The *Caenorhabditis elegans* NK-2 homeobox gene *ceh-22* activates pharyngeal muscle gene expression in combination with *pha-1* and is required for normal pharyngeal development

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SUMMARY

Pharyngeal muscle development in the nematode *Caenorhabditis elegans* appears to share similarities with cardiac muscle development in other species. We have previously described CEH-22, an NK-2 class homeodomain transcription factor similar to *Drosophila* tinman and vertebrate Nkx2-5, which is expressed exclusively in the pharyngeal muscles. In vitro, CEH-22 binds the enhancer from *myo-2*, a pharyngeal muscle-specific myosin heavy chain gene. In this paper, we examine the role CEH-22 plays in pharyngeal muscle development and gene activation by (a) ectopically expressing *ceh-22* in transgenic *C. elegans* and (b) examining the phenotype of a *ceh-22* loss-of-function mutant. These experiments indicate that CEH-22 is an activator of *myo-2* expression and that it is required for normal pharyngeal muscle development. However, *ceh-22* is necessary for neither formation of the pharyngeal muscles, nor for *myo-2* expression. Our data suggest parallel and potentially compensating pathways contribute to pharyngeal muscle differentiation. We also examine the relationship between *ceh-22* and the pharyngeal organ-specific differentiation gene *pha-1*. Mutations in *ceh-22* and *pha-1* have strongly synergistic effects on pharyngeal muscle gene expression; in addition, a *pha-1* mutation enhances the lethal phenotype caused by a mutation in *ceh-22*. Wild-type *pha-1* is not required for the onset of *ceh-22* expression but it appears necessary for maintained expression of *ceh-22*.

Key words: *ceh-22*, pharynx, myogenesis, *Caenorhabditis elegans*

INTRODUCTION

The NK-2 family is a phylogenetically conserved group of homeobox genes having diverse functions and expression patterns. This family was initially identified by Kim and Nirenberg (1989) in *Drosophila*. Additional family members have subsequently been cloned from a variety of vertebrate and invertebrate species (reviewed in Harvey, 1996). NK-2 family members are best distinguished by the presence of a tyrosine at homeodomain position 54 and, in some cases, islands of conserved sequence outside of the homeodomain (Harvey, 1996).

Two NK-2 homeobox genes, *tinman* (*tin*) and Nkx2-5, have been shown to be important regulators of heart development in insects and vertebrates, respectively. The *Drosophila* gene *tin* is expressed in heart precursors and *tin* mutants completely lack a heart (Bodmer et al., 1990; Azpiazu and Frasch, 1993; Bodmer, 1993). Likewise the vertebrate Nkx2-5 genes are expressed in early cardiac progenitors (Lints et al., 1993; Tonissen et al., 1994; Schultheiss et al., 1995; Chen and Fishman, 1996; Shiojima et al., 1996) and Nkx2-5 mutant mice have defects in heart morphogenesis (Lyons et al., 1995). The *tin* and Nkx2-5 mutant phenotypes indicate NK-2 homeobox genes are critical for heart development in widely diverged phyla and suggest these genes function in a conserved mechanism. The functions of *tin* and Nkx2-5 in heart development are of particular interest since little is known of the mechanisms controlling cardiac myogenesis (recently reviewed in Mably and Liew, 1996).

Several lines of evidence suggest the contractile function and development of nematode pharyngeal muscle is similar to cardiac muscle in other species. The *Caenorhabditis elegans* pharynx is a rhythmically contracting organ. Like vertebrate cardiac muscle, pharyngeal muscle contraction does not require nervous system input (Avery and Horvitz, 1989), nor does pharyngeal muscle development involve the MyoD family of myogenic regulatory factors (Krause et al., 1990; Chen et al., 1994). More recently, we have described the NK-2 homeobox gene *ceh-22*, which is expressed exclusively in *C. elegans* pharyngeal muscles (Okkema and Fire, 1994). The CEH-22 homeodomain shares 67% identity to that of mouse Nkx2-5 and we suggest its function may be conserved with Nkx2-5 and *tin*.

CEH-22 was initially identified in a screen for factors controlling expression of the pharyngeal muscle-specific myosin gene, *myo-2* (Okkema and Fire, 1994). *myo-2* expression is
regulated by a combination of cell-type-specific and organ-specific signals that target distinct regulatory sequences within the myo-2 enhancer, termed the B and C subelements. The organ-specific C subelement enhances gene expression in all pharyngeal cell types, muscle and non-muscle, whereas the cell-type-specific B subelement functions exclusively in pharyngeal muscle. CEH-22 binds the B subelement and it is expressed only in the pharyngeal muscles where B activates transcription. The DNA-binding specificity and expression pattern of CEH-22 are consistent with the possibility that ceh-22 is a key component of a pathway activating cell-type-specific gene expression in pharyngeal muscle.

