha-4* mutants (data not shown).

pha-4 mutations do not disrupt early blastomere specification or positional information

One possible explanation for the absence of a pharynx in *pha-4* mutants could be a change in the identity of the EMS blastomere. Normally, EMS descendants have two functions in pharyngeal development: they produce pharyngeal cells of the posterior pharynx, and they also signal to ABa (or its descendants) to make pharyngeal cells of the anterior pharynx (Sulston et al., 1983; Priess and Thomson, 1987). Therefore, an EMS transformation would be predicted to affect formation of the entire pharynx, as well as other EMS-derived structures. We used three experimental approaches to ask whether *pha-4* affects EMS blastomere specification. First, we followed cell lineages in *pha-4* mutant embryos. We found that the cleavage pattern of all *pha-4* blastomeres is normal to the 28-cell stage of embryogenesis, just prior to gastrulation. In addition, EMS descendants that produce pharyngeal and intestinal precursors develop normally up to the 200-cell stage. These cells divide at the right time and in the right orientation, and they move internally into the embryo as in wild type. In addition, MSpaapp, a cell lineally and spatially close to the pharyngeal precursors, undergoes its normal programmed cell death at the 200-cell stage (*n*=3). Thus, mutations in the *pha-4* gene do not perturb the cell divisions and gastrulation movements of EMS descendants that normally would generate pharyngeal cells.

We next asked whether EMS generates its normal battery of non-pharyngeal tissues in *pha-4* mutant embryos. In the wild type, EMS divides to produce two founder cells: E, which gives rise solely to the intestine, and MS, which generates multiple cell types (Sulston et al., 1983). In *pha-4* larvae, E produces intestinal cells normally: twenty intestinal cells that stain with the antibody 1CB4 (Okomoto and Thomson, 1985) are made in *pha-4* mutants (Fig. 3C,D). Non-pharyngeal tissues that derive from the MS blastomere are also present in *pha-4* embryos. Specifically, both intestinal muscles are made, one of which is produced by MS (Fig. 3A,B), as are the somatic gonadal precursors (Fig. 2), coelomocytes (data not shown) and body wall muscles. Because body wall muscles are generated from other blastomeres as well as MS, three approaches were used to determine whether all MS-derived body wall muscles are present in *pha-4* homozygotes. First, the total number of body wall muscle nuclei was counted in intact *pha-4* embryos, and found to be similar to wild-type (Fig. 5G,H). Second, the production of body wall muscles by an isolated EMS blastomere was assessed. EMS blastomeres from *pha-4* embryos and their wild-type siblings were allowed to develop after all other cells were destroyed by laser ablation. Both mutant and wild-type operated embryos produced approximately the same number of body wall muscles (data not shown), as assayed by staining with an antibody that recognizes body wall muscle myosin (mAb 5.6, Miller et al., 1983). Third, the body wall muscle precursors P2, MSp and MSap were killed by laser ablation, leaving ABp and MSaa as the only muscle precursors. In the intact embryo, ABp and MSaa each generates four muscle cells. In addition, MSaa normally produces 20 cells of the pharynx and valve, indicating that the four MSaa-derived body wall muscles are closely related to the pharyngeal cells by cell lineage (Sulston et al., 1983). Operated embryos from *pha-4* mutants each have 7-8 muscle cells, consistent with ABp and MSaa generating their normal complement of body wall muscles (*n*=12, range 6-9). Therefore, EMS blastomeres from *pha-4* mutant embryos generate the proper number and type of non-pharyngeal cells, including cells closely related to pharyngeal cells by cell lineage.

To address further whether EMS is properly specified in *pha-4* mutants, we determined whether early blastomere signalling occurs between EMS- and ABa-derived cells. First, we

determined whether *pha-4* mutants produce cell types that are normally made by ABa and that depend on signalling. The buccal cavity, the IL2 neurons and some epidermal cells all require the MS blastomere and *glp-1* activity for their proper specification (Priess et al., 1987; Hutter and Schnabel, 1994; J. Priess personal communication; S. E. M., unpublished observations). As described above, these three structures are made correctly in *pha-4* homozygotes. Second, we examined when signalling occurs in wild-type embryos. EMS, or its descendants, were ablated with a laser microbeam in wild-type embryos. The operated embryos were allowed to develop, and then scored for the presence of a pharynx by Nomarski DIC microscopy and by antibody staining for pharyngeal muscle. When either EMS (Fig. 6A,B) or its daughter MS (Fig. 6C,D) is killed, no pharyngeal muscle is made (0/6 and 0/13 operated embryos stained positively, respectively). By contrast, ablation of E results in a full pharynx being formed, including the ABa-derived anterior pharynx, and the MS-derived posterior pharynx and valve (8/8, Fig. 6E,F). Ablation of both MS and E is indistinguishable from MS alone (0/7 operated embryos produced pharyngeal muscle; data not shown). These results argue that MS, not E, is responsible for signalling to ABa descendants to make pharyngeal cells. However, pharyngeal cells are produced by ABa when the daughters of MS, namely

