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Rescue of *Caenorhabditis elegans* pharyngeal development by a vertebrate heart specification gene

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ABSTRACT **Development of pharyngeal muscle in nematodes and cardiac muscle in vertebrates and insects involves the related homeobox genes *ceh-22*, *nkx2.5*, and *tinman*, respectively. To determine whether the nematode and vertebrate genes perform similar functions, we examined activity of the zebrafish *nkx2.5* gene in transgenic *Caenorhabditis elegans*. Here, we report that ectopic expression of *nkx2.5* in *C. elegans* body wall muscle can directly activate expression of both the endogenous *myo-2* gene, a *ceh-22* target normally expressed only in pharyngeal muscle, and a synthetic reporter construct controlled by a multimerized CEH-22 binding site. *nkx2.5* also efficiently rescues a *ceh-22* mutant when expressed in pharyngeal muscle. Together, these results indicate that *nkx2.5* and *ceh-22* provide a single conserved molecular function. Further, they suggest that an evolutionarily conserved mechanism underlies heart development in vertebrates and insects and pharyngeal development in nematodes.**

Heart development in animals as distantly related as insects and vertebrates involves related NK-2 family homeobox genes (reviewed in ref. 1). In *Drosophila*, the *tinman* homeobox gene is expressed in cardiac precursors, and *tinman* mutants completely lack a heart (2–4). Likewise in vertebrates, the *nkx2.5* homeobox gene is expressed in myocardial precursors (5–9), and mouse *Nkx2–5* mutants exhibit defects in cardiac morphogenesis and gene expression (10). This remarkable molecular conservation suggests a common mechanism controls heart development in a wide variety of species.

Nematodes have no heart or defined circulatory system; however, existing evidence suggests the nematode pharynx, a rhythmically contracting organ involved in feeding, shares functional and molecular similarities with the heart in other species. Pharyngeal muscle function, like that of vertebrate cardiac muscle, does not require nervous system input (11). Likewise at the molecular level, pharyngeal muscle development does not involve the MyoD family of myogenic regulatory factors (12, 13). Instead, pharyngeal muscle development requires the *tinman/nkx2.5*-related homeobox gene *ceh-22. ceh-22* is expressed exclusively in pharyngeal muscle, where it binds the enhancer of the pharyngeal muscle-specific *myo-2* gene, and a *ceh-22* mutant displays defects in pharyngeal morphology and function (13, 14).

Is there a relationship between heart development in insects and vertebrates and pharyngeal development in nematodes? To address this question, we have examined the ability of the zebrafish *nkx2.5* gene (8, 9) to substitute for *ceh-22* in transgenic *C. elegans*. We have found that, like *ceh-22* (15), *nkx2.5* can activate expression of *myo-2* when expressed ectopically in body wall muscle and that *nkx2.5* can rescue a *ceh-22* mutant when expressed in pharyngeal muscle. These results indicate that *ceh-22* and *nkx2.5* share a common function and suggest that development of the nematode pharynx and the vertebrate heart may involve a conserved regulatory mechanism.

MATERIALS AND METHODS

Plasmids and *C. elegans* Transformation Techniques. To produce *unc-54*::*nkx2.5*, an *nkx2.5* cDNA, PCR amplified from adult zebrafish heart using primers derived from the published sequence (8, 9) was inserted downstream of the *unc-54* promoter in pPD30.38 (16) using an *Nco*I site within the *nkx2.5* 5′-untranslated region. To produce *ceh-22*::*nkx2.5* (designated plasmid pOK102.01) or *ceh-22*::*ceh-22* (designated plasmid pOK102.05), cDNAs were inserted downstream of the *ceh-22* promoter from pOK29.02 (14).

