Development 124, 3965-3973 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV5149

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

Peter G. Okkema[1,2,*], Eunju Ha[1], Christina Haun[1], Wei Chen[1] and Andrew Fire[2]

[1]Department of Biological Sciences, University of Illinois at Chicago (M/C 567), 900 S. Ashland Avenue, Chicago, IL 60607, USA
[2]Department of Embryology, Carnegie Institution of Washington, 115 W. University Parkway, Baltimore, MD 21210, USA

*Author for correspondence (e-mail: okkema@uic.edu)

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 *Xho*I site within the *ceh-22* 5′-UTR (Okkema and Fire, 1994). The control plasmid pOK49.31, containing the *unc-54::ceh-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:

BC1381*dpy-18(e364)/eT1 III; sDf29 unc-46(e177)/eT1 V*
BC2511*dpy-18(e364)/eT1 III; sDf35 unc-60(e677) dpy-11(e224)/eT1 V*
BW163*ctDf1 V/DnT1 {unc-?(n754) let-? (IV;V)*
BC2617*dpy-18(e364)/eT1 III; mDf1/eT1 V*
MT5813*nDf42/DnT1 [unc-?(n754) let-? (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. deVroomen and R. Plasterk (Netherlands Cancer Institute) using nested primers near the 3′ end of *ceh-22*. A PCR-based sib-selection protocol (Plasterk, 1995) was used to isolate a single deletion allele of *ceh-22*, designated *cc8266*. 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 phenotypic 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 (c187) 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 immunofluoresence 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, F_1 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 F_1 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

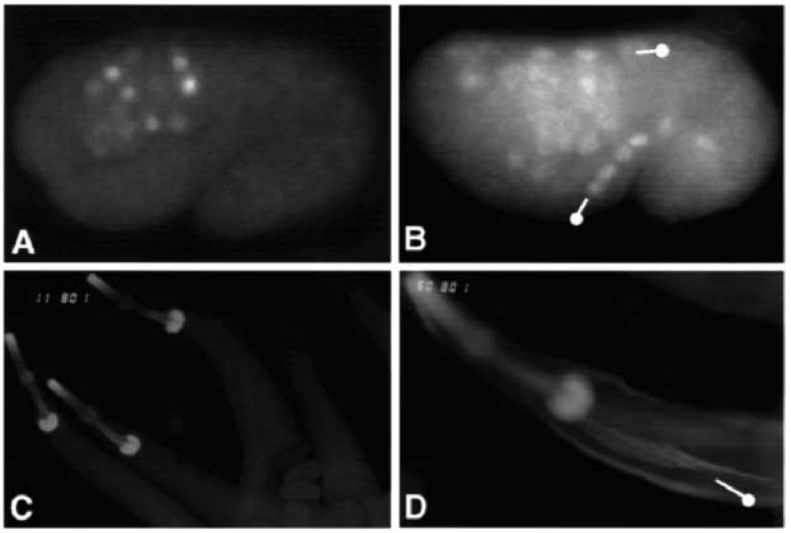

Fig. 1. Ectopic expression of CEH-22 activates *myo-2* expression. (A) Wild-type embryo (at the $1\frac{1}{2}$ fold stage) stained with the anti-CEH-22 antibody c187 (Okkema and Fire, 1994). CEH-22 accumulation is restricted to the pharyngeal muscle nuclei. (B) Transgenic embryo containing *unc-54::ceh-22* 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(ΔHD)* 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.

pattern suggests CEH-22 could be a key determinant of cell-type-specific expression of *myo-2*. To determine whether CEH-22 is sufficient to activate pharyngeal muscle-specific gene expression in a different muscle type, we expressed *ceh-22* mRNA ectopically using the promoter from the body wall muscle-specific *unc-54* myosin heavy chain gene (Mello and Fire, 1995). A transgenic line containing the *unc-54::ceh-22* fusion expressed CEH-22 protein in body wall muscle cell nuclei at a level similar to the endogenous level of CEH-22 in the pharynx (Fig. 1A,B).

We found that ectopic expression of CEH-22 in this manner could activate expression of the endogenous *myo-2* gene in body wall muscle (Fig. 1D). Ectopic CEH-22 was also capable of activating a *myo-2::lacZ* fusion, which is normally expressed exclusively in the pharynx (Table 1).

