**Regulation of Organogenesis by the Caenorhabditis elegans FoxA Protein PHA-4**

**J. Gaudet and S. E. Mango***

The *pha-4* locus encodes a forkhead box A (FoxA/HNF3) transcription factor homolog that specifies organ identity for *Caenorhabditis elegans* pharyngeal cells. We used microarrays to identify pharyngeal genes and analyzed those genes to determine which were direct PHA-4 targets. Our data suggest that PHA-4 directly activates most or all pharyngeal genes. Furthermore, the relative affinity of PHA-4 for different TRTTKRY (R = A/G, K = T/G, Y = T/C) elements modulates the onset of gene expression, providing a mechanism to activate pharyngeal genes at different developmental stages. We suggest that direct transcriptional regulation of entire gene networks may be a common feature of organ identity genes.

Cells acquire distinct fates during development in response to a host of regulators, many of which encode transcription factors. A long-standing issue has been the nature of the target genes controlled by these transcriptional regulators: Do they activate a few genes at the top of a hierarchy, or do they activate many genes that function at multiple stages throughout development? This question has been difficult to resolve because identification of downstream genes has relied largely on genetic approaches, which demand easily observed phenotypes. We used a genomic approach to investigate how the predicted transcription factor PHA-4 promotes development of an organ, the *Caenorhabditis elegans* pharynx.

The *C. elegans* pharynx is a neuromuscular organ that pumps food in from the environment and initiates digestion. The precursors of the pharynx are generated by distinct genetic programs and cell lineages and are assembled into a 92-cell primordium that undergoes complex patterning and morphogenesis (1–3). Ultimately, the precursors acquire different fates within the pharynx, including muscles, neurons, glands, structural cells, valves, and epithelia (4, 5).

The *pha-4* loss-of-function phenotype and the expression pattern of *pha-4* reveal that all cells of the pharynx, regardless of their distinct ancestry or ultimate cell fate, share a common pharyngeal identity that is specified by *pha-4* (6–8). *pha-4* encodes a protein that most closely resembles forkhead box A (FoxA) transcription factors (6, 8), which have been implicated in gut development in other organisms (e.g., *Drosophila* Forkhead and mammalian FoxA2) (9). Few target genes are known for FoxA members. For example, the only identified direct target of PHA-4 is the pharyngeal myosin gene myo-2, indicating that PHA-4 can function late in development (8). We identified and analyzed PHA-4 targets to study how FoxA proteins function during organogenesis.

**Identification of pharyngeal genes.** To identify pharyngeally expressed genes, we probed *C. elegans* microarrays (10) with labeled complementary DNA from embryos that produced either excess pharyngeal cells (*par-1* mutants) (11) or no pharyngeal cells (*skn-1* mutants) (12). We performed three independent experiments with microarrays that contained 62% of the *C. elegans* genome (11,917 out of 19,099 predicted genes) and identified 240 candidate pharyngeal genes with an average *par-1/skn-1* ratio ≥2.0 (13).

Three lines of evidence indicate that our microarray experiments selectively identified pharyngeally expressed genes. First, the microarrays contained 18 genes previously shown to be expressed in the embryonic pharynx. We identified 15 of these genes (13), including genes expressed at low levels (e.g., *eh-22*) (14). Second, a search of messenger RNA (mRNA) in situ hybridization data demonstrated that 59 out of 70 of our positives were pharyngeally expressed (15). Third, we constructed green fluorescent protein (GFP) reporters for 10 randomly chosen microarray positives and found that 8 out of 10 were expressed predominantly or exclusively in the pharynx (13). In total, the expression patterns of 96 of our microarray positives were characterized here or elsewhere, and 82% (79 out of 96) were selectively expressed in the pharynx (13).

**Pharyngeal expression depends on predicted PHA-4 binding sites.** To determine whether pharyngeally expressed genes were direct PHA-4 targets, we searched for predicted PHA-4 binding sites within our microarray positive reporters. The consensus binding site for PHA-4 orthologs is TRTTKRY (R = A/G, K = T/G, Y = T/C) (16), and PHA-4 binds a TRTTKRY element in the promoter of *myo-2*, the only known direct target of PHA-4 (8). All eight of our pharyngeally expressed reporters carried three or more TRTTKRY elements within their likely regulatory elements, and two of our nonpharyngeal reporters had one or no element (13).