Two transgenic lines containing *unc-54*::*nkx2.5* were isolated by microinjection into wild-type N2 *C. elegans*, using the cotransformation marker pRF4 (17). To produce *ceh-22(cc8266)* mutant lines bearing either *ceh-22*::*nkx2.5* or *ceh-22*::*ceh-22* expression vectors, the balanced heterozygous strain OK 0060 [*ceh-22(cc8266)/unc-42(e270) sma-1(e30)*] was transformed with each construct using the pRF4 marker; two transformed homozygous *ceh-22(cc8266)* lines segregating from each of these initial heterozygous transformants were identified and characterized.

Antibody Staining and Expression of *lacZ* Reporters. Expression of MYO-2 protein was examined by staining with the monoclonal antibody 9.2.1 [kindly provided by D. M. Miller (18)] following whole mount fixation of mixed-stage populations of nematodes (19, 20). Primary antibodies were detected by immunofluoresence microscopy using Texas red-conjugated goat-anti mouse IgG (Jackson ImmunoResearch) secondary antibodies. To assay function of *lacZ* reporters, F_1 expression assays were carried out as previously described (21); plasmids pOK21.43 (multimerized *B* sub-element reporter) and pOK29.02 (*ceh-22*::*lacZ*) 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.

RESULTS

Zebrafish *nkx2.5* Can Activate *myo-2* Expression When Expressed in *C. elegans* Body Wall Muscle. In wild-type *C. elegans*, *ceh-22* is expressed exclusively in pharyngeal muscle where it activates expression of the pharyngeal muscle-specific myosin heavy chain gene *myo-2* (14). Ectopic expression of *ceh-22* in body wall muscle can activate expression of *myo-2* (15); because *myo-2* is normally never expressed in body wall muscle, this ectopic expression assay provides a sensitive test for *ceh-22* function. To determine whether zebrafish *nkx2.5* can function similarly to *ceh-22*, we expressed *nkx2.5* in *C. elegans* body wall muscle and examined expression of the endogenous

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myo-2 gene by antibody staining. Two transgenic lines expressing an *nkx2.5* cDNA under the control of the *unc-54* body wall muscle-specific promoter were generated. In both lines, we detected *myo-2* expression in the body wall muscles (Fig. 1 *A* and *B*). These results show that *nkx2.5* can function like *ceh-22* to induce *myo-2* expression.

We next asked whether Nkx2.5 directly interacts with the same sequences recognized by CEH-22 by examining expression of a reporter gene under the control of multimerized CEH-22 binding sites. CEH-22 binds a region within the *myo-2* enhancer termed the *B* sub-element (14). In wild-type animals, a *lacZ* reporter under control of a synthetic enhancer consisting of four copies of a 28-bp *B* sub-element oligonucleotide is expressed specifically in pharyngeal muscle; only occasional expression is observed outside the pharynx (Table 1; ref. 14). In a transgenic strain bearing the *unc-54*::*nkx2.5* expression construct, we found a significant increase in the number of animals expressing β-galactosidase in body wall muscle (from 2.5 to 16.5%) (Table 1; Fig. 1*C*). To rule out the possibility that Nkx2.5 was indirectly enhancing expression of *myo-2* or the *B* sub-element reporter by activating ectopic expression of the *ceh-22* gene, we examined expression of a *ceh-22*::*lacZ* fusion in animals bearing the *unc-54*::*nkx2.5* transgene. Expression of β-galactosidase was limited to pharyngeal muscle (Table 1), a pattern identical to that observed in wild-type animals (14). Thus, these data together indicate that Nkx2.5, like CEH-22, activates transcription by interacting directly with the *B* sub-element of the *myo-2* enhancer.