Although CEH-22 could activate *myo-2* in body wall muscle, it did not appear to do this in every cell in which it was expressed. This observation was particularly evident in transgenic embryos, where CEH-22 was readily detectable in body wall muscle nuclei (Fig. 1). However, we never observed ectopic *myo-2* expression in these embryos; expression was observed only in adult animals. These results suggest either that CEH-22 may require additional adult-specific components or modifications to activate *myo-2*, or alternatively that factors present in the embryo might block CEH-22 function.

To determine the extent of pharyngeal muscle differentiation induced by ectopic CEH-22, we examined expression of additional pharyngeal-specific markers (Table 1). The promoter for the second pharyngeal myosin gene *myo-1* was found to be 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.

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

	Ectopic expression construct	
Pharyngeal muscle marker	*unc-54::ceh-22*	*unc-54::ceh-22(ΔHD)*
endogenous *myo-2*	+	–
myo-2::lacZ	+	–
myo-1::lacZ	+	–
ceh-22::lacZ	–	n.d.
3NB12 antigen	–	n.d.

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(ΔHD)*] 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

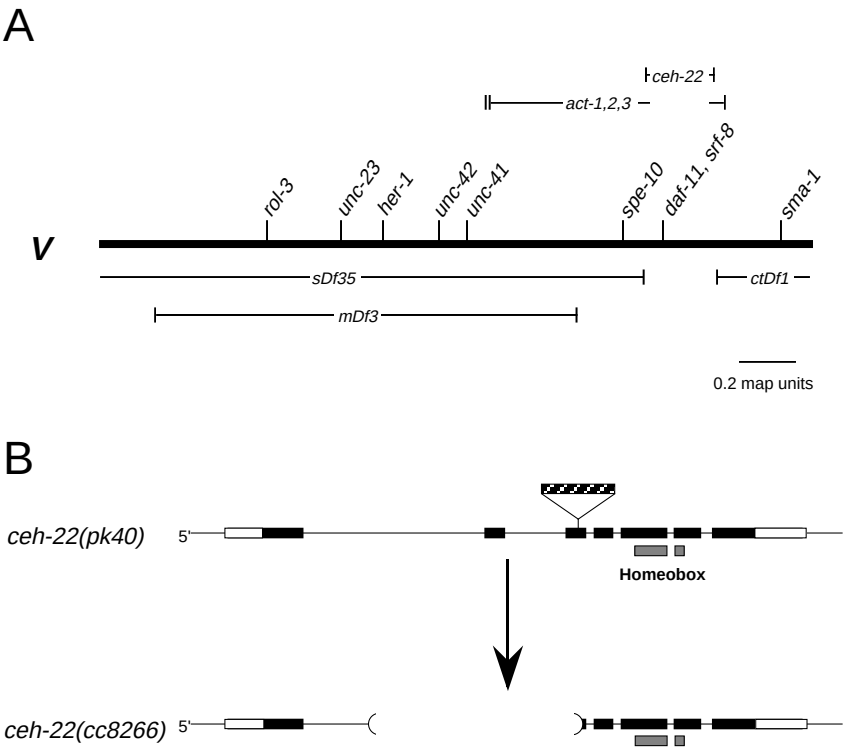

Fig. 2. Genetic map and genomic organization of *ceh-22* mutations. (A) Genetic map indicating the position of *ceh-22* between the endpoints of deficiencies *sDf35* and *ctDf1* and various linked markers. (B) Genomic structure of *ceh-22* mutations. Filled boxes represent coding exons; empty boxes are 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.

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)*

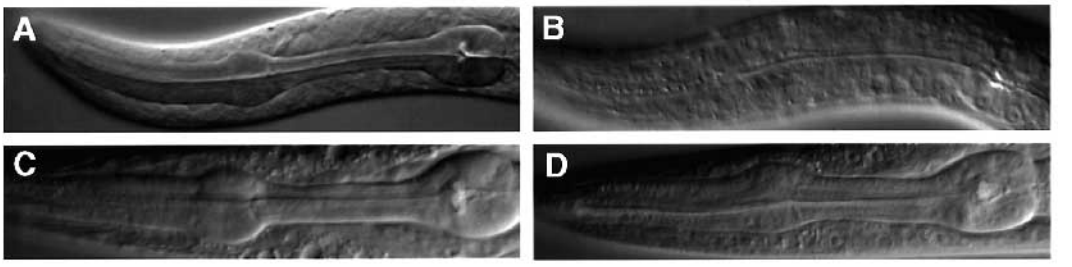

Fig. 3. *ceh-22(cc8266)* mutants have morphological defects in the pharynx. Differential Interference Contrast micrographs of wild-type and *ceh-22(cc8266)* mutant pharynges: (A) wild-type L1; (B) arrested *ceh-22(cc8266)* L1; (C) wild-type L4; (D) *ceh-22(cc8266)* L4. Note the thicker and asymmetric appearance of the pharynx in D.