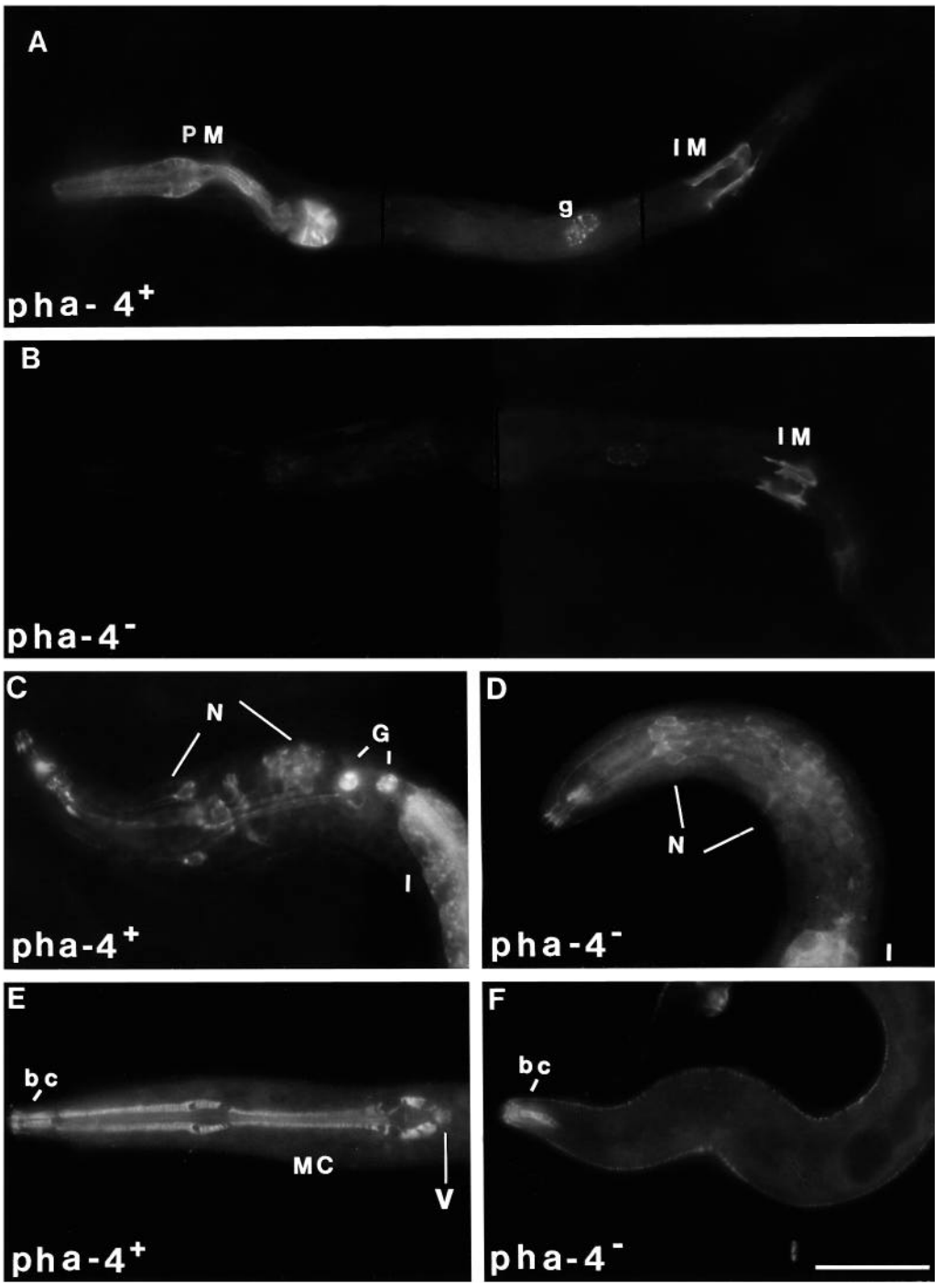

Fig. 3. Differentiated pharyngeal cells are absent in *pha-4* animals. *pha-4*$^{+}$ (A,C,E) and *pha-4*$^{-}$ (B,D,F) larvae stained with antibodies specific for pharyngeal muscle (PM), pharyngeal glands (G), pharyngeal marginal cells (MC) and the pharyngeal-intestinal valve (V). (A,B) Whole larvae; (C-F) larvae heads. Anterior is to the left, dorsal is up. Also shown are non-pharyngeal cells: the intestinal muscles (IM), the germ line (g), the intestine (I), neurons (N), including the six IL2 neurons that reside anterior to the nerve ring (left-most neurons), and the buccal cavity (bc). The germ line is normal in *pha-4* mutants, but not shown here. Unelongated *pha-4* embryos also produce two intestinal muscles, intestine, neurons and germ cells (S. E. M., unpublished observations). Mutant animals were *pha-4(q400)* (B,D) and *fog-2 pha-4(q490)* (F). Bar, ~20 μm for A and B. Bar, ~10 μm for C-F.

MSa and MSp, are ablated at the 15-cell stage (27/29; Fig. 6G and H). These results suggest that, in the wild type, inductive signalling occurs at or by the 15-cell stage, by either MS, or its daughters MSa and MSp. As shown above, MSa and MSp blastomeres from *pha-4* mutants appear to be normal: they generate normal cell lineage patterns and produce normal non-pharyngeal cell types. Thus, the production of induced ABa cell types and the wild-type MS pattern of development argue that signalling between EMS and ABa descendants is likely to occur in *pha-4* mutant embryos. Taken together, the normal EMS cell lineage, the production of non-pharyngeal cell types and the intercellular signalling to ABa descendants argue that EMS is properly specified in *pha-4* mutants. Therefore, the lack of a pharynx in *pha-4* embryos cannot be explained as a defect in EMS that subsequently affects cells from both ABa and EMS.

We also considered the possibility that *pha-4* mutations disrupt positional information, analogous to mutations in the homeotic genes (McGinnis and Krumlauf, 1992). This type of alteration would be expected to interfere with the formation of many anterior structures, not just the pharynx. This possibility seems unlikely, however, since non-pharyngeal cells of the head are made in *pha-4* homozygotes. Specifically, *pha-4* larvae have a buccal cavity (60/60, Fig. 3E,F) and an excretory cell (56/57; Fig. 5A,B), which is required for osmoregulation (Nelson and Riddle, 1984). Normally, these structures lie next to the pharynx: the buccal cavity links the anterior pharynx to the exterior environment and the excretory cell flanks the posterior pharynx. The six IL2 neurons (Fig. 3C,D) and the amphid neurons (Fig. 5C,D), which normally surround the pharynx, are also present in *pha-4* larvae. Finally, *pha-4* embryos have the normal number of epidermal cells, and these are positioned correctly around the head and body on the dorsal and lateral surfaces of the embryo, at a stage when the pharyngeal primordium is clearly absent (Fig. 5E,F). Later, these epidermal cells often fail to migrate to their proper ventral positions (see below). These data indicate that non-pharyngeal cells that are normally in close proximity to the pharynx are present in *pha-4* animals. Therefore, *pha-4* is not required to specify regional identity in the *C. elegans* head. Rather, *pha-4* mutations appear to affect cells because of their affiliation to an organ, the pharynx. Either specification of the pharyngeal precursors or their assembly into a primordium may depend on *pha-4* function.

