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RESEARCH ARTICLE

Contribution of the Amino and Carboxyl Termini for PHA-4/FoxA Function in *Caenorhabditis elegans*

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FoxA transcription factors are central regulators of gut development in all animals that have been studied. Here we examine the sole *Caenorhabditis elegans* FoxA protein, which is called *pha-4*. We describe the molecular characterization of five *pha-4* mutations and characterize their associated phenotypes. Two nonsense mutations are predicted to truncate PHA-4 after the DNA binding domain and remove the conserved carboxyl terminus. Surprisingly, animals harboring these mutations are viable, provided the mutant mRNAs are stabilized by inactivating the nonsense-mediated decay pathway. Two additional nonsense mutations reveal that the DNA binding domain is critical for activity. A missense mutation predicted to alter the PHA-4 amino terminus leads to a dramatic reduction in *pha-4* activity even though the protein is expressed appropriately. We suggest that the PHA-4 amino terminus is essential for PHA-4 function in vivo, possibly as a transactivation domain, and can compensate for loss of the carboxyl terminus. We also provide evidence for autoregulation by PHA-4. *Developmental Dynamics 234:346–354, 2005.*

Key words: PHA-4; FoxA; winged helix; fork head; activation domain; *C. elegans*; embryogenesis; organogenesis; pharynx

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INTRODUCTION

The digestive tract consists of an epithelial tube that is subdivided into foregut, midgut, and hindgut. Although the morphologies and cell types of the mature digestive tract vary from organism to organism, the network of transcription factors that controls initial patterning of the gut tube is remarkably conserved. This network includes forkhead box (Fox) factors, Nkx2 homeobox proteins, GATA zinc finger factors, and T box proteins (reviewed in Stainier, 2002). Here we focus on *pha-4*, a *C. elegans Fox* homologue, and its role in foregut development.

The Fox proteins are characterized by conservation over 110 amino acids that encompass the DNA binding domain (Clark et al., 1993). Fox factors have been further subdivided into 17 classes (A to Q) on the basis of additional sequence conservation (Kaestner et al., 2000). The FoxA class is critical to form the gut in *Drosophila*, vertebrates and *C. elegans* (reviewed in Zaret 1999, 2002). In mouse, which has three *FoxA* genes, the foregut and midgut are particularly sensitive to *FoxA2* activity while the hindgut appears to rely on *FoxA1* and *FoxA2*. In *Drosophila fork head/FoxA* mutants, the foregut is transformed into head ectoderm and the midgut disintegrates (Jurgens and Weigel, 1988). These phenotypes resemble *C. elegans pha-4/FoxA* mutants, in which foregut and hindgut cells are transformed into ectoderm (Mango et al., 1994; Horner et al., 1998). The phenotypes observed for *FoxA* orthologues in different species suggest that the subdivision of the digestive tract into foregut, midgut, and hindgut is evolutionarily ancient and relies on *FoxA* proteins.

While the DNA binding domain of FoxA3 has been crystallized and its interaction with DNA investigated

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(Clark et al., 1993; Gajiwala and Burley, 2000), less is known about the function of regions adjacent to the DNA binding domain. Deletion studies identified sequences at the amino and carboxyl terminal regions that affected activation by murine FoxA2 in HepG2 hepatoma cells (Pani et al., 1992; Qian and Costa, 1995). FoxA2 lacking both amino and carboxyl terminal regions behaved as a dominant negative (Vallet et al., 1995). Of the two regions, the carboxyl terminus appeared particularly important for two reasons. First, a great reduction in transactivation was observed when these sequences were removed from FoxA2 (Pani et al., 1992). Second, sequences from the carboxyl, but not the amino, terminus of Pintallavis/FoxA from *X. laevis* could function as an activation domain in yeast when fused to a heterologous DNA binding domain (Morgan et al., 1996).

How does the FoxA carboxyl terminus activate transcription? This region contains two blocks of sequences termed activation domain II and III (ADII and ADIII), which are conserved among vertebrate *FoxA* genes (Pani et al., 1992). Sequences encompassing ADIII have been implicated in binding histones H3/H4 in vitro (Cirillo et al., 2002). Based on this interaction, as well as FoxA interactions with chromatin in vitro and a similar mode of binding DNA for FoxA compared to linker histones (Clark et al., 1993), FoxA has been proposed to open chromatin by displacing linker histones (Cirillo et al., 1998, 2002; Shim et al., 1998; Cirillo and Zaret, 1999). Chromatin opening early in development, when FoxA2 is known to associate with at least one of its targets in vivo (McPherson et al., 1993; Chaya et al., 2001), may be critical to allow additional regulatory factors to bind and activate transcription. This is an intriguing model that remains to be tested in vivo.

In the present work, we complement the in vitro studies by analyzing the FoxA protein PHA-4 in *C. elegans* embryos. We use mutant alleles of *pha-4* to determine the contribution of regions flanking the DNA binding domain for *pha-4* activity in vivo. This approach has the advantage of investigating *pha-4* when it is expressed within its natural context of cell type and developmental stage, and therefore under appropriate regulation. Surprisingly, we find that the carboxyl terminus of PHA-4 is not essential for viability but that the amino terminus plays a critical role. Our results suggest that PHA-4 carries two activation domains, one in the amino terminus that is essential for PHA-4 activity and a second one in the carboxyl terminus that plays a secondary role.