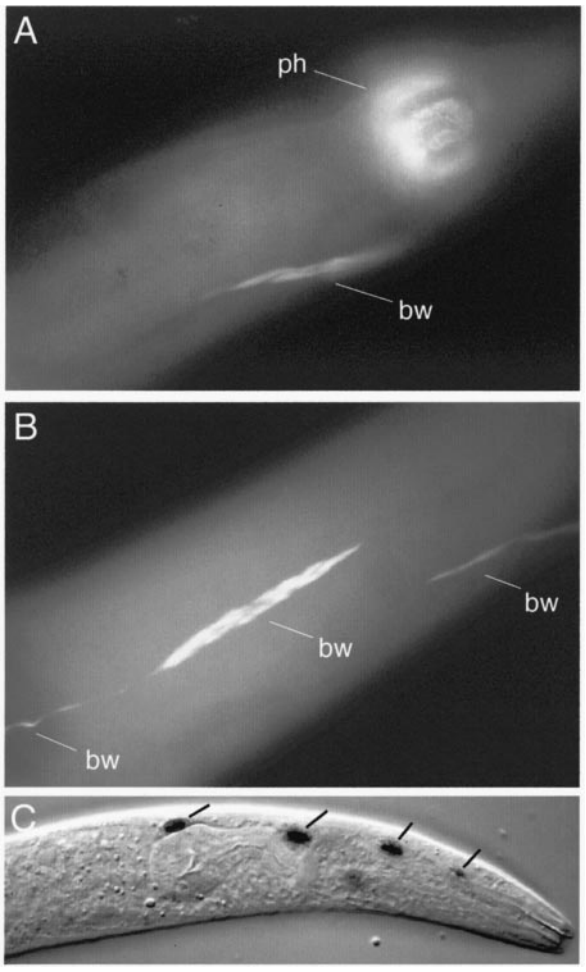


FIG. 1. Expression of zebrafish *nkx2.5* in *C. elegans* body wall muscle activates expression of pharyngeal muscle-specific genes. (*A* and *B*) Adult animals bearing the *unc-54*::*nkx2.5* expression vector stained with the anti-MYO-2 antibody, 9.2.1. MYO-2 protein accumulates in pharyngeal muscles (ph) and body wall muscles (bw). Animals ectopically expressing either *ceh-22* or *nkx2.5* generally express MYO-2 in only a few (approximately 1–5) body wall muscle cells (15); the numbers of MYO-2 positive body wall muscles are similar in animals expressing either *ceh-22* or *nkx2.5*. (C) An adult animal bearing the *unc-54*::*nkx2.5* transgene and expressing a *B* sub-element::*lacZ* reporter in body wall muscles stained with X-Gal. β-Galactosidase staining accumulates in body wall muscle nuclei (marked by black bars).

Table 1. Expression of *lacZ* reporters in body wall muscle

Construct	Percent F_1 transformants expressing β-galactosidase in body wall muscle*	
	Wild type (N2)	OK0027†
B sub-element::*lacZ*	2.5% (n = 118)	16.5% (n = 151)
ceh-22::*lacZ*	ND	0% (n = 69)

**lacZ* reporters were injected into the germ line of adult hermaphrodites, and the entire F_1 brood was stained to detect β-galactosidase expression (21). Using this "F_1 expression assay," both reporters are strongly expressed in pharyngeal muscle (14). The total number of F_1 transformants (n), recognized by pharyngeal expression of β-galactosidase, was counted, and they were then examined for ectopic β-galactosidase expression in body wall muscle.

†The *unc-54*::*nkx2.5* fusion in strain OK 0027 is maintained as an extrachromosomal array that is segregated to 55% of F_1 progeny; therefore, approximately 30% of the F_1 transformants bearing the *unc-54*::*nkx2.5* fusion express *B* sub-element::*lacZ* in body wall muscle.