Two additional loci affecting gene expression in the pharyngeal muscles have also been identified. Mutations in the genes pha-1 and pha-4 interfere with differentiation of all pharyngeal cell types, including muscle (Schnabel and Schnabel, 1990; Mango et al., 1994). pha-4 mutations block pharyngeal development at an early step, prior to formation of the pharyngeal primordium, and they eliminate expression of ceh-22 (Mango et al., 1994; E. Ha and P. Okkema, unpublished). pha-1 mutations block pharyngeal differentiation later, after formation of the primordium, but the mechanism by which pha-1 promotes pharyngeal muscle gene expression and its relationship to ceh-22 are unknown. pha-1 shares weak similarity with basic leucine zipper transcription factors (Granato et al., 1994).

In this paper, we have examined the role ceh-22 plays in pharyngeal muscle gene expression by characterizing the effect of ectopically expressing ceh-22 and by characterizing a ceh-22 loss-of-function mutant. We have also demonstrated that ceh-22 and pha-1 function in closely related processes to promote pharyngeal muscle gene expression and that, although pha-1 is not required for the onset of ceh-22 gene expression, it does function to maintain ceh-22 expression.

**MATERIALS AND METHODS**

**Plasmids and general methods for nucleic acid manipulation**

Standard methods were used to manipulate plasmids DNAs and oligonucleotides (Ausubel et al., 1990). To express ceh-22 in body wall muscle, plasmid pOK48.25 was constructed by ligating the unc-54 promoter from pPD30.38 (Mello and Fire, 1995) to a ceh-22 cDNA at an XhoI site within the ceh-22 5′-UTR (Okkema and Fire, 1994). The control plasmid pOK49.31, containing the unc-54::veh-22(ΔHD), is identical to pOK48.25, except that it contains an in-frame deletion removing CEH-22 amino acids 217-264. The plasmid pCW2.1 containing a ceh-22::gfp reporter was constructed by substituting the gfp coding sequence from pPD95.75 (A. Fire, G. Seydoux, J. Ahnn and S. Xu, unpublished) for the lacZ coding sequence from the previously characterized ceh-22::lacZ reporter pOK29.02 (Okkema and Fire, 1994). Sequences of plasmid constructs are available from the authors.

**Mapping ceh-22**

From physical map data, ceh-22 was placed on chromosome V in the interval between her-1 and the act-1,2,3 gene cluster (Okkema and Fire, 1994). To more precisely map ceh-22, embryos homozygous for chromosomal deficiencies were subjected to polymerase chain reaction (PCR) using ceh-22-specific primers according to Chen et al. (1992); primers from the unlinked myo-2 gene were included as a positive control for successful PCR. Embryos homozygous for deficiencies were isolated as described by Ahnn and Fire (1994) from the following strains:

- BC1381dpv-1(e364)/eT1 III; sd92 unc-46(e177)/eT1 V
- BC251dpv-1(e364)/eT1 III; sd35 unc-60(e677) dpy-11(e224)/eT1 V
- BW163ctDfl V/DnT1 [unc-7(n754) let-2? (IV;V)]
- BC2617dpv-1(e364)/eT1 III; mDf1/eT1 V
- MT5813DnDf42/DnT1 [unc-7(n754) let-2? (IV;V)]

**Handling of nematodes and isolation of ceh-22 mutants**

Transgenic lines containing the plasmid pOK48.25 were isolated by microinjection with the co-transformation marker pRF4 (Mello et al., 1991). A transgenic line expressing ceh-22::gfp was obtained by co-injecting pCW2.1 with pRF4; the resulting extrachromosomal array was chromosomally integrated by gamma irradiation (Mello and Fire, 1995).

A strain containing the Tc1 insertion ceh-22(pk40) was kindly isolated from a frozen transposon insertion mutant bank by M. deVrooomen and R. Plasterk (Netherlands Cancer Institute) using nested primers near the 3′ end of ceh-22. A PCR-based sub-selection protocol (Plasterk, 1995) was used to isolate a single deletion allele of ceh-22, designated ce8266. This allele was out-crossed extensively to wild-type (N2) and the closely linked markers dpy-11(e224) and vab-8(e1017) were recombined on and off the ceh-22(cc8266) chromosome to eliminate linked mutations.