Pharynx-specific expression of eight out of eight reporter constructs depended on TRTTKRY elements (Fig. 1A). For every promoter analyzed, elimination of one or more TRTTKRY elements resulted in either a complete loss of pharyngeal GFP (Fig. 1A, i through vi) or a substantial decrease in expression and a delay in the observed onset of expression (Fig. 1A, vii and viii). Moreover, pharyngeal expression of each of three additional genes [avr-15 (17), H30A04.1/cat-20 (18), and *peb-1* (19)] was dependent on an intron containing two or more TRTTKRY elements (13). We also constructed GFP reporters for one additional microarray positive and one gene with a borderline *par-1/skn-1* ratio (=1.9), neither of which contained any TRTTKRY elements in its upstream region. Neither reporter was expressed in the pharynx (13).

Our findings suggest that expression in the pharynx is critically dependent on TRTTKRY elements for genes expressed either early (e.g., *M05B5.2*) or later (e.g., *ZK816.4*) in pharynx development. Because our reporter constructs relied on randomly selected genes, our findings suggest that many, perhaps all, genes selectively expressed in the pharynx depend on TRTTKRY elements.

**PHA-4 functions during early and late pharyngeal development.** Based on the known expression patterns of *C. elegans* Fox genes, PHA-4 is the best candidate to recognize the pharyngeal TRTTKRY elements. Previous studies showed that *pha-4* functions early in embryogenesis (7). If PHA-4 directly activates most or all pharyngeal genes, then PHA-4 should also be required during later pharynx development. To test this idea, we engineered a temperature-sensitive configuration of *pha-4* [pha-4(53)] and shifted these animals from permissive to restrictive temperatures at different developmental time points (13).

We found that *pha-4* activity was required throughout development for viability (Fig. 2A). Shifting *pha-4(53)* to a restrictive temperature any time after the formation of pharyngeal precursors, but before the pharynx was fully developed (early and late embryos), resulted in 100% lethality. Late embryos shifted to restrictive temperatures arrested as first stage (L1) larvae.
with a “stuffed” pharynx phenotype (20) that resembled that of animals with defects in pharyngeal pumping (21). We also observed substantial lethality among pha-4(ts) animals shifted as L1’s, after pharynx development was complete (Fig. 2A). Adults that developed from these L1’s were small and unhealthy, consistent with a feeding defect (20). These data demonstrate that pha-4 activity is required during both early and late times in embryonic development.

To characterize the temperature-sensitive phenotype further, we analyzed expression of three of our reporters. For D2096.6 and C44H4.1 reporters, a reduction in PHA-4 activity in the mature pharynx resulted in a reduction of pharyngeal GFP. Conversely, restoration of PHA-4 activity was sufficient to reactivate pharyngeal expression (Fig. 2, B through H). This effect was specific because a reporter expressed in nonpharyngeal cells (coelomocytes) showed no change in expression when animals were shifted to a restrictive temperature.

The third pharynx reporter, myo-2::GFP, showed no appreciable decrease in expression. myo-2 expression is probably maintained by the combined actions of other transcription factors, including CEH-22 and PEB-1 (14, 19). This hypothesis is consistent with our finding that the PHA-4 site of myo-2 is important for initial expression of a myo-2 reporter but not for later expression (Fig. 1A).

**PHA-4 binds TRTTKRY elements in vitro.** A second line of evidence that indicates that TTTKRY elements are recognized by PHA-4 is that PHA-4 binds this consensus in vitro. We tested the ability of four different TRTTKRY sequences to compete against a labeled TGTTTGC-bearing probe for binding to PHA-4 in electrophoretic mobility shift assays (EMSAs) (8, 13). The TGTTTGC-bearing sequence represented a characterized PHA-4 site from the myo-2 promoter (myo-2-WT) (8). We tested three more sites identified in our reporter assays: TGGTTGAC from M05B5.2 (myo-2-M), TGGTTTG from D2096.6 (myo-2-D), and TGGTTTGT from ceh-22 (myo-2-ceh). These sequences were placed within the context of myo-2–wild-type (myo-2-WT) flanking sequences to minimize any effects of sequences outside the TTTKRY core. Each of these TTTKRY sequences competed specifically against myo-2-WT compared to a nonspecific competitor (myo-2-non, TGAACAG) (8) (Fig. 3A).

Examination of in vitro binding curves revealed that PHA-4 bound different TTTKRY sequences with different affinities. Quantitative analyses demonstrated that myo-2-ceh had four times less affinity for PHA-4 than did myo-2-WT (paired t test of the log of the median inhibitory concentration [IC50], $P = 0.023$) (Fig. 3C), whereas the myo-2-WT, myo-2-M, and myo-2-D sites had comparable affinities. We observed differences between the myo-2-WT site (TGGTTTG) and the D site (TGGTTTGT) when we used endogenous flanking sequence from D2096.6 rather than myo-2. We performed EMSA with labeled myo-2-WT, as above, and two cold competitors: D2096-WT (wild-type D2096.6 sequence, which includes TGGTTTG) and D2096-mut (D2096.6 sequence mutated to include TGGTTTG) (Fig. 3B). In the context of endogenous D2096.6 promoter sequence, we observed that D2096-mut had a twofold higher affinity than D2096-WT (Fig. 3D) (paired t test of log [IC50], $P = 0.015$). Our ability to detect this difference in the context of D2096.6, but not in the myo-2 sequence, suggests that sequences flanking TTTKRY influence the binding affinity of PHA-4.