***nkx2.5* Can Substitute for *ceh-22* During Normal Pharyngeal Development.** In addition to its role in *myo-2* activation, CEH-22 likely regulates other genes required for pharyngeal development. Indeed, a *ceh-22* mutant exhibits profound contractile and morphological defects in the pharynx, despite expressing *myo-2* nearly as well as wild type (15). To examine the extent to which Nkx2.5 and CEH-22 are functionally equivalent, we asked if expression of *nkx2.5* in pharyngeal muscle could rescue a *ceh-22* mutant. The loss-of-function mutation *ceh-22(cc8266)* causes pharyngeal muscle defects that interfere with feeding, resulting in a lethal/slow growth phenotype (15). Approximately one-quarter of *ceh-22(cc8266)* mutants arrest shortly after hatching, whereas the remaining mutants grow slowly to adulthood. These mutant adults exhibit characteristic morphological and contractile defects in the pharynx and have a starved appearance typical of feeding defective mutants. We placed the *nkx2.5* cDNA under control of the *ceh-22* promoter and introduced this fusion (designated *ceh-22*::*nkx2.5*) into *ceh-22(cc8266)* mutants. For comparison, we also introduced a similar fusion containing the *ceh-22* cDNA under the control of the *ceh-22* promoter (designated *ceh-22*::*ceh-22*) into *ceh-22(cc8266)* mutants.

We tested whether expression of *nkx2.5* or *ceh-22* could rescue the larval arrest/slow growth phenotype by picking synchronized embryos and counting the number of animals reaching adulthood after 4 days at 20°C (Table 2). Ninety-eight percent of the wild-type embryos reached adulthood under these conditions. In comparison, only 36% of the untransformed *ceh-22(cc8266)* mutants reached adult stage, and these adults had a thin, pale appearance indicative of poor feeding (22). For *ceh-22(cc8266)* mutants transformed with *ceh-22*::*nkx2.5* or *ceh-22*::*ceh-22,* 68% and 77% of the animals reached adulthood within 4 days, respectively, and all of these transformed adults had a well fed appearance. The animals that did not reach adulthood likely did not contain the transforming DNA, which in *C. elegans* is maintained as a semi-stable extrachromosomal array (17); however, we cannot rule out the possibility that neither *nkx2.5* nor *ceh-22* completely rescued the mutant phenotype. Nonetheless, the ability of the *ceh-22*::*nkx2.5* transgene to rescue *ceh-22(cc8266)* growth defects nearly as well as *ceh-22*::*ceh-22* was quite striking.

Table 2. *nkx2.5* and *ceh-22* rescue the growth defects in *ceh-22(cc8266)*

Genotype	Percent animals reaching adult after 4 days at 20°C*	*n*
Wild type (N2)	98	305
ceh-22(cc8266)	36	230
ceh-22(cc8266); cuEx(ceh-22::*nkx2.5* cDNA)	68	104
ceh-22(cc8266); cuEx(ceh-22::*ceh-22* cDNA)	77	203

*Embryos segregating from the indicated strains were picked to fresh plates and incubated at 20°C. After 4 days, the number of animals reaching adulthood was determined. Neither *ceh-22*::*ceh-22* nor *ceh-22*::*nkx2.5* rescued a partially penetrant sterile phenotype exhibited by *ceh-22(cc8266)* (ref. 15; data not shown). This phenotype is rescued by transformation with a *ceh-22* genomic DNA fragment; thus, we believe the *ceh-22*::*ceh-22* and *ceh-22*::*nkx2.5* may be missing sequences necessary for rescue of sterility.

We also examined the pharyngeal morphology of representative adult *ceh-22(cc8266)* mutants and *ceh-22(cc8266)* animals bearing either the *ceh-22*::*nkx2.5* or *ceh-22*::*ceh-22* fusion by Nomarski microscopy (Fig. 2). Nine of nine untransformed *ceh-22(cc8266)* mutants displayed an abnormal pharyngeal morphology (Fig. 2*B*). The pharynx appeared thicker than wild type, particularly in the region where the isthmus joins the terminal bulb; in addition, the grinder, a cuticular structure located in the lumen of the pharynx, appeared in an abnormal orientation and did not invert completely during pharyngeal pumping. In contrast, nearly all of the *ceh-22(cc8266)* animals bearing *ceh-22*::*nkx2.5* or *ceh-22*::*ceh-22* displayed a normal pharyngeal morphology (Fig. 2 *C* and *D*); only 1/17 and 1/19 of these adults, respectively, appeared abnormal, whereas the pharyngeal contractions and inversion of the grinder appeared normal in all transgenic animals.