The requirement for *pha-4* activity cannot be by-passed by mutations that give rise to ectopic pharyngeal cells

To determine whether *pha-4* activity is absolutely required for pharyngeal development, double mutant combinations were made between *pha-4* and mutations that give rise to extra, ectopic pharyngeal cells, namely *par-3*, *mex-1* and *pie-1*. Each of these maternal-effect mutants generates extra pharyngeal cells from blastomeres that normally do not contribute to the pharynx (Kemphues et al., 1988; Mello et al., 1992). Embryos from mothers homozygous for each of the maternal-effect mutants and heterozygous for *pha-4* were allowed to develop, and then examined for the presence of pharyngeal tissue by Nomarski DIC microscopy and by staining for pharyngeal myosin. For each double mutant combination, the requirement for *pha-4* activity could not be by-passed by these maternal-effect mutations (Fig. 7A-F), suggesting that *pha-4* activity is critical for development of the pharynx.

A pathway for pharyngeal development

To date, only three mutants have been identified that fail to make a differentiated pharynx: *skn-1* (Bowerman et al., 1992), *pha-1* (Schnabel and Schnabel, 1990) and *pha-4* (this paper). In embryos carrying mutations in the maternal-effect gene *skn-1*, cell types normally made by EMS are altered or absent, and instead EMS develops like its niece, a cell called C (Bowerman et al., 1992). Embryos carrying mutations in the zygotic gene *pha-1* make a pharyngeal primordium. However, the primordium neither elongates normally nor differentiates fully (Schnabel and Schnabel, 1990). The single mutant phenotypes of these three genes suggest that *skn-1* acts before *pha-4* to influence the identity of the EMS blastomere, and

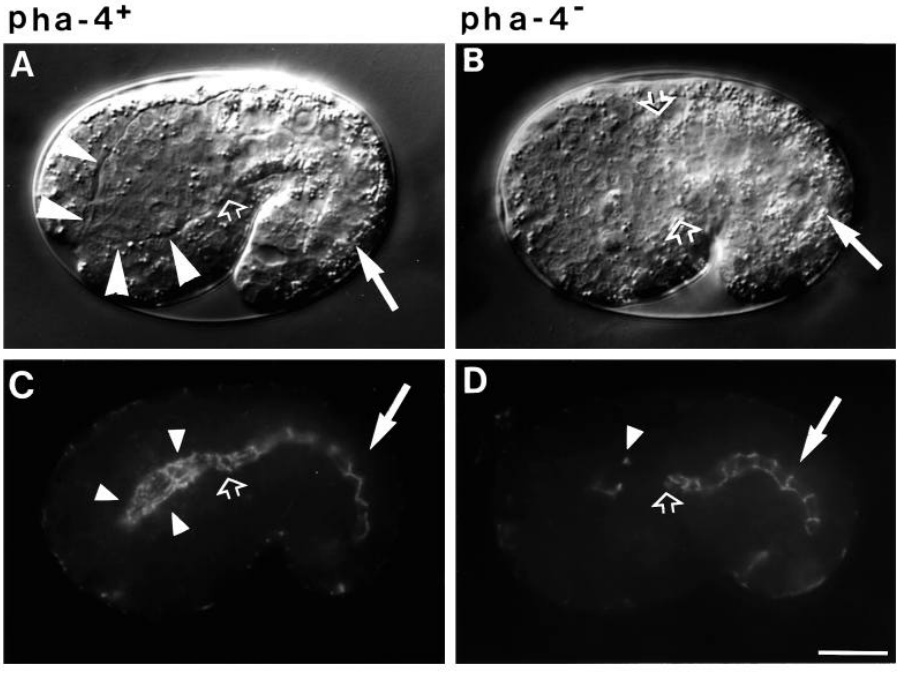

Fig. 4. *pha-4* embryos fail to make a normal pharyngeal primordium. *pha-4*$^{+}$ (A,C) and *pha-4*$^{-}$ (B,D) embryos viewed by Nomarski optics (A,B) and by staining with the monoclonal antibody MH27, which recognizes adherens junctions (Francis and Waterston, 1991). (A) Arrowheads point to the basement membrane surrounding the developing pharynx. (C) Arrowheads indicate adherens junctions found in the developing wild-type pharynx. In *pha-4* embryos some MH27 staining is apparent, but at a greatly reduced level and in a disorganized configuration (arrowheads in D). The intestine is indicated by closed arrows, and the anterior tip of the intestine by open arrows. Bar, ~10 μm.

that *pha-1* acts after *pha-4* during the process of pharyngeal morphogenesis and differentiation. To test this model, double mutant combinations were made between *pha-4* and either *skn-1* or *pha-1*.

In wild-type embryos, one daughter of EMS, the MS blastomere, produces many pharyngeal cells and body wall muscles (Sulston et al., 1983). In *skn-1* embryos, MS produces body wall muscles and ectopic epidermal cells (Bowerman et al., 1992; Bowerman, personal communication). In *pha-4* mutant embryos, no cells with an epidermal morphology are generated by EMS (data not shown). Rather, EMS produces cells that morphologically resemble neurons (Fig. 2 and Materials and Methods). To examine what cell types are made by the MS blastomere in *skn-1*; *pha-4* double mutants, early embryos were isolated from *skn-1*; *pha-4*/+ mothers and all but the MS blastomere killed by laser ablation. The operated embryos were allowed to develop and stained with a fluorescent lectin that recognizes the cuticle secreted by epidermal cells (Mello et al., 1992). The majority of these partial embryos produced epidermal cells (18/21), similar to embryos mutant for *skn-1* alone (14/17). In contrast, isolated MS blastomeres from wild-type embryos or *pha-4* single mutants do not produce epidermal cells (0/9). These data are consistent with *pha-4* acting after *skn-1*.