homozygotes 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 germline; 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 F_1 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)*

Enhancer*	Wild type (N2) [*n*]	*ceh-22(cc8266)* [*n*]
B+B	52% [66]	4% [26]
C+C	43% [95]	35% [20]
C	0% [178]	29% [17]

*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 pheno-

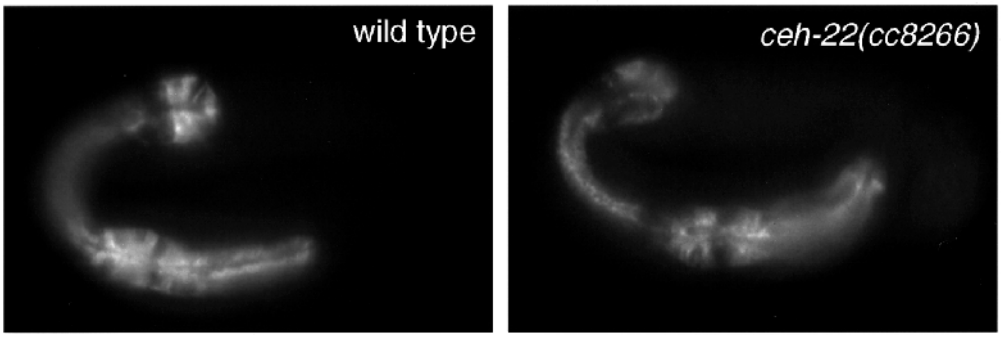

Fig. 4. MYO-2 is expressed in *ceh-22(cc8266)*. Late-stage wild-type (N2) and *ceh-22(cc8266)* embryos stained with the antibody 9.2.1 to detect MYO-2 protein. The intensity of staining is similar in both wild type and *ceh-22(cc8266)*. The staining appears less evenly distributed in the *ceh-22(cc8266)* muscles.

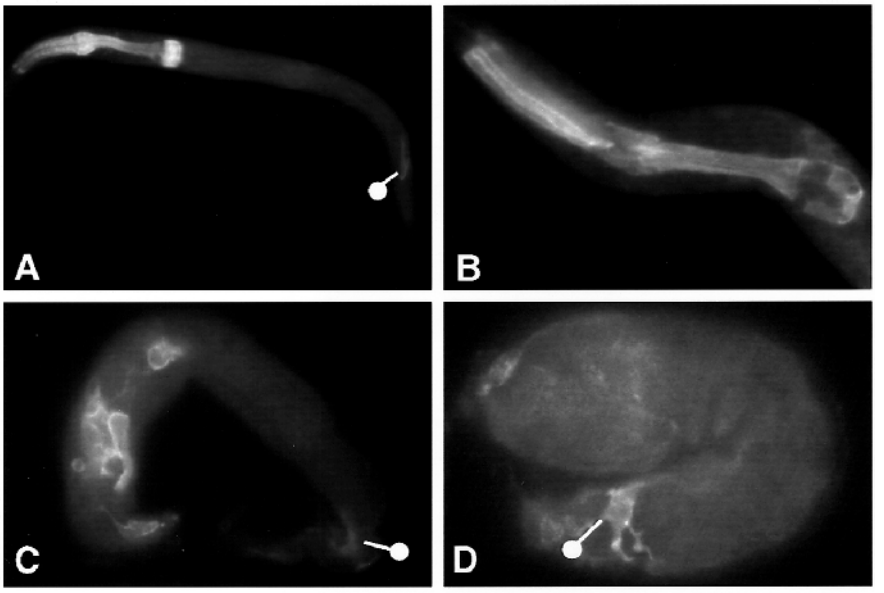

Fig. 5. 3NB12 antigen is not expressed in the *pha-1(e2123ts); ceh-22(cc8266)* double mutant. The 3NB12 antigen is strongly expressed in the pharyngeal muscles of (A) wild type (N2), and in (B) *ceh-22(cc8266)* and (C) *pha-1(e2123ts)* single mutants grown at 25˚C; however pharyngeal staining is almost undetectable in the (D) *pha-1(e2123ts); ceh-22(cc8266)* double mutant grown at 25˚C. Markers indicate two cells posterior to the intestine that also stain with 3NB12; staining in these cells was unaffected in any of these mutants and was used as a positive control for antibody staining.