**Onset of expression is modulated by altering the affinity of TTTKRY elements for PHA-4.** The affinity of TTTKRY elements for PHA-4 influences the onset of expression of six out of six GFP reporters in a manner consistent with the in vitro binding data (Fig. 4A). We generated “up” mutations (from a low- to high-affinity site) and “down” mutations in TTTKRY elements of our GFP reporters. Predicted down mutations in M05B5.2 or T05E11.3 promoters (from TGGTTGAC or TGGTTTG to TGGTTTTG, respectively) resulted in a delay of 2 to 3 hours in the onset of pharyngeal expression as compared to unaltered reporters (Fig. 4, A through E). Conversely, when predicted weaker elements were mutated to TGGTTTG, we noticed a shift of 1 to 3 hours in the onset of expression to earlier stages (Fig. 4A and F through I) (22). Despite differences in the onset of expression, alterations in the sequence of TTTKRY elements did not affect the strength of GFP fluorescence in later stages.

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**Fig. 1. Pharyngeal expression requires TTTKRY elements.** (A) Diagrams of promoter fragments used to drive expression of a GFP::His2B reporter (13). Triangles represent TTTKRY elements on the top or bottom strand, and X’s indicate eliminated TTTKRY elements. Numbers indicate the 5’ end relative to the predicted start codon of each gene. “Pharynx expression” is the relative strength of pharyngeal GFP at its peak in embryogenesis, assessed visually. For M05B5.2 and myo-2, notes in parentheses indicate the relative onset of GFP expression (22). ceh-22, myo-2, and tph-1 are known to be expressed in the pharynx (14, 32–34). (B) Through (F) Embryos and larvae expressing GFP. Anterior is left, and dorsal is up. Brackets indicate pharyngeal cells. (B) Pharyngeal expression of the −196 T05E11.3::GFP reporter with a wild-type predicted PHA-4 site [TGTTTGC; shown in (Ai)]. (C) GFP expression of the −196 T05E11.3::GFP reporter with a disrupted site [TGATGCC; shown in (Ai)]. (D) Pharyngeal expression of the −535 D2096.6::GFP reporter containing the wild-type predicted PHA-4 site [TGTTTGT; shown in (Aii)]. (E) GFP expression of the −535 D2096.6::GFP reporter with a disrupted site [TCCGCCG; shown in (Aii)]. Faint GFP fluorescence is detected in epidermal and gut (arrowhead) cells.
If the affinity of PHA-4 for TRTTKRY elements regulates onset of expression, then a decrease in PHA-4 levels should mimic a down mutation of a PHA-4 binding site. We tested this prediction with the early-expressed, panpharyngeal M05B5.2 reporter in our pha-4(ts) strain (15). In animals grown at an intermediate temperature (20°C), the pharynx was formed, but was often disorganized, suggesting that PHA-4 was present at insufficient levels to promote proper pharynx development (20). Under these conditions, expression of the M05B5.2 reporter was delayed as compared to the same strain grown at a permissive temperature (Fig. 5, A through D). Eventually, expression of this reporter reached a level equivalent to that of unshifted controls. This result is consistent with our model and suggests that PHA-4 is critical for controlling the onset of pharyngeal gene expression.

The effect of affinity on the onset of pharyngeal expression may be generally true. An analysis of the promoters of our microarray positives revealed a striking correlation between the rank of positive genes (according to their differential expression in microarray experiments) and the probability of a gene having a high-affinity PHA-4 binding site in its promoter (Fig. 5E). Genes that were highly ranked were more likely to contain a moderate- to high-affinity site than were lower ranked genes (χ² goodness-of-fit, P = 0.07). Conversely, lower ranked genes were slightly more likely to contain only a low-affinity PHA-4 site. We suggest that this correlation was revealed because we isolated RNA from embryos at mid-stages of pharyngeal development, when the abundance of a given mRNA reflected the onset of its expression.