To examine the *pha-1*; *pha-4* double mutant phenotype, we took advantage of the strong temperature-sensitive allele, *pha-1(e2123)* (Schnabel and Schnabel, 1990). When raised at restrictive temperature, animals carrying *pha-1(e2123)* make a pharyngeal primordium and some differentiation occurs: the pharynx is surrounded by a basement membrane, 95% of all embryos are stained by the pharyngeal muscle antibody 3NB12 and some pharyngeal morphogenesis occurs such that there is often a central lumen (Fig. 7G; Schnabel and Schnabel, 1990). However, pharynges from *pha-1* mutants rarely elongate fully, nor do they express later differentiation markers (Schnabel and Schnabel, 1990). When embryos from *pha-1*; *pha-4*/+ mothers are raised at restrictive temperature, only 71% make any pharyngeal muscle, as assayed by 3NB12 staining (Fig. 7H). Since only a quarter of these embryos should be homozygous for *pha-4*, this result indicates that the *pha-1*; *pha-4* double mutant cannot produce pharyngeal muscle. In addition, these *pha-1;pha-4* double mutants exhibit a small amount of MH27 staining in the head, as do *pha-4* single mutants (36/36, data not shown and Fig. 4D). These data suggest that *pha-4* acts before *pha-1*. These results are consistent with a pathway for pharyngeal development whereby *pha-4* acts after *skn-1* and before *pha-1*.

Non-pharyngeal defects in *pha-4* mutants

Animals homozygous for *pha-4* mutations show two non-pharyngeal defects with incomplete penetrance. First, some animals fail to make a normal rectum. The wild-type rectum is composed of a series of rings that link the intestine to the anus (Sulston et al., 1983). The cells of the rectum derive from ABp, which does not contribute to the pharynx. In strong *pha-4* mutants, the posterior rectal cells are present in almost all animals (96%, *n*=56). Anterior rectal cells, however, are often missing: only 21% of *pha-4*(*q490*) embryos have rectal intestinal valve cells (*n*=42), and 73% have rectal epithelial cells (*n*=49, data not shown).

To address whether the absence of the rectal-intestinal valve cells in *pha-4* homozygotes is due to a direct requirement for *pha-4* activity, or an indirect consequence of the pha-

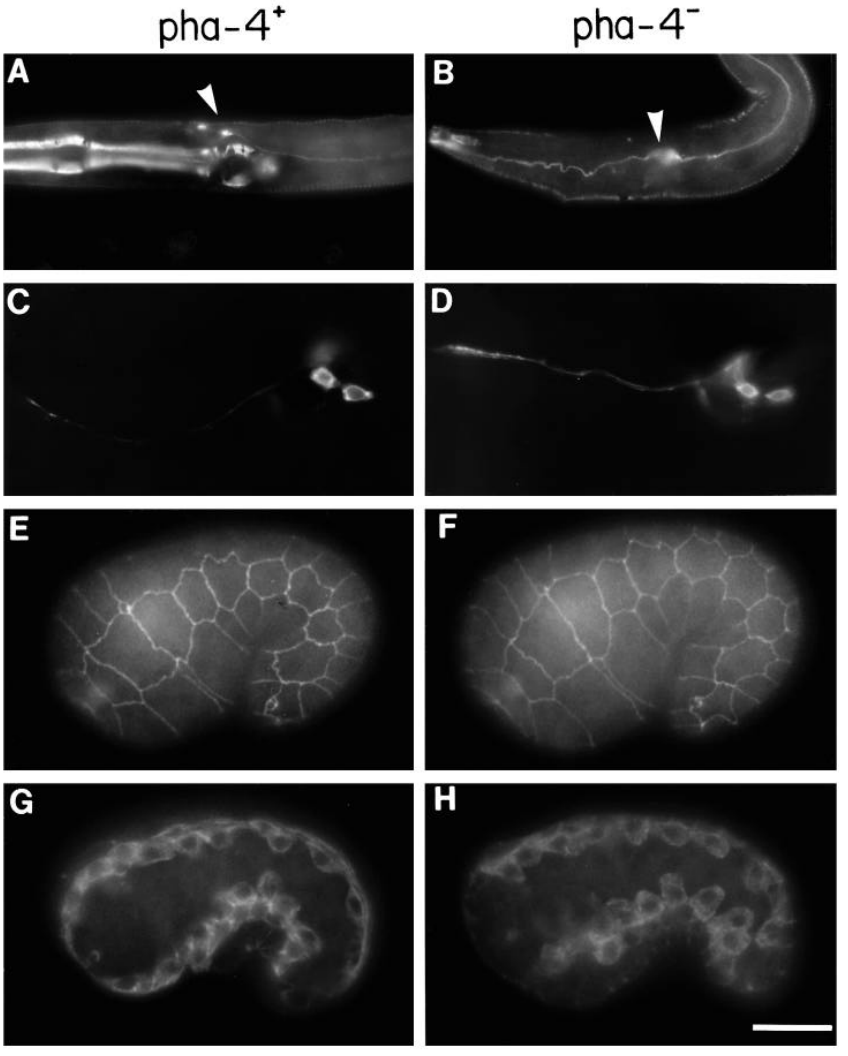

Fig. 5. Non-pharyngeal cell types are made normally in *pha-4* homozygotes. *pha-4*$^+$ (A,C,E) and *pha-4*$^-$ (B,D,F) animals have an excretory cell (A,B, arrowhead), amphid neurons (C,D), epidermal cells (E,F) and body wall muscles (G,H). Wild-type embryos have 81 body wall muscle nuclei (Sulston et al., 1983), compared to an average of 83 for *pha-4* embryos (*n*=6, range=79-86). Ten of the twelve amphid neurons stain brightly in *pha-4*$^-$ larvae (*n*=6) and their *pha-4*$^+$siblings (*n*=2). Only two amphids are visible in this focal plane. Mutant animals were *pha-4(q400)* (B) and *fog-2 pha-4(q490)* (D,F). Bar, ~20 μm in A and B, ~15 μm in C and D, and ~10 μm in E-H.

ryngeal defect, ABa and EMS, the blastomeres that normally produce pharyngeal cells, were ablated with a laser microbeam. The operated embryos were then scored for the ability to make rectal-intestinal valve cells. All partial embryos from wild-type mothers made valve cells (20/20). Thus, descendants of ABa and EMS, including pharyngeal cells, are not required to produce valve cells in wild-type embryos. In contrast, only 80% of the embryos from *pha-4*/+ mothers made rectal-intestinal valve cells (17/21; Fig. 8A,B). This result suggests that the rectal defect observed in *pha-4* mutants is not a consequence of the absence of a pharynx, but rather that the *pha-4* gene plays a more direct role in the production of the valve cells.