All heterotypic defects of ceh-22(cc8266) could be rescued by transformation with the plasmid pOK101.07. This plasmid contains a 7.2 kb fragment of ceh-22 genomic DNA (base-pairs 13403-20578 of the sequenced cosmid F29F11; Accession number, Z73974). ceh-22 is the only predicted gene completely contained within this fragment.

**Antibody staining and expression of lacZ reporters**

CEH-22 protein expression was examined in ceh-22(pk40) and ceh-22(cc8266) mutant embryos by staining with a rabbit polyclonal antibody (c87) raised against amino acids 79-346 of CEH-22 (Okkema and Fire, 1994) permeabilized embryos in the preparation were identified by double staining with the monoclonal antibody 5-6, which recognizes body wall muscle cells (Miller et al., 1986; Ardizzi and Epstein, 1987).

Expression of MYO-2 protein and 3NB12 antigen was examined by staining with the monoclonal antibodies 9.2.1 [kindly provided by D. M. Miller (Miller et al., 1986; Ardizzi and Epstein, 1987)] or 3NB12 [kindly provided by B. Bowerman (Okamoto and Thomson, 1985)] following whole-mount fixation of mixed-stage populations of nematodes (Miller and Shakes, 1995; Finney and Ruvkun, 1990). Primary antibodies were detected by immunofluorescence microscopy using fluorescein-conjugated goat anti-rabbit IgG (Cappel) and Texas red-conjugated goat-anti mouse IgG (Jackson Immuno Research) secondary antibodies.

To assay function of lacZ reporters, F1 expression assays were carried out as previously described (Okkema et al., 1993): plasmid DNAs were microinjected at 100 μg/ml into the germ line of adult hermaphrodites and F1 progeny stained for β-galactosidase activity as late larvae or adults (Fire, 1993). The enhancer assay constructs [pOK8.45 (B+B), pOK8.49 (C+C), and pOK3.21 (C)], myo-1::lacZ [pOK10.38], myo-2::lacZ [pPD20.97], and ceh-22::lacZ [pOK29.02] were previously described (Okkema et al., 1993; Okkema and Fire, 1994).

**RESULTS**

Ectopic expression of ceh-22 activates pharyngeal muscle-specific genes

In wild-type animals, CEH-22 is expressed exclusively in pharyngeal muscle (Okkema and Fire, 1994). This expression
Fig. 1. Ectopic expression of CEH-22 activates myo-2 expression. (A) Wild-type embryo (at the 1-fold stage) stained with the anti-CEH-22 antibody c187 (Okkema and Fire, 1994). CEH-22 accumulation is restricted to the pharyngeal muscle nuclei. (B) Transgenic embryo containing unc-54::ceh-22 at a similar stage to A, stained with c187. Groups of body wall muscle nuclei containing CEH-22 are marked; CEH-22 expression in the pharynx can be seen in a lower focal plane. (C) Adult worms from a transgenic strain containing unc-54::ceh-22(AHD) stained with the anti-MYO-2 antibody 9.2.1 (Miller et al., 1986). MYO-2 expression is only detected in the pharynx. (D) An adult from a transgenic strain containing unc-54::ceh-22 stained with 9.2.1. MYO-2 is detected in the pharynx and several body wall muscles. MYO-2 appears to be incorporated into myofilaments in body wall muscles in a pattern similar the UNC-54 myosin (Miller et al., 1983). This expression has no apparent deleterious effect on myofilament organization.

Table 1. Expression of pharyngeal muscle-specific markers in body wall muscles of C. elegans ectopically expressing wild-type or mutant CEH-22

<table>
<thead>
<tr>
<th>Pharyngeal muscle marker</th>
<th>Ectopic expression construct</th>
<th>unc-54::ceh-22</th>
<th>unc-54::ceh-22(AHD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>endogenous myo-2</td>
<td></td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>myo-2::lacZ</td>
<td></td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>myo-1::lacZ</td>
<td></td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>ceh-22::lacZ</td>
<td>−</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>3NB12 antigen</td>
<td>−</td>
<td>n.d.</td>
<td></td>
</tr>
</tbody>
</table>

induced in body wall muscle [as judged using a myo-1::lacZ fusion (Okkema et al., 1993)]. By contrast, two markers of earlier pharyngeal muscle differentiation, an antigen recognized by the monoclonal antibody 3NB12 (Priess and Thompson, 1987) and a ceh-22::lacZ fusion (Okkema and Fire, 1994), were not expressed. We conclude that ectopic CEH-22 is capable of inducing aspects of pharyngeal muscle differentiation in body wall muscle but that it does not result in a complete transformation to a pharyngeal muscle phenotype.