**Affinity model for pharyngeal expression.** We propose that (i) PHA-4 directly regulates a wide array of genes expressed at multiple stages of pharyngeal development, and (ii) the affinity of TRTTKRY elements for PHA-4 regulates the relative onset of the expression of target genes. Two observations suggest that temporal regulation by PHA-4 is important in vivo. First, mutations that alter the affinity of TRTTKRY elements shift the onset of GFP expression by 1 to 3 hours, which is a substantial effect given the rapid development of *C. elegans* embryos (<14 hours). Second, using our pha-4(ts) strain, we observed a concordance between the temperature at which we first observed lethality and the temperature at which we first detected delayed GFP expression (Fig. 5) (20).

We propose that high-affinity sites bind PHA-4 in early embryos whereas low-affinity sites may not bind until later, after PHA-4 levels increase. Consistent with this idea, PHA-4 is expressed at low levels at the ~50-cell stage of embryogenesis and increases as development progresses (6).

Moreover, 15 out of 23 genes expressed during early pharyngeal development (as determined by mRNA in situ hybridization (15) or our reporters) carry high-affinity sites, and the remaining 8 genes carry sites whose affinity has not been tested. This affinity model is reminiscent of the threshold response of genes to a gradient of bicoid along the anterior-posterior axis or dorsal activity along the dorsoventral axis of the *Drosophila* embryo (23, 24). PHA-4 target genes may demonstrate temporally, rather than spatially, restricted threshold responses so that genes required for early aspects of pharynx development (e.g., regulators) are expressed before those required for later aspects of development (e.g., differentiation markers).

The affinity of a PHA-4 binding site does not correlate with the absolute onset of pharyngeal gene expression. The most striking example of this discrepancy is the different behavior of T05E11.3 and myo-2 reporters. Both genes depend on a TGTTTGC site for expression in the pharynx, but T05E11.3 expression is first visible in early embryos, and myo-2 expression is not visible until ~3 to 4 hours later (Fig. 4). These data suggest that a high-affinity PHA-4 site may be necessary but not sufficient for early activation of transcription. In some cases, the availability of other activators may determine the onset of expression. For example,
Implications for other developmental regulators. FoxA proteins in many species may directly regulate most or all genes within an organ. Both mammalian and Drosophila orthologs of PHA-4 are required for early aspects of digestive tract formation and also directly activate late-acting genes (16, 25–27). Moreover, heterozygous Foxa/+ mice have numerous abnormalities, demonstrating that vertebrae are sensitive to the dosage of FoxA proteins, consistent with the model presented here (28, 29). More generally, direct regulation of an entire genetic network may be a hallmark of organ identity factors. The selector gene SCALLOPED directly activates genes that function at two distinct steps of the regulatory hierarchy leading to wing formation in Drosophila (30). Similarly, the paired-type homeodomain factor Pax6/Clonyless is both necessary and sufficient for specification of eye formation in Drosophila, where it directly activates the expression of both early- and late-acting genes (31). These observations suggest that organ identity factors may use a strategy similar to that of PHA-4 to establish gene expression during organogenesis.

Fig. 3. PHA-4 binds to different TRT TKRY elements with different affinities. (A and B) EMSA with radiolabeled duplex (myo2-WT) and varying amounts of cold competitor duplex. F, free probe; M, probe + reticulocyte lysate; and P, probe + PHA-4. (A) Amounts of cold competitor were, from left to right: 10×, 100×, 300×, 600×, 1000×, and 3000× molar excess relative to the probe. Cold competitor duplexes differ from myo2-WT only in the PHA-4 site, as indicated. Experiments shown were performed using the same reagents to allow a comparison of relative binding affinities. (B) Amounts of cold competitor were, from left to right: 100×, 300×, 1000×, and 3000× molar excess relative to the probe. (C and D) Quantitation of competition reactions provided estimates of the IC50. IC50 values are expressed as fold excess of competitor and are inversely proportional to binding affinity (35). (C and D) IC50 values from three independent replicates of the experiments in (A and B), respectively.

Fig. 4. The onset of expression in the pharynx can be altered by changes in the affinity of TRT TKRY elements for PHA-4. (A) Diagrams of promoter fragments used to drive expression of a GFP::HIS2B reporter. Vertical lines represent TRT TKRY elements that were not mutated in these experiments or whose relative affinity for PHA-4 has not been determined in vitro. Triangles represent TRT TKRY elements mutated in these experiments: black, gray, and open triangles represent high-, TGT TGTGC, medium-, TGT T TGT, and low- (TATT TGT) affinity PHA-4 binding sequences, respectively. "Onset" refers to the developmental stage at which expression of GFP in the pharynx is first detected (22). (B through M) Embryos expressing GFP; brackets indicate the developing region of the embryo. (B through D) Pharyngeal expression of the −196 T05E11.3::GFP reporter containing the wild-type predicted PHA-4 site [TGT TGTGC; shown in (A)ii]. (E through G) Pharyngeal expression of the −196 T05E11.3::GFP reporter containing a mutated site [TGT TGT; shown in (A)ii]. (H through J) Pharyngeal expression of the −535 D2096.6::GFP reporter containing the wild-type predicted PHA-4 site [TGT TGTGC; shown in (A)ii]. (K through M) Expression of the −535 D2096.6::GFP reporter containing a mutated site [TGT T TGTGC; shown in (A)ii].