DISCUSSION

Our results demonstrate that zebrafish *nkx2.5* and *ceh-22* are functionally interchangeable in *C. elegans*. Nkx2.5 can bind sites within the chromosome normally targeted by CEH-22 and interact productively and specifically with the *C. elegans* transcriptional machinery. Mutational analysis of the *B* subelement of *myo-2* indicates that CEH-22 interacts with additional *C. elegans* factors to regulate gene expression (14). Whatever the nature of the interactions with these other as yet unidentified proteins, Nkx2.5 seems capable of reproducing them. Amino acid sequence identity between Nkx2.5 and CEH-22 is greatest within the homeodomain (68% identity), although two short regions of similarity are also found in conserved positions upstream and downstream of the homeodomain (Fig. 3). The region of conservation downstream of the homeodomain is part of an NK2-specific domain like that found in Nkx2.5 (1, 8, 9); however, deletion of this divergent NK2 domain from CEH-22 has no detectable effect on its ability to activate *myo-2* when expressed in body wall muscle (R. Reardon and P.G.O., unpublished work). It therefore seems likely that the homeodomain and perhaps the conserved upstream peptide are the primary determinants mediating function of CEH-22 and Nkx2.5, although other regions of low primary sequence identity but high functional homology may also contribute to activity.

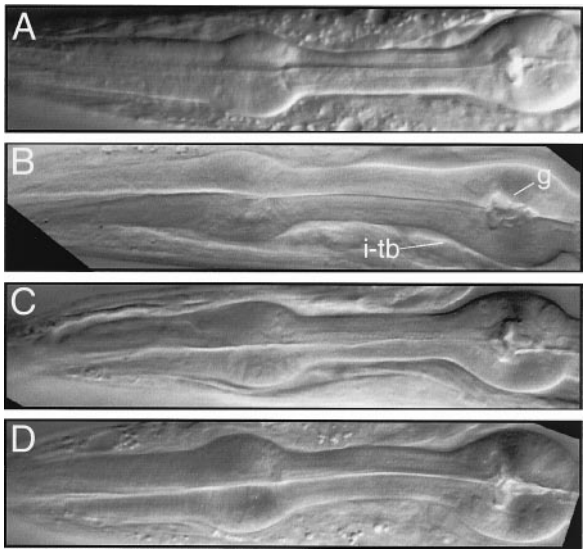
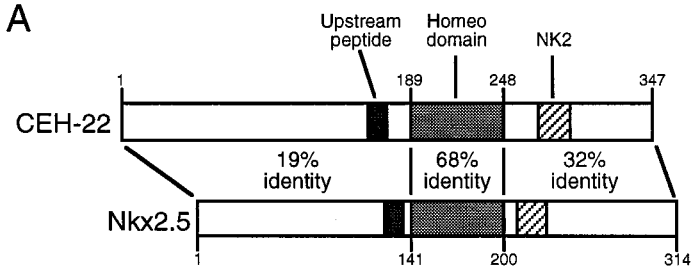


FIG. 2. Expression of *nkx2.5* or *ceh-22* in pharyngeal muscle rescues a *ceh-22* mutant. Nomarski micrographs of the adult pharynx in wild type (*A*), a *ceh-22(cc8266)* mutant (*B*), and a *ceh-22(cc8266)* mutant rescued with either the *ceh-22*::*nkx2.5* (*C*) or the *ceh-22*::*ceh-22* (*D*) expression vectors. The poorly formed grinder (g) and thickened junction of the pharyngeal isthmus and terminal bulb (i-tb) characteristic of *ceh-22(cc8266)* are marked in *B*.