As a control, we tested whether CEH-22-mediated activation of pharyngeal muscle genes was dependent upon the ability of CEH-22 to bind DNA. We had previously shown that a CEH-22 deletion removing homeodomain helix 3 fails to bind DNA in vitro (Okkema and Fire, 1994). Expression of this protein in body wall muscle [using the construct unc-54::ceh-22(AHD)] did not induce either the myo-2::lacZ, myo-1::lacZ, or the endogenous myo-2 gene (Fig. 1C, Table 1).

ceh-22 is required for normal pharyngeal development

To understand the role played by ceh-22 in pharyngeal muscle development, we have isolated and characterized a ceh-22 mutant. ceh-22 was mapped to a small region on chromosome V between the endpoints of sDf35 and ctDf1 (see Materials and Methods). None of the previously reported mutations in the ceh-22 region result in pharyngeal defects.

To obtain a ceh-22 mutant, a reverse genetic approach was used (Plasterk, 1995). A strain containing a Tc1 transposon insertion into ceh-22 was kindly provided by M. deVroomen and R. Plasterk (Netherlands Cancer Institute). This insertion allele, ceh-22(pk40) (Fig. 2B), does not disrupt ceh-22 gene function: CEH-22 protein expression in ceh-22(pk40) homozygotes is indistinguishable from wild type (data not shown), and these animals have no apparent pharyngeal muscle defects. As has been seen with other exonic Tc1 insertions (Rushforth et al., 1993), the pk40 insertion is likely to be efficiently spliced from the ceh-22 pre-mRNA. For this reason, it is generally necessary to generate imprecise excision (deletion) alleles to study the loss-of-function phenotypes.

ceh-22(pk40) was used to generate a second allele, ceh-22(cc8266), which contains a 1248 bp deletion removing...
exon 2 and the 5' half of exon 3 (Fig. 2B; see Materials and Methods). Animals homozygous for this allele showed defects in pharyngeal muscle development and gene expression (see below). These animals failed to express detectable CEH-22 protein (0/27 permeabilized ceh-22(cc8266) homozygous embryos stain with polyclonal antibodies raised against CEH-22). Although no CEH-22 protein was detected, it should be stressed that the homeobox has not been deleted in the mutant and a truncated ceh-22 mRNA was detectable in homozygous embryos (data not shown). Based upon the structure of the intron/exon boundaries remaining in ceh-22(cc8266), the expected structure for this mRNA would contain exon 1 spliced directly to exon 4. In this case, the transcript would contain a frameshift mutation upstream of the homeobox and translation would not produce a functional DNA-binding protein. Nonetheless, we cannot be sure that ceh-22(cc8266) completely eliminates gene function.

ceh-22(cc8266) was outcrossed extensively and recombined with closely linked markers (see Materials and Methods), and the resulting mutant phenotype examined. ceh-22(cc8266) results in a recessive, partially penetrant larval lethal phenotype. Approximately 25% of ceh-22(cc8266) homozygotes arrested shortly after hatching as L1 larvae. These arrested larvae had thin pharynges displaying only very feeble muscle contractions and the basement membrane surrounding the pharynx appeared indistinct when viewed by Nomarski microscopy (Fig. 3B). It is likely these severely affected animals arrested because of an inability to feed. Interestingly, the strongest pharyngeal contractions in these mutant animals occurred in the posterior bulb of the pharynx in the region of the m6 muscles, a muscle cell type that does not normally express ceh-22 (Okkema and Fire, 1994).

The remaining ceh-22(cc8266) homozygotes grew slowly to adulthood but appeared thin and starved. In these ceh-22(cc8266) adults, the pharynx was misshapen and, in contrast to the thin pharynges found in arrested L1s, the isthmus of the pharynx was reproducibly thicker than in wild type (Fig. 3D). Approximately 50% of the ceh-22(cc8266)

Fig. 2. Genetic map and genomic organization of ceh-22 mutations. (A) Genetic map indicating the position of ceh-22 between the endpoints of deficiencies sDf35 and ctDf1 and various linked markers. (B) Genomic structure of ceh-22 mutations. Filled boxes represent coding exons; empty boxes are untranslated regions. The pk40 Tc1 insertion (hatched bar) is located in exon 3 after base-pair 2774 (as numbered in Okkema and Fire, 1994); ceh-22(cc8266) deletes base pairs 1543-2791.