Fig. 5. Reduction of pha-4 activity reduces early pharyngeal expression. (A through D) Expression of the −521 M05B5.2::GFP::HIS2B reporter in pha-4(ts) embryos incubated at permissive [24° (A) and (B)] and intermediate temperatures [20° (C) and (D)]. Brackets indicate the location of the developing pharynx. (E) Frequency of TRT TKRY elements in the upstream regions of microarray positives. Genes are ranked according to their differential expression in our microarray experiment; 1 is the highest rank (par-1/skn-1 = 25.0), and 240 is the lowest (par-1/skn-1 = 2.0). Bars show the percentage of genes in each rank group with one or more TRT TKRY elements. Black bars represent the frequency of genes with high- and moderate-affinity sites (defined by in vivo and in vitro experiments as TGT TGTGC, TGT T TGT, and TATT TGT). Shaded bars represent the frequency of genes containing only a low-affinity PHA-4 site (defined by in vivo and in vitro experiments as TATT TGT).
Tunneling Spectroscopy of the Elementary Excitations in a One-Dimensional Wire

O. M. Auslaender,1* A. Yacoby,1 R. de Picciotto,2 K. W. Baldwin,2 L. N. Pfeiffer,2 K. W. West2

The collective excitation spectrum of interacting electrons in one dimension has been measured by controlling the energy and momentum of electrons tunneling between two closely spaced, parallel quantum wires in a GaAs/AlGaAs heterostructure while measuring the resulting conductance. The excitation spectrum deviates from the noninteracting spectrum, attesting to the importance of Coulomb interactions. An observed 30% enhancement of the excitation velocity relative to noninteracting electrons with the same density, a parameter determined experimentally, is consistent with theories on interacting electrons in one dimension. In short wires, 6 and 2 micrometers long, finite size effects, resulting from the breaking of translational invariance, are observed.

Electronic systems such as metals contain a vast number of mobile electrons. Surprisingly, despite the Coulomb repulsion between them, many electronic properties of metals can be well described in terms of independent particles with a finite lifetime, each carrying charge e and spin half \( \frac{1}{2} \). Most importantly, their excitation spectrum, which for non-interacting electrons is simply determined by the underlying band mass \( m \), is only slightly modified by the Coulomb interactions. However, such a simple description of electronic systems, known as Landau Fermi-liquid theory (1), is valid only in two and three spatial dimensions. In one-dimensional (1D) metals, where electrons are forced to move on a line, the single-particle description completely breaks down by even the slightest Coulomb repulsion. Instead, the excitations of an interacting 1D system, being fluctuations of the charge and spin densities, are well described by collective modes that involve the entire electron population (3). These collective modes are decoupled into two separate kinds: collective spin modes and collective charge modes. Coulomb interactions couple primarily to the latter, thereby strongly influencing their dispersion. Conversely, the excitation spectrum of the spin modes, typically unaffected by interactions, remains similar to the noninteracting case. For example, low-energy charge excitations propagate with velocity \( v_p \) = \( v_F \), where \( v_F = \hbar k_F/m \) is the Fermi velocity (\( \hbar \) is Planck’s constant, and \( k_F \) is the Fermi wave vector). The parameter \( \epsilon \), which characterizes the interactions, is given by \( g = \frac{1}{U(U/[2E_F])} \), where \( U \) is the Coulomb interaction energy between particles in the wire and \( E_F \) is the Fermi energy \( (4) \). Thus, the stronger the Coulomb repulsion is, the larger the propagation velocity is. Although interactions fundamentally alter the excitation spectrum of a 1D wire, they do not affect its conductance (5–10). In fact, a clean 1D wire has universal conductance irrespective of its length, density, and dispersion \( \epsilon \), rem-

1Department of Condensed Matter Physics, Weizmann Institute of Science, Rehovot 76100, Israel. 2Bell Labs, Lucent Technologies, 700 Mountain Avenue, Murray Hill, NJ 07974, USA.

*To whom correspondence should be addressed. E-mail: ophil.auslaender@weizmann.ac.il