RESULTS

Sequence Features of PHA-4

The PHA-4 protein shares the same general organization as other FoxA proteins with a central DNA binding domain flanked by amino and carboxyl terminal regions (Fig. 1A). The PHA-4 carboxyl terminus is more highly conserved with *C. briggsae* PHA-4 than is the amino terminus (68% identical, 79% similar compared to 43% identical, 52% similar, Fig. 1B). In addition, the PHA-4 carboxyl terminus shares sequence similarities with other transcription factors. The most striking example is *ceh-43*, a *C. elegans* Distal-less transcription factor homologue, which is 56% similar to *pha-4* over 86 amino acids (aa; Fig. 1B; Azzaria et al., 1996; Burglin and Aspock, 1999). Within this conserved region are two small blocks of sequence that resemble mammalian ADIII activation domain sequences; no ADII-like sequences are present in either *pha-4* or *ceh-43* (Fig. 1B,C).

Interestingly, aa388–408 within the PHA-4 carboxyl terminus are predicted to be alpha helical by the COILS program (Fig. 1B; Lupas et al., 1991; Lupas, 1996). FoxA1 (but not 2 or 3) and *C. briggsae* PHA-4 are also predicted to contain alpha helices in the carboxyl terminus. The FoxA1 predicted alpha helix (aa407–421) is not included in the histone binding segment and lies immediately proximal to ADII. Taken together, the sequence features of the PHA-4 carboxyl terminus suggest that this region is important for PHA-4 function.

The Carboxyl Terminus Is Not Essential for PHA-4 Function and Viability

To investigate the role of the PHA-4 carboxyl terminus in vivo, we took advantage of mutations in the endogenous *pha-4* locus that were predicted to generate truncated PHA-4 proteins. These mutations enabled us to examine *pha-4* within its native setting and avoid using extragenic constructs that could be subject to inappropriate regulation. Two nonsense mutations, *pha-4(q500)* and *pha-4(zu225)*, were predicted to terminate translation at aa349 and aa384, respectively, thereby removing both the ADIII-like regions and the predicted alpha helix (Fig. 1A). Embryos homozygous for the carboxyl terminal mutations arrested with a "weak" Pha-4 phenotype in which a higher proportion of embryos completed embryonic morphogenesis and made a few pharyngeal cells compared to embryos carrying a null *pha-4* mutation (Mango et al., 1994). The weak phenotypes associated with the carboxyl terminal mutations suggested that some mutant protein was synthesized and functional.

Most nonsense mutations produce little mutant protein due to the action of the nonsense-mediated decay (NMD) system of mRNA surveillance (reviewed in Hilleren and Parker, 1999; Mango, 2001). NMD recognizes and eliminates mRNAs carrying nonsense mutations leading to mRNAs that are unstable and rapidly degraded. Therefore, it was likely that the phenotypes associated with *pha-4(q500)* and *pha-4(zu225)* reflected lower mRNA levels as well as protein alterations. In *C. elegans*, mRNA surveillance requires the products of the *smg* genes (Hodgkin et al., 1989; Pulak and Anderson, 1993; Cali et al., 1999) and mutations in any one of seven *smg* genes stabilize nonsense-containing mRNAs (Pulak and Anderson, 1993). To examine the phenotype associated with the truncated PHA-4 proteins expressed at normal levels, we generated *smg-3; pha-4* double mutants (Tables 1 and 2, Fig. 2).

Strikingly, *pha-4(q500)* and *pha-4(zu225)* mutants were each viable when *smg-3* was inactivated (Tables 1 and 2). Double mutants no longer died as pharynx-less embryos or first stage larvae, but rather gave rise to adults with functioning pharynges that could be maintained as a homozygous strain (Fig. 2). Normally, the mature pharynx contains seven cell types: muscles, epithelia, marginal cells, glands, arcade cells, valves, and neurons (Albertson and Thom-