Fig. 3. ceh-22(cc8266) mutants have morphological defects in the pharynx. Differential Interference Contrast micrographs of wild-type and ceh-22(cc8266) mutant pharynges: (A) wild-type L1; (B) arrested ceh-22(cc8266) L1; (C) wild-type L4; (D) ceh-22(cc8266) L4. Note the thicker and asymmetric appearance of the pharynx in D.
homoygotes reaching adulthood were sterile. In the most severe case, the gonad did not elongate and the number of germ cells appeared reduced (data not shown). It is not known whether ceh-22 is expressed in the larval gonad or germine; the basis for this sterility is unknown. The remaining cc8266 adults were fertile; a homozygous ceh-22(cc8266) strain could be maintained as a slowly growing stock. Both the pharyngeal and fertility defects were rescued by transformation with an 7.2 kb genomic DNA fragment containing ceh-22 (see Materials and Methods).

**CEH-22 is required for function of the myo-2 enhancer B subelement**

To understand the role ceh-22 plays in regulating genes in the pharynx, we examined expression of various reporter constructs and pharyngeal muscle-specific genes in ceh-22(cc8266) homozygotes. CEH-22 protein binds a pharyngeal muscle-specific regulatory element from the myo-2 enhancer termed the B subelement (Okkema and Fire, 1994). In wild type, an enhancer consisting of two copies of the B subelement (B+B enhancer) strongly activated pharyngeal muscle transcription (Table 2). 34 of 66 wild-type transformants expressed a B+B enhancer driven construct in pharyngeal muscle and most of these animals expressed the reporter in many pharyngeal muscle cells. In comparison, the B+B enhancer was almost completely inactive in ceh-22(cc8266). Only 1 of 26 F1 transformants expressed the reporter in pharyngeal muscle and this animal expressed the reporter in only a single pharyngeal muscle cell.

**Alternative mechanisms activate myo-2 expression in ceh-22 mutant animals**

In contrast to the reduced function of the B subelement, ceh-22(cc8266) animals exhibit an apparent increase in function of C subelement (Table 2), a distinct organ-specific regulatory element of the myo-2 enhancer believed to respond to signals controlling pharyngeal organogenesis (Okkema and Fire, 1994). This increase was most evident in reporter constructs with a single copy of the C subelement: in wild-type animals, a single copy of C was not sufficient for enhancer function; however, in ceh-22(cc8266) a single copy of C functioned as a pharyngeal enhancer. These results suggest either that wild-type ceh-22 negatively regulates C function or that ceh-22(cc8266), animals compensate for loss of B subelement function by hyperactivating the organ-specific pathway for pharyngeal muscle gene activation.

Despite the loss of B subelement function, expression of the endogenous myo-2 gene appeared normal in ceh-22(cc8266).

**Table 2. Activity of B and C subelements in wild type and ceh-22(cc8266)**

<table>
<thead>
<tr>
<th>Enhancer*</th>
<th>Wild type (N2) [n]</th>
<th>ceh-22(cc8266) [n]</th>
</tr>
</thead>
<tbody>
<tr>
<td>B+B</td>
<td>52% [66]</td>
<td>4% [26]</td>
</tr>
<tr>
<td>C+C</td>
<td>43% [95]</td>
<td>35% [20]</td>
</tr>
<tr>
<td>C</td>
<td>0% [178]</td>
<td>29% [17]</td>
</tr>
</tbody>
</table>

*Enhancers were assayed upstream of a myo-3::lacZ fusion as previously described (Okkema and Fire, 1994). The myo-3::lacZ is expressed in body wall muscle providing an internal control for the number of transformed animals; pharyngeal enhancer activity is reported as the percentage of total β-gal-positive transformants (n) that express β-gal in the pharynx.

No apparent alteration in the intensity or the timing of MYO-2 protein expression was observed when the mutant was stained with anti-MYO-2 antibodies. Abundant MYO-2 was detectable by the three-fold stage of development in both wild type and ceh-22(cc8266) (Fig. 4). Mutant animals had a subtle but reproducible disorganization in the pattern of MYO-2 staining in the pharyngeal muscles. This appearance could be due to either a small change in the timing or the level of myo-2 expression, or changes in expression of other ceh-22-regulated genes. Staining with the antibody 3NB12 (see below) and expression of a ceh-22::lacZ reporter (data not shown) likewise appeared normal in ceh-22(cc8266).

**Synergistic interaction between ceh-22 and pha-1**

Mutations in the pha-1 gene cause a block in differentiation of all pharyngeal cell types resulting in a late embryonic lethal phenotype (Schnabel and Schnabel, 1990). The pharyngeal primordium forms normally with a distinct basement membrane, but the primordium does not elongate and assume its normal morphology. Pharyngeal muscle expression of the antigen recognized by the antibody 3NB12, which in wild type is first expressed at the comma stage, is normal in pha-1 mutant pharyngeal muscle. However, expression of MYO-2, which in wild type is first detectable at the three-fold stage, is substantially reduced. The temperature-sensitive allele pha-1(e2123ts) undergoes normal pharyngeal development at 16°C, but has a strong pha-1 mutant phenotype when grown at 25°C.