A second non-pharyngeal defect associated with *pha-4* is that some mutant embryos fail to elongate normally (see Table I). Morphogenesis during wild-type embryogenesis begins when epidermal cells located dorsally, migrate ventrally to enclose the embryo. After enclosure, the epidermal cells constrict circumferentially to squeeze the ellipsoid embryo into a long, thin worm (Priess and Hirsh, 1986). In *pha-4* embryos, epidermal cells are initially found in the correct location on the dorsal and lateral surfaces. However, these cells fail to migrate properly to their ventral positions. Thus, the elongation defect differs qualitatively from the pharyngeal and valve cell defects, in which specific cell types are not generated. Because of the failure of epidermal cells to enclose, internal cells are squeezed out ventrally when the epidermal cells constrict, and the embryo ruptures (Fig. 8C,D). Other migrations, such as the intercalation of dorsal epidermal cells, are unaffected by *pha-4* mutations.

The elongation defect may reflect a direct requirement for *pha-4* activity during epidermal migration. Alternatively, this effect may be an indirect consequence of the absence of a pharynx. Consistent with the latter hypothesis, other mutants with altered pharyngeal development, namely *glp-1* and *pha-1*, also have morphogenetic defects (Priess et al., 1987;

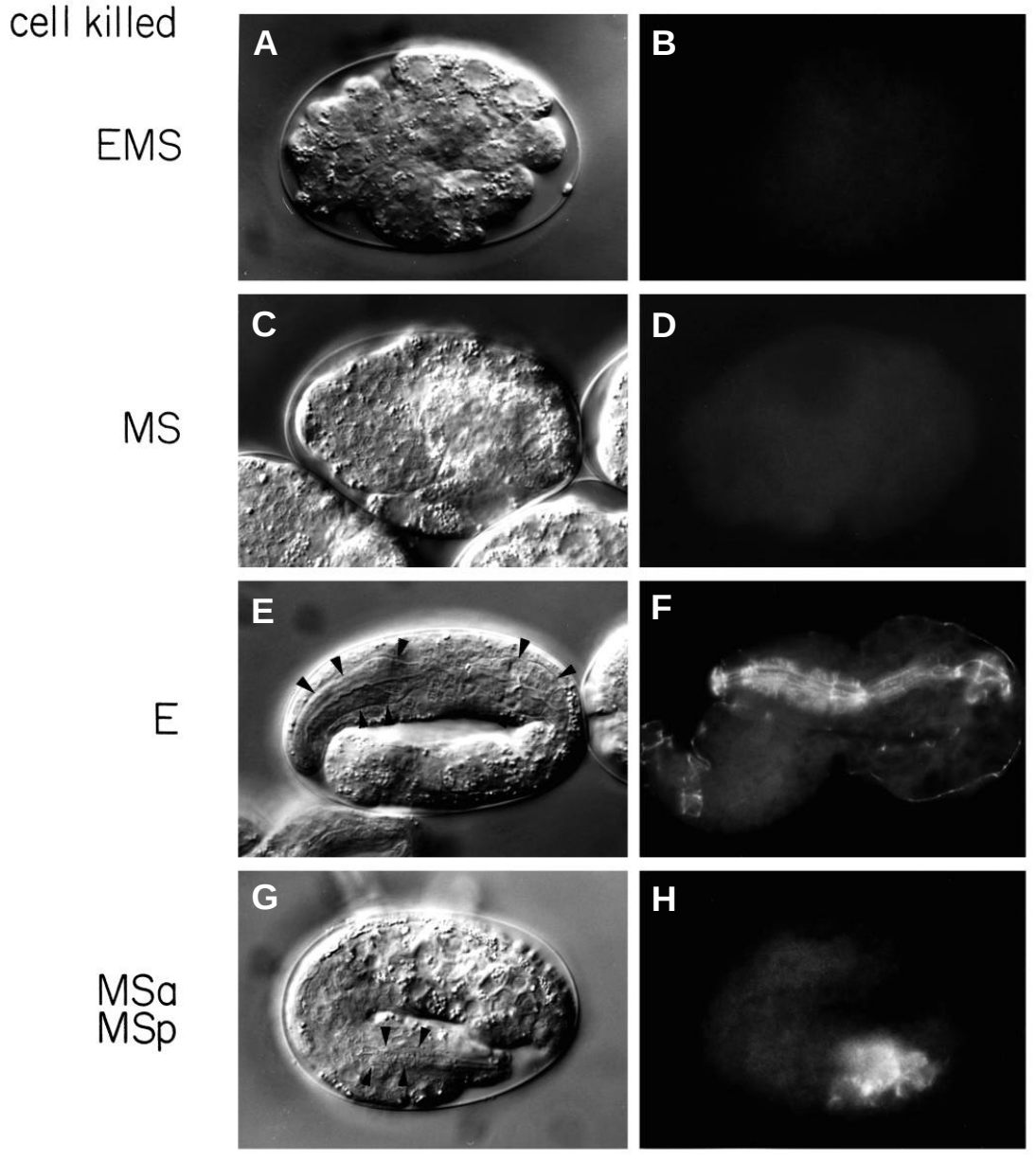

Fig. 6. Pharyngeal signalling occurs at or by the 15-cell stage in wild-type embryos. The following blastomeres were ablated in wild-type embryos: (A,B) EMS at the 4-cell stage; (C,D) MS at the 8-stage; (E,F) E at the 8-cell stage, (G,H) MSa and MSp at the 15-cell stage. Ablated embryos developed overnight, and examined by Nomarski microscopy (left panels) or by staining for pharyngeal muscle (right panels) for the presence of a pharynx. Embryos were stained with 3NB12 (A-E, G,H) or a combination of 9.2.1, which stains pharyngeal muscle, and MH27, which stains adherens junctions (F). Bar, ~10 μm.

Schnabel and Schnabel, 1990). We cannot test whether the migration defect reflects a direct or indirect requirement for *pha-4* activity, since laser ablation of ABa and EMS in wild-type embryos has drastic effects on morphogenesis.