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)* at 25°C (data not shown). 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 the same set of pharyngeal muscle cells as *ceh-22* (m3, m4, m5 and m7), whereas *myo-2* 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

Genotype	% reaching L4/adult at 16˚C*
Wild type	100
pha-1(e2123ts)	70
ceh-22(cc8266)	75
pha-1(e2123ts); ceh-22(cc8266)	8

*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 $1\frac{1}{2}$-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

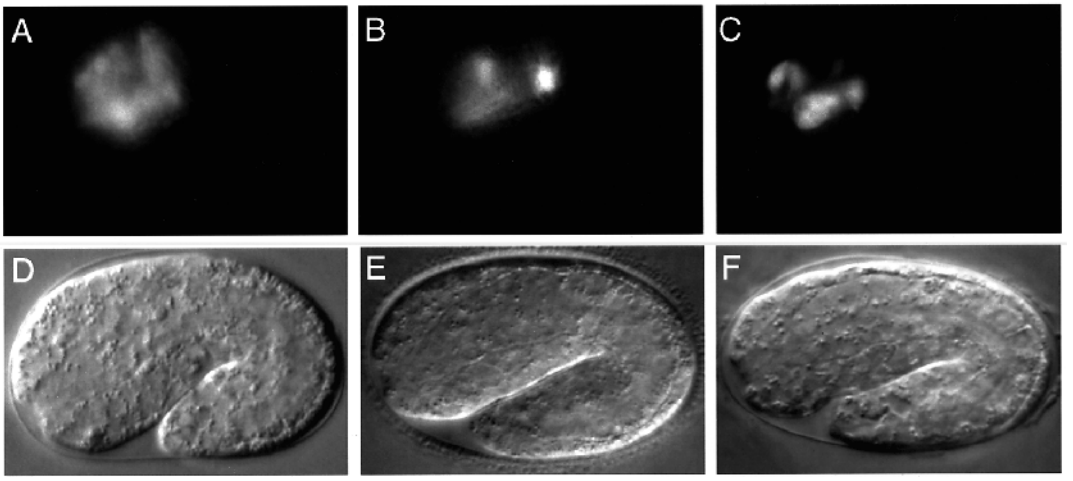

Fig. 6. *ceh-22::gfp* is initiated normally in *pha-1* mutants. Fluorescence (A-C) and Nomarski microscopy (D-F) of 1½-fold stage embryos expressing *ceh-22::gfp*. (A,D) Wild type; (B,E) *pha-1(e2123ts)* grown at 16°C; (C,F) *pha-1(e2123ts)* grown at 25 °C. The wild-type embryo in A and D is homozygous for *cuIs1*, which contains a chromosomally integrated array of the *ceh-22::gfp* reporter; the *pha-1* mutants are segregating from a strain that is *pha-1(e2123ts) vab-7(e1562); cuIs1/+* and may be heterozygous for the *ceh-22::gfp* reporter.

completely removes the *pha-1* gene (data not shown; Schnabel and Schnabel, 1990).

DISCUSSION

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

The *ceh-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 CEH-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 CEH-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 CEH-22 does not autoactivate its own expression, nor does it induce expression of the 3NB12 antigen. Thus CEH-22 can activate multiple components of pharyngeal muscle differentiation, but it does not result in a complete transformation to pharyngeal muscle fate.

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

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

Temperature	Percentage of animals expressing strong GFP*
16°C	62% (*n*=34)
25°C	0% (*n*=30)

*Embryos segregating from *pha-1(e2123ts) vab-7(e1562); cuIs1/+* 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. *cuIs1* is a chromosomally integrated array containing the *ceh-22::gfp* reporter plasmid pCW2.1.

†Four of these terminally arrested embryos displayed very weak GFP expression in the pharynx.

Despite accumulating in embryonic body wall muscle nuclei, CEH-22 does not activate *myo-2* in these embryos. Therefore it seems likely that CEH-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 CEH-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 *ceh-22* loss-of-function phenotype confirms this gene plays a critical role in pharyngeal development. In severely affected *ceh-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 *ceh-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 *ceh-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 *ceh-22* mutants.

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

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 function using antisense RNA. Therefore we believe that *ceh-22(cc8266)* is likely either a strong loss-of-function or a null allele.