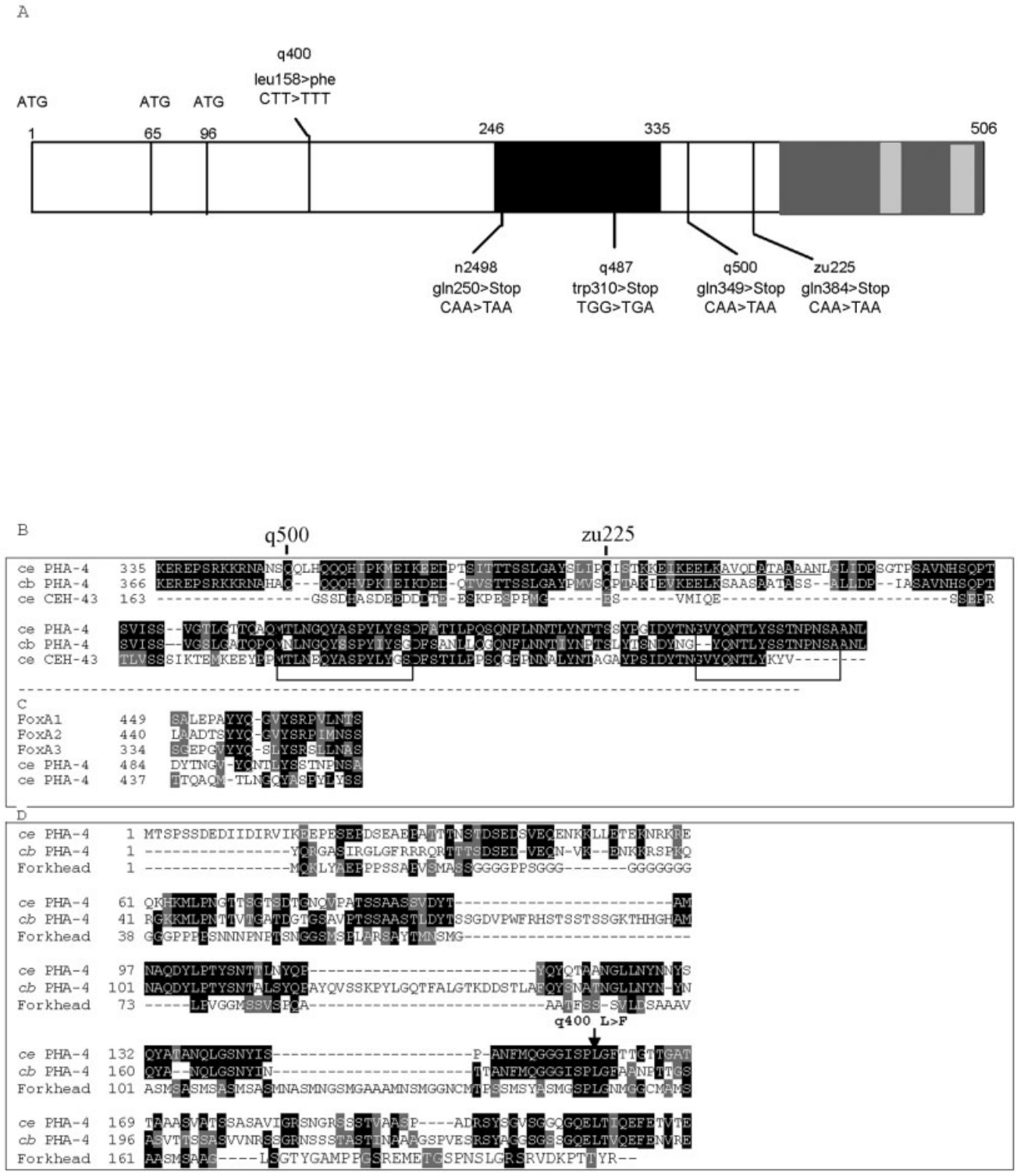

Fig. 1. Location of PHA-4 mutations relative to conserved sequences. **A:** ATG represents the position of the three different translational start codons (Azzaria et al., 1996). The conserved DNA binding domain is indicated by a black box. Conserved sequences between PHA-4 and CEH-43 are shaded dark grey with two imbedded ADIII-like (Pani et al., 1992) sequences in light grey. The five mutations discussed in this study are indicated. **B:** Alignment of the carboxyl terminus of PHA-4 with *C. briggsae* PHA-4 and *C. elegans* CEH-43. Identical residues are highlighted in black and similar residues in gray. The ADIII-like regions are boxed and a putative alpha-helix is overlined (aa388–408). The nonsense mutations *q500* and *zu225* indicate the location of the predicted stop codons. Alignments in B, C, and D were performed using Clustal W (Thompson et al., 1994), manually adjusted and shaded with Boxshade (http://www.ch.embnet.org/software/BOX_form.html). **C:** Alignment of ADIII from FoxA proteins with PHA-4 carboxyl terminus. **D:** Alignment of the amino terminus of *C. elegans* PHA-4 with *C. briggsae* PHA-4. The conserved SPLG and *pha-4(q400)* missense mutation are boxed.

TABLE 1. Percentage of Arrested Self-Progeny From Heterozygous Mothers

	Smg-3 +		Smg-3 −	
pha-4 Genotype of parent	%	n	%	n
+/+	0.4	341	0.6	618
zu225/+	27	1,261	2.7	364
q500/+	26	545	<4	462
q487/+	24	212	25	547
n2498/+	24	872	23	688

TABLE 2. Viability Associated With *pha-4; smg-3* Double Mutants

	Arrested embryos		Arrested first stage larvae		
pha-4 Genotype	%	n	%	n	Brood size
q500/*q500*	1.7	482	63	482	88 (1)
zu225/*zu225*	1.7	361	30.2	361	105 (3)
+/+	2.4	404	0.2	404	135 (3)

son, 1976). Using cell type-specific GFP markers, we observed normal expression in *smg-3; pha-4(zu225)* embryos and larvae for pharyngeal muscle, marginal cell, gland and epithelial cell markers (data not shown). Together, these results suggest that a grossly normal pharynx containing appropriate cell types is made in these mutant animals. Similar results were observed when *pha-4(zu225)* was combined *smg-1(cc546ts)* (data not shown).

We considered whether suppression of *pha-4(zu225)* and *pha-4(q500)* by *smg* mutations relied on mRNA read-through of the nonsense mutation as well as mRNA stabilization. In yeast, mutations in some NMD components can suppress certain nonsense mutations by translational read-through (Hilleren and Parker, 1999). Two lines of evidence demonstrate that translational read-through does not account for viability of *smg-3; pha-4* double mutants. First *smg* mutations cannot suppress all *pha-4* nonsense mutations. We combined a *smg-3* mutation with two other *pha-4* nonsense mutations, *pha-4(n2498)* and *pha-4(q487)* (Table 1). *pha-4 (n2498)* converts a CAA/gln to a TAA/stop mutation (as do *pha-4(q500)* and *pha-4(zu225)*) and is predicted to encode a protein that lacks most of the DNA binding domain and the entire carboxyl terminus. *pha-4(q487)* alters a TGG/trp to a TGA/stop codon (Fig. 1; Horner et al., 1998), which would delete the entire carboxyl terminus and part of the DNA binding domain. If *smg-3* mutations permit read-through of nonsense mutations, then homozygous *smg-3;pha-4(n2498)* or *smg-3; pha-4(q487)* animals should be viable. However, *smg-3* was unable to suppress either *pha-4* allele (Table 1). This result revealed that *smg-3* did not lead to appreciable read-through of stop codons and that the PHA-4 DNA binding domain is critical for activity. The second line of evidence suggesting that *smg-3* mutations did not promote translational read-through of *pha-4* stop mutations was based on expression of PHA-4 in vivo. Homozygous *smg-3; pha-4* embryos failed to stain with an antibody that recognizes the PHA-4 carboxyl terminus (Fig. 3C) (Horner et al., 1998). These data demonstrate that phenotypic rescue by *smg* mutations does not depend on translational read-through. Therefore, the conserved PHA-4 carboxyl terminus is not essential for viability.