To determine whether ceh-22 and pha-1 affect related processes in pharyngeal muscle differentiation, we examined the phenotype of a pha-1(e2123ts); ceh-22(cc8266) double mutant embryos. When grown at 25°C, these animals arrested with a terminal phenotype that appeared by initial observations to be an additive combination of the two single mutant phenotypes.
types: the pharyngeal primordium was clearly evident, but it did not elongate and the basement membrane surrounding this primordium had an abnormal appearance characteristic of ceh-22(cc8266).

When molecular markers of pharyngeal muscle differentiation were examined, however, we saw a strongly synergistic interaction between the pha-1 and ceh-22 mutations. The pharyngeal muscles of both pha-1(e2123ts) single mutants grown at 25°C and ceh-22(cc8266) single mutants stained very well with the antibody 3NB12 (Fig. 5; Schnabel and Schnabel, 1990; Granato et al., 1994). In contrast, terminally arrested pha-1(e2123ts); ceh-22(cc8266) double mutants raised at 25°C showed almost no 3NB12 staining in the pharynx (Fig. 5). myo-2 expression also appeared reduced in pha-1(e2123ts); ceh-22(cc8266) double mutants at 25°C showed almost no 3NB12 staining in the pharynx (Fig. 5). This reduction is somewhat more difficult to characterize because myo-2 expression is already substantially decreased in the pha-1 single mutant. In wild type, the 3NB12 antigen is expressed in all pharyngeal cell types (Okkema and Fire, 1994). Interestingly, in the pha-1(e2123ts); ceh-22(cc8266) double mutant, we often saw the greatest amount of myo-2 expression near the position of the m6 pharyngeal muscles, which normally do not express CEH-22.

We saw additional evidence for gene interaction when the double mutant was grown at the permissive temperature, where we observed that pha-1(e2123ts) enhanced the ceh-22 lethal phenotype (Table 3). When wild-type embryos were maintained at 16°C for 5 days, 100% of the animals reached late L4 or early adult stage. Both the ceh-22(cc8266) and pha-1(e2123ts) single mutants grew less vigorously than wild type, but 75% and 70% of these animals reached L4/adulthood in 5 days, respectively. In contrast the pha-1(e2123ts); ceh-22(cc8266) double mutant was almost completely inviable at 16°C; only 8% of the double mutants reached L4/adult. The majority of pha-1(e2123ts); ceh-22(cc8266) double mutants grown at 16°C arrest as L1s with a phenotype similar to severely affected ceh-22 single mutants.

Table 3. pha-1(e2123ts) enhances the ceh-22(cc8266) lethal phenotype

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% reaching L4/adult at 16°C*</th>
</tr>
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<tbody>
<tr>
<td>Wild type</td>
<td>100</td>
</tr>
<tr>
<td>pha-1(e2123ts)</td>
<td>70</td>
</tr>
<tr>
<td>ceh-22(cc8266)</td>
<td>75</td>
</tr>
<tr>
<td>pha-1(e2123ts); ceh-22(cc8266)</td>
<td>8</td>
</tr>
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</table>

*For each genotype, 40 embryos were picked to fresh plates at 16°C and the stage of development scored after 5 days.

pha-1 is not required for the onset of ceh-22 gene expression

To determine whether pha-1 is required for ceh-22 gene expression, we examined expression of a ceh-22::gfp reporter in wild-type animals and in pha-1(e2123ts) mutants grown at 16°C and 25°C. This reporter is expressed identically to the endogenous ceh-22 gene (Okkema and Fire, 1994; data not shown). Expression of the ceh-22::gfp initiated at the lima-bean stage of development in all cases, and it increased until approximately the two-fold stage (Fig. 6 and data not shown). In wild-type animals and pha-1(e2123ts) grown at 16°C, GFP expression remained high throughout the remainder of the life cycle. By contrast, in pha-1(e2123ts) grown at 25°C, ceh-22::gfp expression reached a peak at approximately the 1½-fold stage and decreased with only very weak GFP detected in occasional terminally arrested embryos (Table 4). This effect of pha-1(e2123) appears specific to the ceh-22::gfp fusion since pharyngeal expression of a C subelement driven gfp reporter is not reduced in terminally arrested pha-1 mutant animals (D. Moons and P. Okkema, unpublished). These results suggested that (1) pha-1 is not required for the onset of ceh-22 expression and (2) pha-1 appears to be required for maintaining ceh-22 expression. We observed similar effects of pha-1 using antibodies to examine expression of the endogenous CEH-22 protein and by examining ceh-22::gfp expression in animals homozygous for the chromosomal deficiency tDf2 that
ceh-22 activates pharyngeal muscle genes