DISCUSSION

pha-4 is required to generate the pharyngeal primordium

The *pha-4* gene is required to make a pharyngeal primordium and hence a pharynx during *C. elegans* embryogenesis. In *pha-4* mutants, no mature pharynx is visible by light microscopy and differentiated pharyngeal cells are missing. More importantly, no pharyngeal primordium is formed in *pha-4* mutant embryos. We considered three explanations for the lack of a pharyngeal primordium in *pha-4* mutants: *pha-4* activity might be required to specify the identity of the EMS blastomere, to determine anterior positional information, or to mediate development of the pharyngeal precursors. We found no alteration in EMS development in *pha-4* mutant embryos other than its effect on pharyngeal development and no general defect in anterior development. Instead, the cells affected by *pha-4* mutations are related to each other by virtue of belonging to an organ, the pharynx, and not simply by cell lineage or position.

How are cells specified to become one of the five differentiated cell types that are organized into the pharyngeal organ? A priori one can imagine three possibilities. A cell could first be specified as a pharyngeal precursor, and only secondarily develop into a specific cell type within the pharynx (e. g. pharyngeal muscle). This kind of developmental program is used to generate the *Drosophila* eye. During fly eye development, pluripotent cells in the eye-antennal imaginal disc interact with one another to determine their specific fates within the eye (reviewed by Basler and Hafen, 1991). Alternatively, a cell could first be specified as a particular cell type (e.g. muscle) and only later join the pharynx. This pattern of development is seen during vertebrate limb formation, in which somitic cells that are apparently committed to a myogenic pathway, migrate and populate the developing limb; these cells form the limb skeletal muscles (reviewed by Wachtler and Christ, 1992). Finally, specification as a pharyngeal cell and specification as a particular cell type could occur independently.

The absence of a pharyngeal primordium and the absence of both early and late pharyngeal markers in *pha-4* mutant embryos is consistent with the idea that pharyngeal cells acquire a pharyngeal organ identity prior to differentiating into a specific cell type such as a muscle or neuron. Organ identity may be conceptually analogous to positional or cell type identity. Just as the pharynx is absent from *pha-4* embryos,

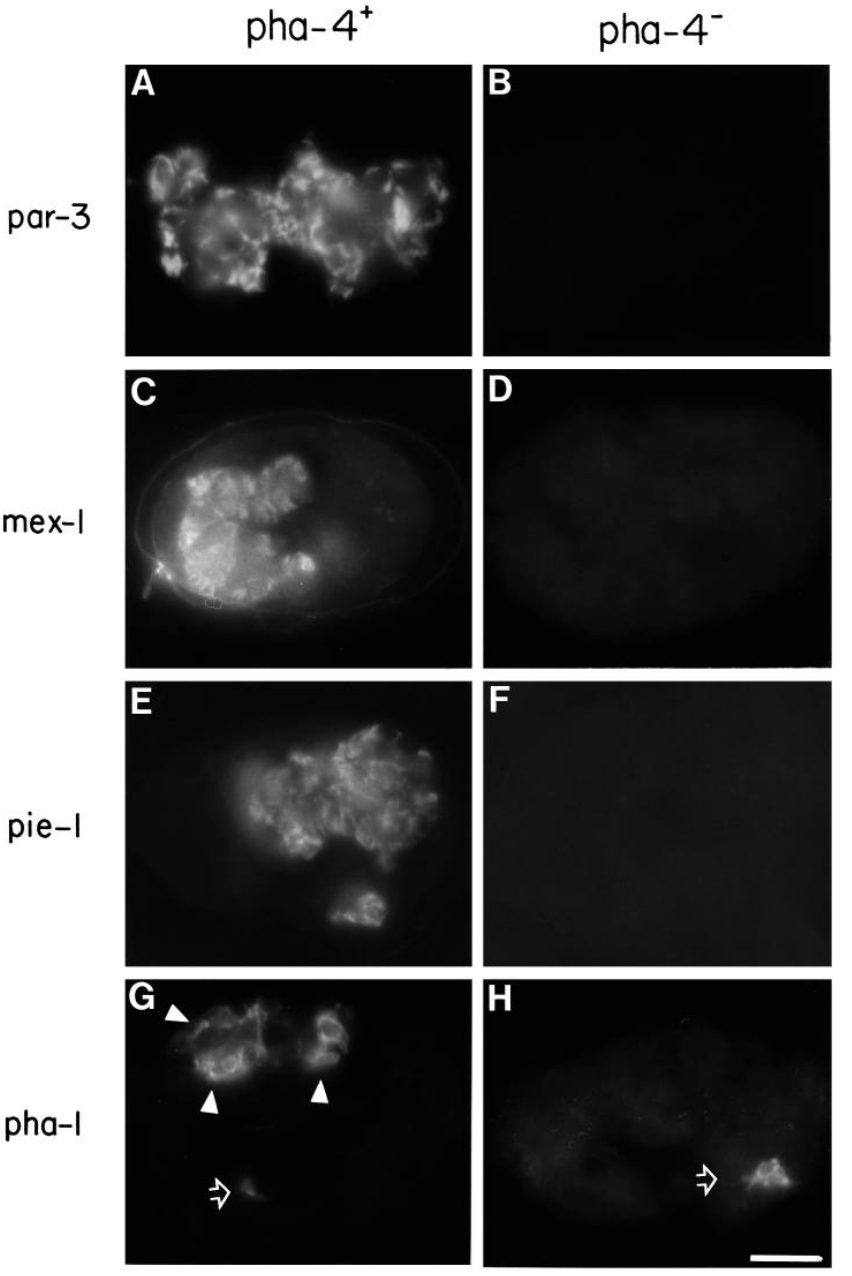

Fig. 7. Phenotypes of double mutants containing *pha-4*: embryos bearing mutations in either (A,B) *par-3*, (C,D) *mex-1* (E,F) *pie-1* or (G,H) *pha-1*, and that were either (A,C,E,G) *pha-4*$^{+}$ or (B,D,F,H) *pha-4*$^{-}$ were scored for pharyngeal development with the pharyngeal muscle antibody 9.2.1 (A-F) or 3NB12 (G,H). In G and H, closed arrowheads indicate the pharynx, open arrows indicate one intestinal muscle that is is the plane of focus. Whereas virtually all *par-3* (100%, *n*=130), *mex-1* (Mello et al., 1992 and data not shown), *pie-1* (98%, *n*=54) and *pha-1* (95%, *n*=122) single mutant embryos produce pharyngeal muscle, only 76% of embryos from *par-3; pha-4/+* mothers (*n*=139), 78% of embryos from *mex-1; pha-1/+* mothers (*n*=60), 69% of embryos from *pie-1; pha-4/+* mothers (*n*=78) and 71% of embryos from *pha-1; pha-4/+* mothers (*n*=397) generate pharyngeal muscle. Bar, ~10 μm.