Phenotypes Associated With PHA-4 Carboxyl Terminal Truncations

Worms harboring PHA-4 carboxyl terminal truncations, though viable, often had visible phenotypes. While some worms looked virtually wild type, others appeared starved, judging by their small size and pale color (data not shown). Phenotypes did not fall easily into two distinct classes but rather showed a range of "sickliness" that could have resulted from abnormal pharynx function. We compared healthy vs. sickly *smg-3; pha-4* animals for pharyngeal morphology and pumping. For pump rates, we examined at least two independent animals per strain, and counted for two 1-min intervals (see Experimental Procedures section). While most healthy mutant animals had normal pharynges, sickly animals had partially or completely stuffed pharynges (Fig. 4A,B). Furthermore, while pump rates were nearly normal in healthy *smg-3; pha-4(q500)* homozygous animals, pump rates were slow and irregular in sickly mutant animals (Fig. 4C). These findings suggested that a portion of *smg-3; pha-4* homozygotes had defects in pharyngeal function.

Because the rate of pharyngeal pumping is regulated by pharyngeal neurons in wild-type animals (Avery and Thomas, 1997), we examined pharyngeal neurons in *smg-3; pha-4 (zu225)* mutants. The positions of the cell bodies of the anterior pharyngeal neurons were somewhat variable, as was the intensity of GFP expression (Fig. 5C, 8/10 animals examined). Similar results were observed with *pha-4(q500)* (data not shown). By contrast, the pharyngeal neurons appeared normal in *smg-3* single mutants (Fig. 5B). We suggest that defects in pharyngeal pumping in *smg-3; pha-4* mutant animals may reflect impaired development or function of pharyngeal neurons.

The carboxyl terminus may also be important for regulation of PHA-4 levels. We devised a sensitive approach to quantify the amount of PHA-4 in pharyngeal nuclei (see Experimental Procedures section). We measured the accumulation of truncated PHA-4 protein in a Smg mutant background, using a temperature-sensitive allele of *smg-1* and a pan-PHA-4 antibody (Fig. 6A,B) (Gaudet and Mango, 2002). This analysis revealed that at the permissive temperature of 24°C, when NMD has been inactivated, PHA-4 protein accumulated to 2.5 higher levels compared to

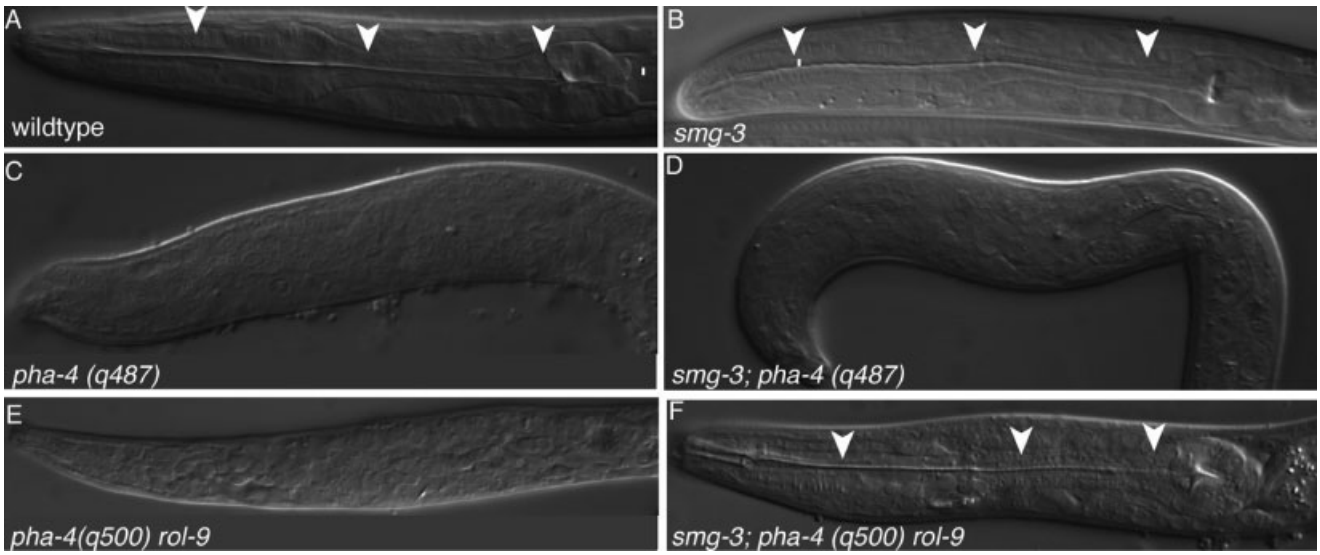

Fig. 2. *smg-3* rescues pharyngeal development in *pha-4* animals lacking carboxyl terminal sequences. First stage (L1) larvae that are Smg-3+ (**A,C,E**) or mutant Smg-3 (**B,D,F**) are shown. Worms are Pha+ (A,B), *pha-4(q487)* (C,D), *pha-4(q500) rol-9* (E,F). The pharynx is denoted by white arrowheads.

smg-1(cc546ts) or wild-type embryos. This result suggests that the carboxyl terminus of PHA-4 may be required to downregulate nuclear PHA-4. Similar observations have been noted for FoxO proteins, which carry a nuclear export signal (NES) within the carboxyl terminus (Biggs et al., 1999). However, we did not observe a predicted NES within PHA-4 using the Signal Scan program (Prestridge, 1991) (data not shown). At the intermediate temperature of 20°C, truncated PHA-4 protein accumulated to the same level as wild-type PHA-4 and yet these embryos arrested at the first larval stage. This result supports the idea that the carboxyl terminus contributes to PHA-4 activity to some extent. Finally, at the restrictive temperature of 15°C, PHA-4 was fourfold lower than controls, presumably because of NMD-mediated degradation of *pha-4* mRNA.