**DISCUSSION**

cenh-22 can activate pharyngeal muscle-specific gene expression in vivo

The cenh-22 gene was identified in an expression screen for factors binding to the myo-2 enhancer (Okkema and Fire, 1994). Both the DNA-binding activity and the expression pattern of Cenh-22 suggested it was likely to be a key regulator of the B subelement of the myo-2 enhancer. Here we provide direct evidence that Cenh-22 can activate myo-2, as well as a second pharyngeal muscle-specific promoter from myo-1, when ectopically expressed in C. elegans body wall muscle. In contrast, ectopic Cenh-22 does not autoactivate its own expression, nor does it induce expression of the 3NB12 antigen. Thus Cenh-22 can activate multiple components of pharyngeal muscle differentiation, but it does not result in a complete transformation to pharyngeal muscle fate.

While Cenh-22 clearly can activate pharyngeal myosins in body wall muscle, it does not do so with complete efficiency.

Despite accumulating in embryonic body wall muscle nuclei, Cenh-22 does not activate myo-2 in these embryos. Therefore it seems likely that Cenh-22 requires interactions with additional factors for efficient activation of pharyngeal myosin expression. Similar conclusions have been drawn from studies of ectopic expression of tin and XNkx2-5 in Drosophila and Xenopus, respectively (Bodmer, 1993; Cleaver et al., 1996). The identity of the cooperating factors is unclear in either system at this point. We have shown previously that B subelement function requires a binding site for at least one factor in addition to Cenh-22 (Okkema and Fire, 1994). Similarly, mouse Nkx2-5 may collaborate with serum response factor to activate cardiac muscle gene expression (Chen et al., 1996).

**ceh-22 loss-of-function phenotype**

The cenh-22 loss-of-function phenotype confirms this gene plays a critical role in pharyngeal development. In severely affected cenh-22(cc8266) mutants, pharyngeal muscle cells form and differentiate; however, the pharyngeal contractions are weak and the pharynx appears thinner and less distinct than in wild type. These animals arrest shortly after hatching with an overall appearance similar to animals hatched in the absence of food. Individuals that develop beyond this L1 arrest can grow to slowly to adulthood; however, they have a thin, pale appearance characteristic of feeding defective mutants (Avery, 1993). The pharynges of cenh-22(cc8266) adults contract more vigorously than the arrested larvae and, in fact, appear thicker than in wild type. This apparent pharyngeal muscle hypertrophy may indicate that, in addition to its role in activating pharyngeal muscle gene expression, wild-type cenh-22 or its targets may limit pharyngeal muscle growth. It is interesting in this regard that we have observed that activity of the organ-specific C subelement is enhanced in cenh-22 mutants.

Transcriptional enhancement by the B subelement is eliminated in cenh-22(cc8266) mutants. However, the myo-2 gene is expressed similarly to wild type. These results suggest that neither cenh-22 nor B subelement function is required for myo-2 expression. Consistent with this observation, a disruption of the Cenh-22 binding site within the context of the intact myo-2

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Table 4. cenh-22::gfp expression is not maintained in terminally arrested pha-1(e2123ts) mutants

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Percentage of animals expressing strong GFP*</th>
</tr>
</thead>
<tbody>
<tr>
<td>16°C</td>
<td>62% (n=34)</td>
</tr>
<tr>
<td>25°C</td>
<td>0% (n=30)</td>
</tr>
</tbody>
</table>

*Embryos segregating from pha-1(e2123ts) vab-7(e1562); cuds1/+ were incubated for approximately 20 hours at 25°C, or 40 hours at 16°C. All animals were examined for GFP expression by fluorescence microscopy. cuds1 is a chromosomally integrated array containing the cenh-22::gfp reporter plasmid pCW21.†Four of these terminally arrested embryos displayed very weak GFP expression in the pharynx.
enhancer and promoter has no detectable effect on expression of a myo-2::lacZ reporter (Okkema and Fire, unpublished). Perhaps these results are not unexpected: the B subelement is one of several sequence elements activating myo-2 expression. The myo-2 enhancer consists of at least three distinct subelements, including B, that contribute to enhancer function (Okkema and Fire, 1994). In addition, myo-2 contains a separable pharyngeal muscle-specific promoter that is sufficient for low level, enhancer-independent gene expression (Okkema et al., 1993). In the absence of CEH-22 and B subelement function, these other regulatory sequences appear sufficient to activate myo-2 expression. The feeding-defective and lethal phenotypes of ceh-22(cc8266) might be due either to subtle defects in myo-2 expression, or to defects in expression of other CEH-22 regulated genes, or to both effects.