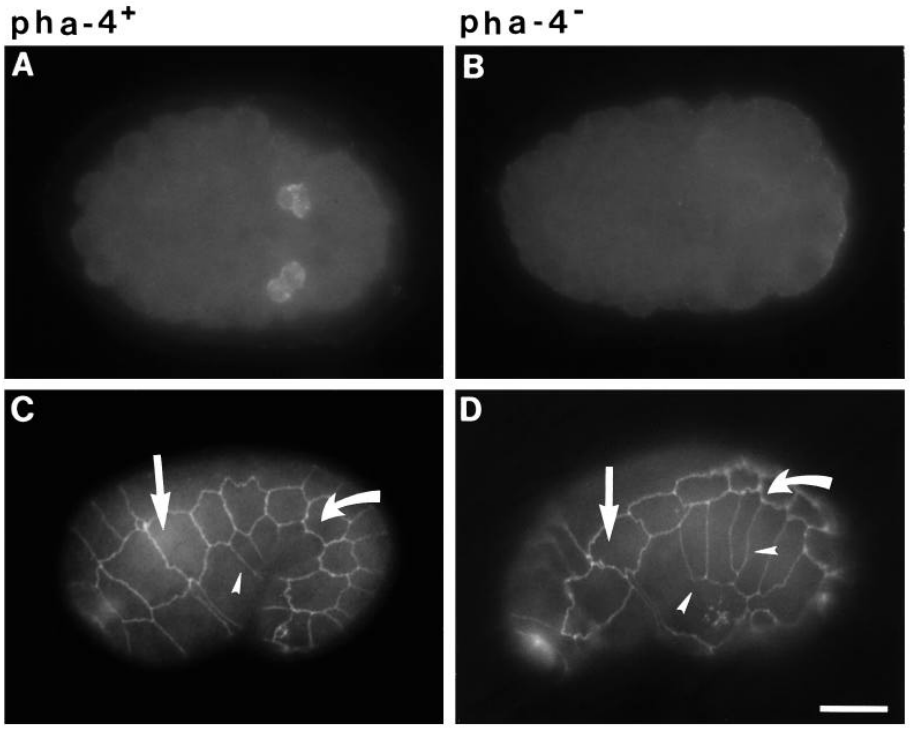

Fig. 8. Non-pharyngeal defects associated with *pha-4* mutations. Embryos were examined for the production of rectal-intestinal valve cells (A,B) and embryonic enclosure (C,D). (A,B) ABa and EMS were ablated with a laser microbeam in embryos from *pha-4/+* mothers. Operated embryos were scored for the presence of valve cells with the monoclonal antibody J126 after developing overnight. (C,D) Epidermal cells were visualized with the monoclonal antibody MH27, which recognizes adherens junctions. Arrows denote epidermal cells that are made correctly in *pha-4*$^{+}$ and *pha-4*$^{-}$ embryos. Arrowheads indicate epidermal cells (C) that have failed to migrate to their correct ventral locations in the *pha-4*$^{-}$ embryo (D). Bar, ~10 μm.

mutants of the *Drosophila* posterior group lack posterior cells (St Johnston and Nüsslein-Volhard, 1992), and mice doubly mutant for *MyoD* and *Myf-5* are missing skeletal myoblasts (Rudnicki et al., 1993). The proposal that cells possess organ identity is supported by the lineage of wild-type pharyngeal cells. During normal embryogenesis, precursors are born that produce only pharyngeal cells; each precursor subsequently gives rise to two or more different cell types within the pharynx (Sulston et al., 1983). Therefore, a precursor is not restricted to a single pharyngeal cell type but appears restricted to the pharyngeal fate.

We envision two possible roles for *pha-4* in pharyngeal development. Wild-type *pha-4* may specify certain cells as 'pharyngeal', or it may regulate assembly of pharyngeal cells into a primordium. If the primary function of *pha-4* is primordium assembly, then assembly must in turn be critical for pharyngeal differentiation. To distinguish between these two roles of *pha-4*, the identity of cells that normally would give rise to pharynx must be assessed in *pha-4* mutants. We have used three methods to address the fate of those cells: 4-dimensional lineage analysis, electron microscopy and cell-type-specific markers. We find that cells normally predicted to be pharyngeal arise in *pha-4* mutants by their typical pattern of cell divisions and that those cells migrate into the embryonic head as usual. However, those cells either fail to differentiate or differentiate as neurons. Current technology does not distinguish between those two fates – at least in this region of the embryo (see also Materials and Methods). We conclude that *pha-4* activity is key for either specification or assembly of the pharyngeal precursors. As a consequence, *pha-4* defines a developmental step that unites the regulation of pharyngeal precursors – whether derived by induction from AB or by autonomous cues from MS.

The *pha-4* phenotype contrasts sharply with that of maternal genes that influence blastomere identity. In the maternal-effect mutants (e.g. *glp-1*, *skn-1*), the normal program of cell division and differentiation of one blastomere is transformed into that of another blastomere, irrespective of the cell types made by that blastomere. For example, in *glp-1* mutant embryos, ABa descendants that would normally generate pharyngeal precursors, assume the identities of their cousins, which do not produce pharyngeal cells (Hutter and Schnabel, 1994; Moskowitz, Gendreau and Rothman, unpublished data). The production of pharyngeal cells by EMS, in contrast, is unaffected by *glp-1* mutations. In *glp-1* mutants, EMS generates its normal complement of pharyngeal cells, which form a fully differentiated half pharynx (Priess et al., 1987). Thus, the maternal genes act at an early point in embryogenesis to influence blastomere fate, whereas *pha-4* is required for the generation of cells in an organ.