The PHA-4 Amino Terminus Is Essential for Pharynx Development and Viability

The PHA-4 amino terminus is essential for PHA-4 activity in vivo. *pha-4(q400)* is a point mutation that converts leucine 159 to phenylalanine (Fig. 1). Mutants carrying this mutation arrest as embryos or L1 larvae and lack a pharynx (Mango et al., 1994). Therefore, *pha-4(q400)* defines a region that is essential for PHA-4 function. Antibody staining of mutant embryos revealed that the few pharyngeal cells produced in *pha-4(q400)* mutants expressed no detectable PHA-4(q400) protein (Fig. 3E). Similarly no PHA-4(q400) protein was ob-

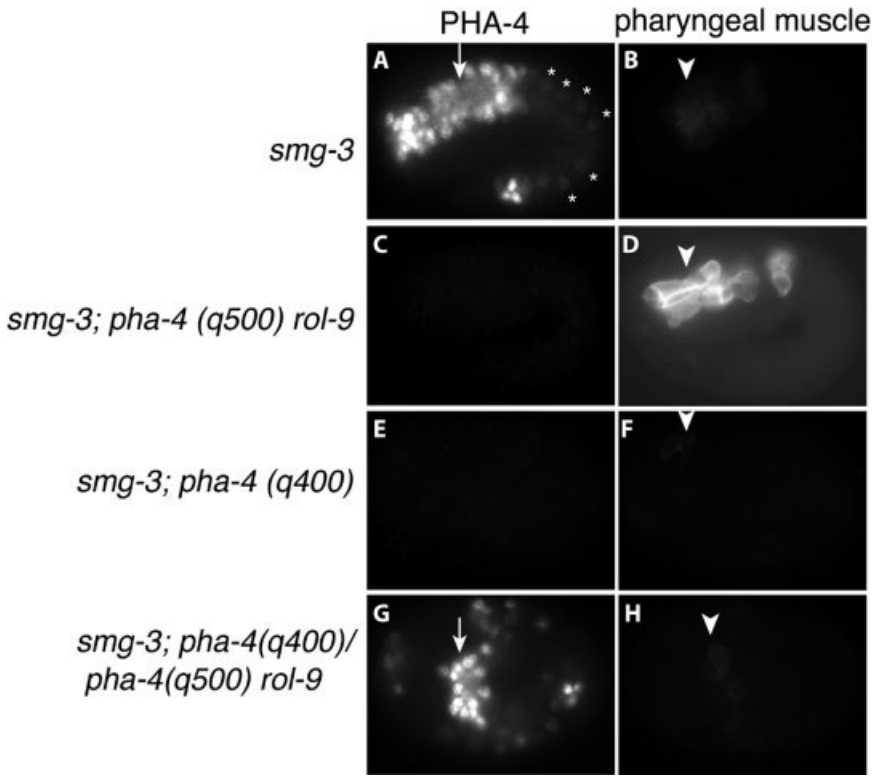

Fig. 3. PHA-4(q400) protein is stable. *smg-3* (**A,B**), *smg-3;pha-4(q500) rol-9* (**C,D**), *smg-3; pha-4(q400)* (**E,F**) *smg-3;pha-4(q400)/pha-4(q500) rol-9* (**G,H**) stained for PHA-4 (A,C,E,G) and pharyngeal muscles (3NB12; B,D,F,H). Arrows indicate PHA-4 staining cells and arrowheads point to the 3NB12 staining of pharyngeal muscle cells. In A, asterisks mark the location of midgut cells that weakly express PHA-4. In *pha-4(q400)* embryos, one or two pharyngeal muscles are produced even though no PHA-4 is detectable. Each embryo is ~50 μm.