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 $1\frac{1}{2}$-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 pregastrulation 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.

We gratefully acknowledge the contribution of R. H. A. Plasterk and co-workers for isolating the Tc1 insertion mutant described in this paper from a mutant collection (funded by grant 5 RO1 RR10082-02 of the NIH-NCRR to R. H. A. P.). We are also indebted to past and present members of our laboratories for advice throughout this work, and to Betsy Goodwin and Jack Thatcher for critical reading of the manuscript. This work was supported by the NIH (R01-GM53996 to P. G. O.; R01-GM37706 to A. F.) and the Carnegie Institution of Washington. A. F. is a Rita Allen Foundation scholar. Some nematode strains used in this work were provided by the *Caenorhabditis* Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR).

REFERENCES

Ahnn, J. and Fire, A. (1994). A screen for genetic loci required for body-wall muscle development during embryogenesis in *Caenorhabditis elegans*. *Genetics* **137**, 483-498.

Ardizzi, J. P. and Epstein, H. F. (1987). Immunochemical localization of myosin heavy chain isoforms and paramyosin in developmentally and structurally diverse muscle cell types of *C. elegans*. *J. Cell Biol.* **105**, 2763-2770.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., Struhl, K. (1990). *Current Protocols in Molecular Biology*. New York: John Wiley and Sons.

Avery, L. (1993). The genetics of feeding in *Caenorhabditis elegans*. *Genetics* **133**, 897-917.

Avery, L. and Horvitz, H. R. (1989). Pharyngeal pumping continues after laser killing of the pharyngeal nervous system of *C. elegans*. *Neuron* **3**, 473-485.

Azpiazu, N. and Frasch, M. (1993). *tinman* and *bagpipe*: two homeo box genes that determine cell fates in the dorsal mesoderm of *Drosophila*. *Genes Dev*. **7**, 1325-1340.

Bodmer, R. (1993). The gene *tinman* is required for specification of the heart and visceral muscles in *Drosophila*. *Development* **118**, 719-729.

Bodmer, R., Jan, L. Y. and Jan, Y. N. (1990). A new homeobox containing gene, *msh-2*, is transiently expressed early during mesoderm formation of *Drosophila*. *Development* **110**, 661-669.

Chen, C. Y., Croissant, J., Majesky, M., Topouziz, S., McQuinn, T., Frankovsky, M. J. and Schwartz, R. J. (1996). Activation of the cardiac alpha-actin promoter depends upon serum response factor, *tinman* homologue, *Nkx-2. 5*, and intact serum response elements. *Dev. Genet.* **19**, 119-130.

Chen, J-N. and Fishman, M. C. (1996). Zebrafish *tinman* homolog demarcates the heart field and initiates myocardial differentiation. *Development* **122**, 3809-3816.

Chen, L., Krause, M., Draper, B., Weintraub, H. and Fire, A. (1992). Body-wall muscle formation in *Caenorhabditis elegans* embryos that lack the MyoD homolog *hlh-1*. *Science* **256**, 240-243.

Chen, L., Krause, M., Sepanski, M. and Fire, **A.** (1994). The *Caenorhabditis elegans* MYOD homolog HLH-1 is essential for proper muscle function and complete morphogenesis. *Development* **120**, 1631-1641.

Cleaver, O. B., Patterson, K. D., Krieg, P. A. (1996). Overexpression of the *tinman*-related genes *XNkx-2. 5* and *XNkx-2. 3* in Xenopus embryos results in myocardial hyperplasia. *Development* **122**, 3549-3556.

Finney, M. and Ruvkun, G. B. (1990). The *unc-86* gene product couples cell lineage and cell identity in *C. elegans*. *Cell* **63**, 895-905.

Fire, A. (1993). Histochemical techniques for locating *Escherichia coli* β-galactosidase activity in transgenic organisms. *Gene Analysis Techniques and Applications* **9**, 151-158

Granato, M., Schnabel, H. and Schnabel, R. (1994). Genesis of an organ: molecular analysis of the *pha-1* gene. *Development* **120**, 3005-3017.

Harvey, R. P. (1996). NK-2 homeobox genes and heart development. *Dev. Biol.* **178**, 203-216.

Kim, Y. and Nirenberg, M. (1989). *Drosophila* NK-homeobox genes. *Proc. Natn. Acad. Sci., USA* **86**, 7716-7720.