pha-4 activity is required to form the rectum

Development of the rectum is often defective in *pha-4* embryos due to the absence of two valve cells that link the intestine to the rectum. In addition, the rectal epithelial cells are occasionally missing. Other cells of the digestive tract that form the buccal cavity, the intestine and the anus appear unaffected. The rectal defect is likely to reflect a direct requirement for *pha-4* activity since laser ablation of ABa and EMS does not inhibit production of valve cells in wild-type embryos, and cannot rescue the valve cell defect in *pha-4* embryos. The observation that *pha-4* activity is required to produce pharyngeal and rectal cells, and no other cell types, suggests that these structures may share a developmental process. One speculative possibility is that the pharynx and rectum may be related to each other evolutionarily. For example, pseudocoelomates such as *C. elegans*, which have a linear digestive system, may have evolved from an organism such as an acoelomate or cnidarian

that had only a single structure to link the intestine to the exterior (Wilmer, 1990). This structure could have been duplicated and the two copies evolved separately into a pharynx and a rectum. This model predicts that other genes will be identified that are restricted to the pharynx and rectum. One candidate is the molecule recognized by the antibody 3NB12. This antibody stains a subset of muscles in the pharynx and also stains cells associated with the rectum (Priess and Thomson, 1987).

Generation of pharyngeal cells during embryogenesis.

We propose that pharyngeal cells are specified in at least three distinct stages. Early in development, the ABa and EMS blastomeres acquire a unique identity that is specified by maternal genes (Bowerman et al., 1992; Hutter and Schnabel, 1994; Mango et al., 1994; Mello et al., 1994; Moskowitz, Gendreau and Rothman, submitted). Subsequently, a subset of ABa and EMS descendants acquire a pharyngeal organ identity and become pharyngeal precursors. These precursor cells assemble into a morphologically distinct pharyngeal primordium. Either specification of the pharyngeal precursors or their assembly into a primordium depends on zygotic activity of the *pha-4* gene (this work). Finally, the pharyngeal precursors are specified as one of five pharyngeal cell types, and the primordium undergoes morphogenesis to produce the mature, functional pharynx. This final step is mediated by other zygotic genes like *pha-1* (Schnabel and Schnabel, 1990; see also Avery, 1993).

One group of pharyngeal precursors is generated by the EMS blastomere (Sulston et al., 1983). The identity of EMS depends on the maternal gene *skn-1* (Bowerman et al., 1992). In the absence of *skn-1* activity, EMS adopts an alternative developmental pathway to produce epidermal cells but no pharyngeal cells (Bowerman et al., 1992). We show that this transformation in blastomere fate occurs whether or not the *pha-4* gene is active. This result, coupled with the early time of *skn-1* action (Bowerman et al., 1992, 1993), argues that *pha-4* acts after *skn-1*. One possibility is that *pha-4* may be regulated directly by *skn-1*. The *skn-1* gene codes for a DNA-binding protein that is likely to be a transcription factor (Bowerman et al., 1992; B. Bowerman, personal communication). Thus, *skn-1* may transcriptionally activate *pha-4*, thereby linking the maternal and zygotic genomes.

A second group of pharyngeal precursors is generated by the ABa blastomere (Sulston et al., 1983). The production of pharyngeal cells by ABa depends on a distinct pathway from that of EMS. Whereas EMS generates pharyngeal cells autonomously and independently of *glp-1* activity (Priess and Thomson, 1987; Bowerman et al., 1992), the production of pharyngeal precursors by ABa depends on signalling from EMS descendants and on *glp-1* (Priess and Thomson, 1987; Priess et al., 1987). Therefore, the generation of pharyngeal cells by these two blastomeres relies on two distinct mechanisms. Because *pha-4* affects pharyngeal cells that derive from both ABa and EMS, these pathways are likely to converge by the time *pha-4* is active.

The *glp-1*-dependent and *skn-1*-dependent pathways of pharyngeal development are different up to at least the 8- to 15-cell stage of embryogenesis. Ablation of MSa and MSp at the 15-cell stage cannot block the production of pharyngeal muscle by ABa, whereas ablation of earlier EMS descendants does block pharyngeal development. Because it is not clear whether cells are killed instantaneously by laser ablation, we cannot determine precisely when signalling between EMS and ABa descendants occurs. However, our conclusion that signalling occurs at or by the 15-cell stage is in good agreement with temperature-shift experiments that predict *glp-1* activity is required until this time (Austin and Kimble, 1987; Priess et al., 1987). Normally, at the 12- to 15-cell stage of embryogenesis, only two of the four ABa descendants give rise to pharyngeal cells. The two ABa descendants that do produce pharyngeal cells have close contacts with MS and its daughters, whereas the two that do not produce pharyngeal cells have little or no contact with these blastomeres. Thus, a simple explanation for why only two of the ABa descendants produce pharyngeal cells is that only these two blastomeres physically contact the signalling cells, MS and its daughters. This hypothesis predicts that the MS-derived signal cannot diffuse throughout the embryo.

The final stages of pharyngeal development depend on activity of the *pha-1* gene (Schnabel and Schnabel, 1990). In *pha-1* single mutants, a pharyngeal primordium is formed, but differentiation and morphogenesis are defective. No pharynx is made in the *pha-1*; *pha-4* double mutant, which is consistent with *pha-4* acting before *pha-1*. Temperature-shift experiments suggest that *pha-1* acts between the 200-cell stage and comma stage of embryogenesis (Schnabel and Schnabel, 1990). Thus, *pha-4* may function at or by the 200-cell stage, which corresponds to the time at which segregation of the pharyngeal precursors occurs. Our antibody staining results are consistent with this hypothesis. Two antibodies, one that recognizes early pharyngeal muscle and another that stains late pharyngeal muscle, show the same small extent of staining in some *pha-4* embryos. Thus, once cells begin to differentiate as pharyngeal muscle, they complete that differentiation program. Therefore, *pha-4* activity is required for a distinct early step in pharyngeal development, and not continuously throughout differentiation.

The discovery of the *pha-4* locus suggests that pharyngeal precursors acquire an organ identity prior to differentiating into a specific cell type within the pharynx. Future experiments will reveal whether the generation of other organs in nematodes, and in other animals, relies on a similar genetic logic.

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