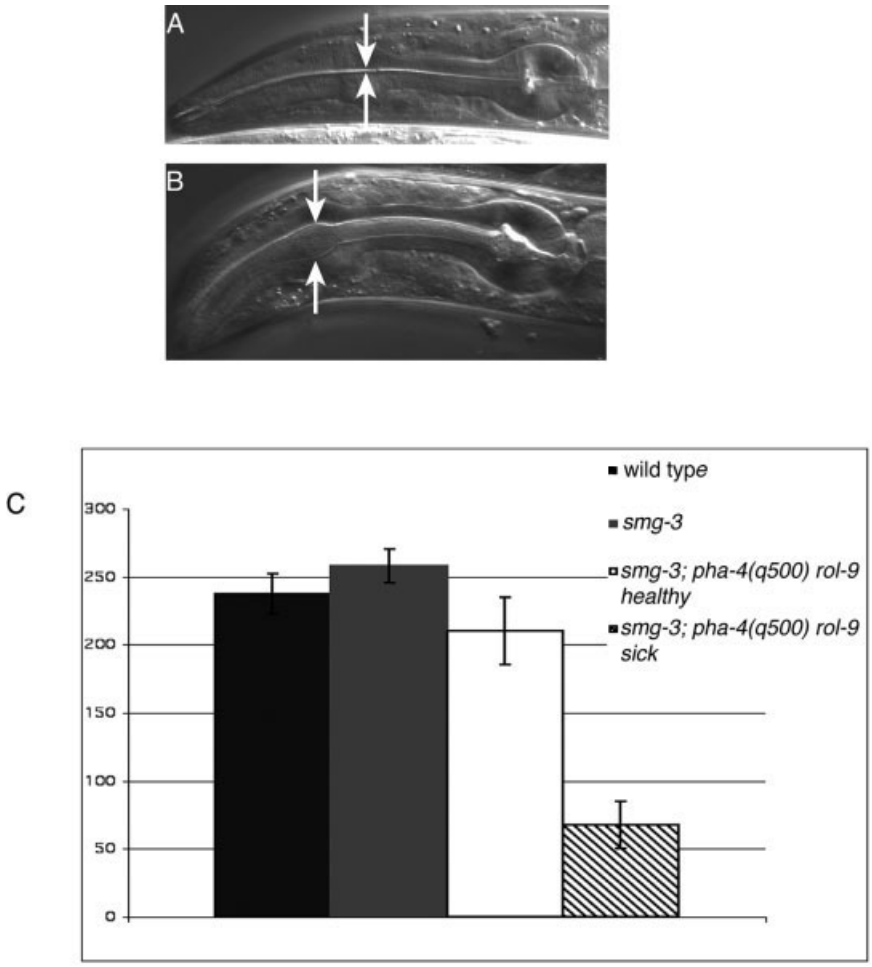

Fig. 4. Pharyngeal phenotypes of *pha-4(q500)* mutants. Pharynges of healthy (**A**) and sickly (**B**) *smg-3; pha-4(q500)* homozygotes. The lumen of the sickly pharynx is stuffed with food (arrows) while food in the healthy pharynx has cleared. **C:** Pharyngeal pump rates (pumps per minute) in wild type (N2, black), *smg-3* (grey), *smg-3; pha-4(q500)rol-9* healthy (white), and *smg-3; pha-4(q500)rol-9* sickly (hatched) animals. Animals were considered sickly by their small, thin, pale appearance.

served in midgut cells, which are generated in mutant animals and express low levels of PHA-4 in wild-type embryos (Fig. 3A) (Horner et al., 1998).

Two models could explain the absence of PHA-4(q400) protein. First, there could be positive autoregulation of *pha-4* during normal development that is disrupted by *pha-4(q400)*. Second, mutant PHA-4(q400) protein could be unstable. To distinguish between these possibilities, we examined PHA-4(q400) in *smg-3; pha-4(q400)/pha-4(q500) rol-9* transheterozygotes, in which pharyngeal development was partially rescued by PHA-4(q500). Since PHA-4(q500) is missing the epitope recognized by the carboxyl-terminal PHA-4 antibody (see above), only PHA-4(q400) protein was available for immunological staining.

smg-3; pha-4(q400)/pha-4(q500) rol-9 embryos expressed PHA-4 protein, revealing that PHA-4(q400) was stable when pharyngeal development was restored (Fig. 3G). The PHA-4 expression in pharyngeal and rectal cells was equivalent to that seen in wild-type embryos (Fig. 3A,G). We conclude that loss of PHA-4 protein associated with *pha-4(q400)* is not due to protein instability. Rather, loss of PHA-4(q400) probably reflects a defect in function and positive feedback (see below).

DISCUSSION

Our analysis of five *pha-4* mutations has revealed three surprising findings concerning PHA-4 protein and function. First, the conserved carboxyl terminus was not essential for PHA-4 activity or worm viability. Animals homozygous for either of two nonsense mutations predicted to truncate PHA-4 after the DNA binding domain were viable and fertile *(q500, zu225)*. These worms were not completely normal, however, suggesting the carboxyl terminus does serve a function, albeit not an essential one. Second, amino terminal sequences were critical for PHA-4 activity. A missense mutation predicted to alter the sequence SPLG to SPFG virtually inactivated PHA-4. This mutation likely pinpoints a region that is critical for PHA-4 activity or structure. Third, two more nonsense mutations located in the DNA binding domain (*n2498* and *q487*) were associated with strong Pha-4 phenotypes, suggesting that the DNA binding domain is essential for function.

The PHA-4 Carboxyl Terminus

The presence of evolutionarily conserved sequences in the carboxyl terminus of PHA-4 had suggested that this region was important for activity. The carboxyl terminus of *C. elegans* PHA-4 is highly similar to *C. briggsae* PHA-4 and *C. elegans ceh-43*/Distalless (Burglin and Aspock, 1999). Two small sequences within this region resemble ADIII from vertebrates, which is required to activate transcription in hepatoma cells. Cirillo and colleagues identified a 47 amino acid block in the carboxyl terminal region of FoxA1 (which encompasses ADIII) that can bind histones and open compacted chromatin in vitro (Cirillo et al., 2002). In addition, the carboxyl terminal region of PHA-4 contains sequences predicted to be alpha-helical. These sequence features suggested that the carboxyl terminus might play a critical role for PHA-4 activity.

We envision three possible models to explain why the PHA-4 carboxyl terminal region is non-essential. First, PHA-4 may harbor two activation domains with one located in the carboxyl terminal region largely redundant and a second one in the amino terminal region. This model agrees with studies with vertebrate Fox proteins that identified two potential activation domains in the amino and carboxyl termini (Pani et al., 1992; Qian and Costa, 1995; Vallet et al., 1995). We favor this proposal since it provides an explanation for the sequence conservation of this region

and for the phenotypes associated with our truncated alleles. If true, this model raises the question of which activation domain is critical for vertebrates in vivo. Previous studies had implicated the carboxyl terminus as the essential region. However, these studies relied on cell lines and artificial FoxA target genes containing multiple copies of a strong FoxA binding site upstream of a basal promoter (Pani et al., 1992; Qian and Costa, 1995). In vivo, FoxA proteins are likely to activate transcription in combination with other factors (Zaret, 1999; Cirillo et al., 2002; Gaudet and Mango, 2002; Ao et al., 2004; Gaudet et al., 2004). Combinatorial control of transcription may function differently from transcription regulated by a single binding site and might, therefore, depend on different FoxA domains.