Similar experiments examining function of murine *Nkx2–5* in *Drosophila* indicate that derivatives of *Nkx2–5* can also partially rescue the *tinman* mutant, suggesting a conservation of function also exists between *tinman* and *Nkx2–5* (R. Bodmer, personal communication; G. Ranganayakulu, R. Harvey, and E. Olson, personal communication). Together these results and ours suggest that a key step in the development of cardiac muscle in vertebrates and insects and pharyngeal muscle in nematodes is controlled by a common factor. Expression of *tinman* and *nkx2.5* in the hearts of *Drosophila* (23, 24) and the chicken, respectively (25), involve upstream signals mediated by transforming growth factor-β superfamily and *wingless* signaling pathways; it will be important to determine whether similar upstream signals regulate *ceh-22* expression in the *C. elegans* pharynx to understand the extent to which the pathways specifying heart and pharyngeal development are conserved.

Although *ceh-22, nkx2.5*, and *tinman* seem to share common function, it is not clear that they are orthologs. These genes are members of the larger family of NK2 homeobox genes and other family members function in a variety of tissues (1). Indeed the CEH-22 homeodomain shares highest sequence identity with mouse *Nkx2–2* and *Drosophila vnd* (14), which are primarily expressed in the developing nervous system (26, 27). It will be interesting to determine whether these or other NK2 family members will also function like *nkx2.5* to activate *myo-2.* It is noteworthy that another *C. elegans* NK2 homeobox gene, *ceh-24*, is expressed outside the pharynx in some neurons and the vulval muscles (28), suggesting certain NK2 family members may be incapable of activating *myo-2.*

The nematode pharynx, vertebrate heart, and insect heart are all muscular tubes that pump liquids, although the specific functions of these organs are very different. What is the significance of the fact that these different organs utilize a common myogenic mechanism? One interesting observation is that in all vertebrates thus far examined, *nkx2.5* (or the related genes *nkx2.3* and *nkx2.7*) is expressed not only in the anterior lateral plate mesoderm, which will give rise to the heart, but also in the anterior endoderm, which will give rise to parts of the anterior digestive tract (5–9). Likewise, *Drosophila tinman* is transiently expressed in an anterior region of the embryo surrounding the stomodeum (2), which gives rise to the mouth, pharynx, and esophagus (29). Perhaps the ancestral function of *ceh-22/tinman/nkx2.5* was to demarcate a "module" (as defined in ref. 30) that gave rise to an anteriorly located contractile tube used for feeding, circulation, or both. The mesodermal component of this field may have been co-opted during evolution to form the very distinct structures found in modern phyla.



B

Homeodomain

```
CEH-22 (189)KRKRRVLFTKAQTYELERRFRSQKYLSAPEREALAMQIRLTPTQVKIWFQNHRYKTKKSH(248)
           .:: :::..::.:::.:::..:::::::::. ::  ..:: ::::::::::.::: :...
Nkx2.5 (141)RRKPRVLFSQAQVYELQRRFKQQKYLSAPERDHLANVLKLTSTQVKIWFQNRRYKCKRQR(200)
```

Upstream peptide

```
CEH-22 (163)IKLEDED(169)
           .:::: :
Nkx2.5 (128)LKLDDAD(134)
```

NK2 domain

```
CEH-22 (276)TRAMPIPMLVRDSSAR(291)
                : . .:.:::::..
Nkx2.5 (213)PRRISVPVLVRDGKPC(228)

Cons        PRRVAVPVLVRDGKPC
```

FIG. 3. Regions of sequence identity between CEH-22 and Nkx2.5. (*A*) Schematic diagram of CEH-22 and Nkx2.5 indicating the positions of conserved sequences (numbered according to the predicted protein sequence). The overall levels of amino acid sequence identity are reported for the homeodomains and for the portions of the proteins outside the homeodomains. (*B*) Sequence alignments between conserved regions of CEH-22 and Nkx2.5. Amino acid identities are indicated by a colon (:) and similarities by a period (.); numbering indicates the location of these sequences within the primary amino acid sequence. A portion of the NK2-specific domain consensus reported in ref. 1 is shown with residues present in both CEH-22 and Nkx2.5 underlined.

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