Krause, M., Fire, A., White Harrison, S., Priess, J. and Weintraub, H. (1990). *CeMyoD* accumulation defines the body wall muscle cell fate during *C. elegans* embryogenesis. *Cell* **63**, 907-919.

Lints, T. J., Parsons, L. M., Hartley, L., Lyons, I. and Harvey, R. P. (1993). *Nkx-2. 5*: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants. *Development* **119**, 419-431.

Lyons, I., Parsons, L. M., Hartley, L., Li, R., Andrews, J. E., Robb, R. and Harvey, R. P. (1995). Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeobox gene *Nkx2-5*. *Genes Dev.* **9**, 1654-1666.

Mably, J. D. and Liew, C. C. (1996). Factors involved in cardiogenesis and the regulation of cardiac-specific gene expression. *Circ. Res.* **79**, 4-13.

Mango, S. E., Lambie, E. J. and Kimble, J. (1994). The *pha-4* gene is required to generate the pharyngeal primordium of *Caenorhabditis elegans*. *Development* **120**, 3019-3031.

Mello C. C., Kramer, J. M., Stinchcomb, D. and Ambros, V. (1991). Efficient gene transfer in *C. elegans*: extrachromosomal maintenance and integration of transforming sequences. *EMBO J.* **10**, 3959-70.

Mello, C. and Fire, A. (1995). DNA transformation. In *Methods in Cell Biology: C. elegans* (ed. D. Shakes and H. Epstein). San Diego, CA: Academic Press.

Miller, D. M. and Shakes, D. C. (1995) Immunofluorescence microscopy. In *Methods in Cell Biology: C. elegans* (ed. D. Shakes and H. Epstein). San Diego, CA: Academic Press.

Miller, D. M., Ortiz, I., Berliner, G. C. and Epstein, H. F. (1983) Differential localization of two myosins within nematode thick filaments. *Cell* **34**, 477-490.

Miller, D. M., Stockdale, F. E. and Karn, J. (1986). Immunological identification of the genes encoding the four myosin heavy chain isoforms of *C. elegans*. *Proc. Natn. Acad. Sci., USA* **83**, 2305-2309.

Okamoto, H. and Thomson, J. N. (1985). Monoclonal antibodies which distinguish certain classes of neuronal and supporting cells in the nervous tissues of the nematode *Caenorhabditis elegans*. *J. Neurosci.* **5**, 643-653.

Okkema, P. G. and Fire, A. (1994) The *Caenorhabditis elegans* NK-2 class homeoprotein CEH-22 is involved in combinatorial activation of gene expression in pharyngeal muscle. *Development* **120**, 2175-2186.

Okkema, P. G., Harrison, S. W., Plunger, V., Aryana, A. and Fire, A. (1993). Sequence requirements for myosin gene expression and regulation in *C. elegans*. *Genetics* **135**, 385-404.

Plasterk, R. H. A. (1995). Reverse genetics: from gene sequence to mutant worm. In *Methods in Cell Biology: C. elegans* (ed. D. Shakes and H. Epstein). San Diego, CA: Academic Press.

Priess, J. and Thompson, N. (1987). Cellular interactions in early *Caenorhabditis elegans* embryos. *Cell* **48**, 241-250.

Rushforth, A. M., Saari, B. and Anderson, P. (1993). Site-selected insertion of the transposon Tc1 into a *Caenorhabditis elegans* myosin light chain gene. *Molec. Cell. Biol.* **13**, 902-910.

Schnabel, H. and Schnabel, R. (1990). An organ-specific differentiation gene, *pha-1*, from *Caenorhabditis elegans*. *Science* **250**, 686-688.

Schultheiss, T. M., Xydas, S. and Lassar, A. B. (1995). Induction of avian cardiac myogenesis by anterior endoderm. *Development* **121**, 4230-4214.

Shiojima, I., Komuro, I., Mizuno, T., Aikawa, R., Akazawa, H., Oka, T., Yamazaki, T. and Yazaki, Y. (1996). Molecular cloning and characterization of human cardiac homeobox gene *Csx1*. *Circ. Res.* **79**, 920-929.

Tonissen, K. F., Drysdale, T. A., Lints, T. J., Harvey, R. P. and Krieg, P. A. (1994). *XNkx-2. 5*, a Xenopus gene related to *Nkx-2. 5* and *tinman*: evidence for a conserved role in cardiac development. *Dev. Biol.* **162**, 325-328.

(Accepted 6 August 1997)