A second explanation for the effects we observe is that the PHA-4 carboxyl terminus is required selectively to activate nonessential pharyngeal genes. For example, most pharyngeal neurons regulate the timing or efficiency of pharyngeal pumping and are not essential for viability (Avery and Horvitz, 1989). The MC neuron is important for rapid pumping (Raizen et al., 1995). Motor neuron M3 and interneuron M5 are required for trapping bacteria in the isthmus (Avery, 1993). The misplacement of pharyngeal neurons and the similar phenotypes between animals carrying truncated PHA-4 and animals lacking pharyngeal neurons suggest that one or more neuronal genes may not be expressed appropriately. However, a survey of genes expressed in pharyngeal neurons did not reveal any likely targets (L.S.K. and S.E.M., unpublished observations).

Third, the PHA-4 carboxyl terminus may not be an activation domain in vivo. The carboxyl terminus could be inert or could fulfill a different function, such as being a repression domain or a regulatory region. Examination of pharyngeally-expressed reporters revealed apparent normal expression levels and onset of expression in *smg-3; pha-4* mutant animals, suggesting the role of the carboxyl terminus may be relatively subtle (data not shown). We note that the carboxyl terminus may be important for regulating the accumulation of PHA-4 in the nucleus since we observed higher than normal PHA-4 levels when NMD was inactivated in *smg-1(ts)* mutants.

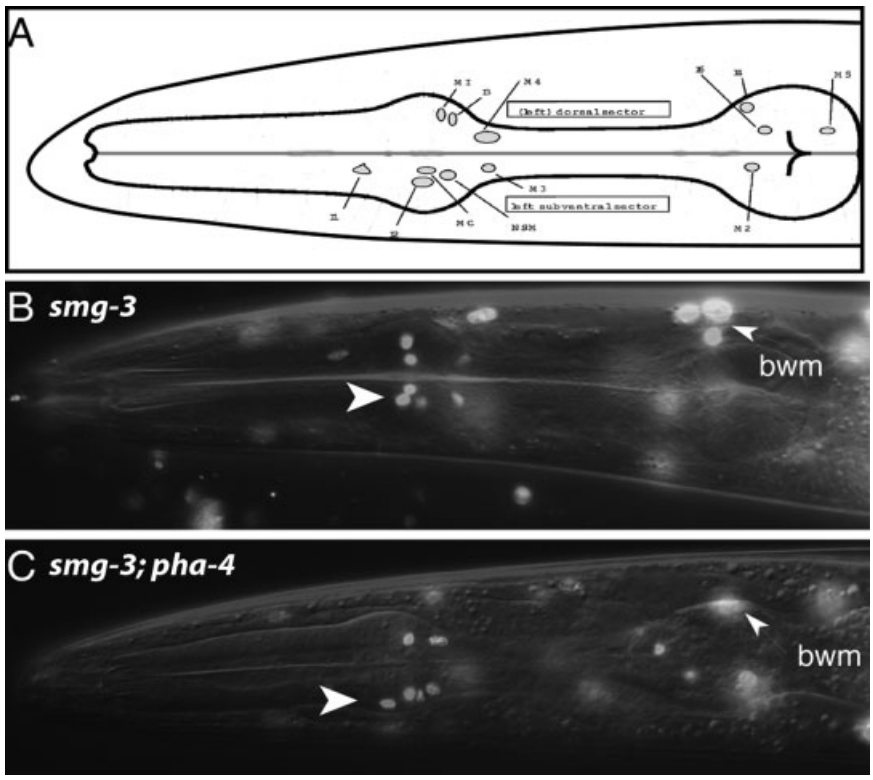

Fig. 5. Mispositioning of pharyngeal neurons in *smg-3; pha-4* mutants. **A:** Diagram of neuronal nuclei in the pharynx. Adapted from a drawing by R. Ellis. I1, I2, I3, pharyngeal interneurons; M3, M4, NSM, MC, pharyngeal motor neurons. See Albertson and Thomson (1976) for a description of pharyngeal cells. GFP in *smg-3* (**B**) or *smg-3; pha-4(zu225)* (**C**) L1 larvae. Pharyngeal neurons visualized with *myo-3::pha-4 ivs1::gfp* (arrowheads; see Experimental Procedures section). Body wall muscles: bwm, arrows.

The PHA-4 Amino Terminus

Given that the carboxyl terminus of PHA-4 was not essential, we searched for conserved regions in the amino terminus of PHA-4. PHA-4 does not share extensive sequence identity with vertebrate FoxA family members in the amino terminal region. Interestingly, sequence analysis shows that the amino terminus of FoxA1 and FoxA2 resembles Axial/FoxA2 in zebrafish (36 and 60% similarity, respectively), whereas FoxA3 appears to have a more distinct amino terminus (25% similar to FoxA1, FoxA2, and Axial). *Drosophila* Fork Head and PHA-4 amino terminal regions are not closely related to vertebrate Fox proteins or to each other. Nevertheless, PHA-4 does contain a short sequence (SPLG) that exists in Fork Head and FoxA2 (Fig. 1D). Intriguingly, SPLG is changed to SPFG by the *pha-4(q400)* mutation. We suggest that the amino terminal region defined by *pha-4(q400)* is critical for function.

pha-4(q400) codes for a stable protein since PHA-4(q400) can be detected in *smg-3; pha-4(400)/pha-4(q500)* transheterozygotes. This result resembles those of other studies that described stable proteins with L>F alterations (Labouesse et al., 1994; Eisenmann and Kim, 1997). For example, *let-60(ga89)/Ras* carries an L>F mutation that is associated with dominant negative phenotypes, and genetic evidence suggests LET-60(ga89) binds its regulators (Han and Sternberg, 1991). An L>F mutation in the transcription factor *lin-26, lin-26(n156)*, confers a weak loss-of-function phenotype and protein is detectable (Labouesse et al., 1996). We suggest that the defect in *pha-4(q400)* is caused by loss of activity not protein.