Is ceh-22(cc8266) a null allele? Traditional genetic tests to answer this question are difficult to apply, because no existing chromosomal deficiencies remove the gene. The deletion in ceh-22(cc8266) does not remove the ceh-22 promoter or homeobox, making it possible that a DNA-binding protein could be produced. ceh-22(cc8266) homozygotes produce a stable ceh-22 mRNA, which may be translated. However, molecular evidence suggests that ceh-22(cc8266) may result in a complete or nearly complete loss of ceh-22 function. Using polyclonal antibodies raised against CEH-22, we cannot detect CEH-22 accumulation in ceh-22(cc8266) mutants. In addition, transcriptional activation by the B subelement of the myo-2 enhancer appears completely eliminated in the mutant. Finally, we have not observed an earlier arrest or more severe pharyngeal defects in preliminary experiments disrupting ceh-22 gene expression. The feeding-defective and lethal phenotype of ceh-22(cc8266) might be due either to subtle defects in myo-2 expression, or to defects in expression of other CEH-22 regulated genes, or to both effects.

### Synthetic interactions between ceh-22 and pha-1

The pha-1 gene is required for terminal differentiation of all pharyngeal cell types (Schnabel and Schnabel, 1990). It is expressed transiently in pharyngeal precursors, as well as body wall muscle precursors, starting at approximately the 100-cell stage (Granato et al., 1994). Temperature-shift experiments suggest that pha-1 is no longer required after the 1½-fold stage of development (Schnabel and Schnabel, 1990). pha-1 encodes a novel protein of unknown biochemical function (Granato et al. 1994). PHA-1 protein does however contain a basic region similar to those found in some families of DNA-binding proteins and thus could function as a transcriptional regulator.

We have observed that the conditional loss-of-function mutation pha-1(e2123ts) strongly enhances the lethal phenotype of ceh-22(cc8266), even when maintained at the permissive temperature. When grown at the non-permissive temperature, the double mutant also has a much more severe defect in pharyngeal muscle gene expression than either single mutant. This synergistic phenotype is most apparent when expression of the endogenous pharyngeal muscle-specific antigen recognized by the antibody 3NB12 is examined.

What then is the relationship between ceh-22 and pha-1? Our results suggest that pha-1 may promote pharyngeal muscle gene expression in part by maintaining ceh-22 gene expression. However, it is unlikely that this is the sole mechanism by which pha-1 functions in pharyngeal muscle. Expression of the 3NB12 antigen appears completely normal in both pha-1 null mutants (Schnabel and Schnabel, 1990) as well as in ceh-22(cc8266). Therefore, we can rule out models in which pha-1 functions upstream of ceh-22 in a linear, unbranched pathway. We believe that pha-1 may also promote pharyngeal muscle gene expression independently of ceh-22 and that the severe defects observed in the double mutant result from disruption of interacting processes activating pharyngeal muscle gene expression. We have tested the simple hypothesis that pha-1 activates transcription in parallel to ceh-22 via the organ-specific C subelement of the myo-2 enhancer (Okkema and Fire, 1994); however, C subelement function appears normal in pha-1 mutants (D. Moons and P. Okkema, unpublished).

### Comparison of ceh-22 mutant phenotype to tin and Nkx2-5 mutants

Mutations in the Drosophila gene tinman result in loss of heart and visceral muscles (Azpiazu and Frasch, 1993; Bodmer, 1993). This severe defect is very different from the phenotype that we observe in ceh-22 mutants. By comparison, the mouse Nkx2-5 mutant phenotype may be more similar to that of ceh-22. In these mutant mice, cardiac muscle cells differentiate but the organ does not undergo complete morphogenesis (Lyons et al., 1995).

These distinct phenotypes of tin mutants may reflect functions that are unique to this gene. In Drosophila, tin is first expressed prior to gastrulation throughout the presumptive mesoderm; only later does it get restricted to heart muscle precursors (Bodmer et al., 1990; Azpiazu and Frasch, 1993). In comparison, ceh-22 and Nkx2-5 are not expressed in the pre-gastrulation mesoderm; these genes are first expressed near the time cells are committing to a specific muscle fate. An interesting possibility is that tin has acquired a role in both the specification and differentiation of the Drosophila heart, whereas ceh-22 and Nkx2-5 function only in pharyngeal muscle and cardiac muscle differentiation, respectively.

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