The lack of expression of PHA-4(q400) suggests that *pha-4* may autoregulate itself. PHA-4(q400) protein was not detected in *smg-3;pha-4(q400)*

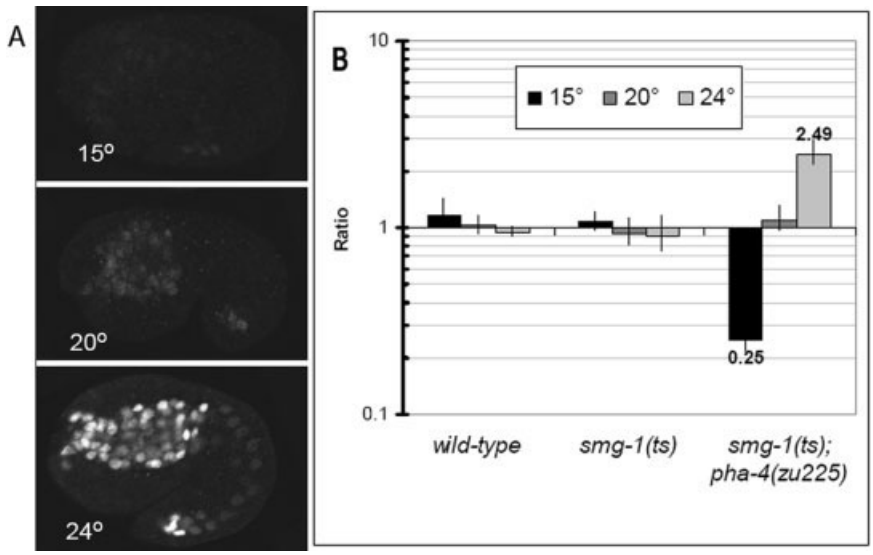

Fig. 6. The carboxyl terminus of PHA-4 regulates PHA-4 levels and activity. **A:** PHA-4 staining in *smg-1(cc546ts); pha-4(zu225)* at 15° (restrictive for *pha-4*), 20°, and 24°C (permissive for *pha-4*). PHA-4 intensity increases with temperature as SMG-1 is compromised. **B:** Normalized ratios of PHA-4 intensity in wild-type, *smg-1(cc456ts)*, and *smg-1(cc546ts); pha-4(zu225)* embryos at mid embryogenesis (1.5-fold stage). $P < 10^{-5}$ for *smg-1(cc546ts); pha-4(zu225)* at 15° and 24°C.

homozygotes, but was detected in *smg-3; pha-4(q400)/pha-4(q500)rol-9* transheterozygotes. This observation raises the intriguing possibility that the amino terminal region is required for positive feedback regulation, and that PHA-4(q500) provides that activity in the transheterozygote. The *pha-4* locus contains two high-affinity (TGTTTGC) and one lower-affinity (TCAAACA) PHA-4 binding sites within the large first intron (L.S.K and S.E.M, unpublished observation). These sites suggest that PHA-4 may directly activate its own transcription, and that activation requires the amino terminus of PHA-4.

EXPERIMENTAL PROCEDURES

Genetics and Strains

C. elegans var. Bristol was maintained at 20°C according to Brenner (1974). All *pha-4* alleles were described previously (Mango et al., 1994; Horner et al., 1998) except for *zu225*, which was a kind gift of J. Priess. Mutations used were: linkage group III (LG III) *smg-3(r867)*, *LG V: unc-51(e1189), fog-2(q71), stu-3(q265), pha-4(n2498), pha-4(q487), pha-4(q500), pha-4(zu225)*, and *rol-9(sc148)*.

Injections

Unless otherwise stated, all injections were performed with 20 ng/μl EcoRI-digested pRF4 (Mello et al., 1991), 5 ng/μl linearized reporter plasmid, and sheared herring sperm DNA to a total of 100 ng/μl.

Characterization of *smg-3; pha-4* Phenotypes

For Table 1, single worms were picked to individual plates, allowed to lay eggs for 24 h, then removed. Plates were incubated another 24 h and dead embryos and sick L1 larvae counted. Larvae that could not be unambiguously categorized were left on the plate for another 24 h. Larvae were considered to be "sick" if they were not moving, S-shaped, pale, and small. A sample of animals was mounted for differential interference contrast microscopy to confirm their phenotype. For Figure 5, adult worms were mounted without anesthetic and examined under the light microscope.

Immunostaining

Embryos were stained as described previously (Horner et al., 1998; Kaltenbach et al., 2000). Antibodies used were 3NB12 [pharyngeal muscle (Priess and Thomson, 1987) at 1:20] and α-carboxyl PHA-4 [all pharyngeal cells (Horner et al., 1998) at 1:20]. GST-tagged ATG3-PHA-4 was used to generate affinity-purified rabbit pan-PHA-4 polyclonal antibodies by Covance and used at a dilution of 1:2,000.

Pump Rates

Pharynx pumping was recorded with a Sony miniDV handycam and a dissecting microscope at 5x magnification. Images were projected with Final Cut Pro and analyzed in slow motion (for wild type, *smg-3* and healthy *smg-3; pha-4(q500) rol-9*) or normal speed for sickly *smg-3; pha-4(q500)rol-9)*. For each strain, at least two independent animals were counted for two 1-min intervals.

Quantitation of Nuclear PHA-4

In situ antibody staining was performed as described in (Horner et al., 1998). Single layer confocal images were taken using a Ziess LSM510. Intensity of PHA-4 staining was quantified in four nuclei of ten 1.5-fold embryos using ImageJ. The intensity of PHA-4, after background subtraction, was normalized to the intensity of PHA-4 in ten *par-1* mutant embryos (Gaudet and Mango, 2002) on the same slide.

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