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Divergent roles for *NK-2* class homeobox genes in cardiogenesis in flies and mice

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SUMMARY

Recent evidence suggests that cardiogenesis in organisms as diverse as insects and vertebrates is controlled by an ancient and evolutionarily conserved transcriptional pathway. In *Drosophila*, the *NK-2* class homeobox gene *tinman (tin)* is expressed in cardiac and visceral mesodermal progenitors and is essential for their specification. In vertebrates, the *tin* homologue *Nkx2-5/Csx* and related genes are expressed in early cardiac and visceral mesodermal progenitors. To test for an early cardiogenic function for *Nkx2-5* and to examine whether cardiogenic mechanisms are conserved, we introduced the mouse *Nkx2-5* gene and various mutant and chimeric derivatives into the *Drosophila* germline, and tested for their ability to rescue the *tin* mutant phenotype. While *tin* itself strongly rescued both heart and visceral mesoderm, *Nkx2-5* rescued only visceral mesoderm. Other vertebrate 'non-cardiac' *NK-2* genes rescued neither. We mapped the cardiogenic domain of tin to a unique region at its N terminus and, when transferred to Nkx2-5, this region conferred a strong ability to rescue heart. Thus, the cardiac and visceral mesodermal functions of *NK-2* homeogenes are separable in the *Drosophila* assay. The results suggest that, while *tin* and *Nkx2-5* show close functional kinship, their mode of deployment in cardiogenesis has diverged possibly because of differences in their interactions with accessory factors. The distinct cardiogenic programs in vertebrates and flies may be built upon a common and perhaps more ancient program for specification of visceral muscle.

Key words: Mouse, *Drosophila*, Heart, Cardiogenesis, *tinman*, *Nkx2-5*

INTRODUCTION

Molecular and genetic analysis of developmental genes in a variety of organisms has led to the notion that certain regulatory pathways have retained a dedicated function during evolution (Manak and Scott, 1994). Functional conservation between apparent cognates has recently been tested in a *Drosophila* transgenic assay. Mammalian *Hox* genes, which specify positional identity along the anterior/posterior body axis, and the *Pax6* gene, required for eye development in mice and humans, can mimic the dominant activities of their *Drosophila* counterparts (Malicki et al., 1990; McGinnis et al., 1990; Zhao et al., 1993; Bachiller et al., 1994; Halder et al., 1995; Lutz et al., 1996). Likewise, mammalian bone morphogenetic protein 4 (BMP-4), a member of the TGF-β superfamily, and its direct inhibitor, chordin, have equivalent functions to *Drosophila* decapentaplegic (Dpp) and short gastrulation (sog), respectively, in dorsoventral axis formation (Holley et al., 1995).

The finding that *NK-2* class homeobox genes are expressed in the developing hearts of both *Drosophila* and vertebrates has prompted a deeper questioning into the mechanism of heart evolution and of the genetic pathways that underlie heart specification in embryos (for reviews see Olson and Srivastava, 1996; Harvey, 1996; Fishman and Olson, 1997). The structurally diverse hearts of invertebrates and vertebrates have, in the past, been considered examples of convergent evolution (Martin, 1980). However, new information on their embryonic origins from paired mesodermal progenitor populations and analogous modes of induction, hint at a deeper relationship (Bodmer, 1995; Harvey, 1996).

The *Drosophila* heart, or dorsal vessel, is a simple linear tube composed of inner muscular (cardial) and outer pericardial cell layers. Cellular haemolymph is drawn in through numerous valvular openings (ostia) in the posterior wall, and is pumped by unidirectional peristalsis out through a thinner anterior 'aorta'. Cardial cells are structurally similar to cardiomyocytes of the vertebrate heart, with intercalating disk-like adherens junctions linking myofilaments of neighbouring cells. In the embryo, the dorsal vessel is formed from paired mesodermal progenitor cells which arise in dorsal mesoderm and migrate to the dorsal midline. The TGF-β superfamily member Dpp is essential for specification of cardiogenic precursors in dorsal

mesoderm (Frasch, 1995), mediating an inductive interaction with ectoderm.

Although the hearts of vertebrates are far more complex, they develop through a simple linear heart tube intermediate. Heart formation in vertebrates is also initiated in response to inductive signalling, in this case from endoderm (for a review see Nascone and Mercola, 1996). Members of the BMP family (BMP-2 and BMP-4), cognates of *Drosophila* Dpp, are key mediators of induction, in concert with other factors (Schultheiss et al., 1996; Lough et al., 1996). Committed cardioblasts initiate expression of contractile protein genes and converge at the ventral midline to form a linear tube, which begins to beat. The heart tube then initiates looping morphogenesis, a process specific to vertebrates in which the future ventricular region adopts a sweeping rightward curvature. In birds and mammals, looping brings chamber primordia and vessels into their correct juxtaposition for subsequent remodelling into a multichambered organ (Olson and Srivastava, 1996). Neural crest and proepicardial cells then migrate into the heart from extracardiac sites, contributing to aortopulmonary septation and the coronary circulation, respectively.

Recent studies show that members of the NK-2 class of homeodomain transcription factors are expressed in both vertebrate and invertebrate hearts, and play key roles in their specification and differentiation (Bodmer et al., 1990; Lints et al., 1993; Komuro and Izumo, 1993; Tonissen et al., 1994; Evans et al., 1995; Buchberger et al 1996; Chen and Fishman 1996; Lee et al., 1996; Reecy et al., 1997). In *Drosophila*, the *NK-2* gene *tinman* (*tin*) is expressed in all cells of the blastula fated to become trunk mesoderm, then during invagination and lateral spreading of those cells at gastrulation (stage 7). As cells move dorsally during germ band extension (stage 8), *tin* expression is lost from ventral mesoderm, while being maintained in dorsal mesoderm by the inductive activity of Dpp (Frasch, 1995). During this second phase of expression, *tin* is proposed to perform all of its cell fate specification functions in the dorsal mesoderm from which cardiac, visceral and dorsal somatic muscle lineages arise (Azpiazu and Frasch, 1993; Bodmer, 1993).

In later embryonic development, *tin* expression becomes restricted only to heart. To date, only a single direct downstream target gene of the tin protein has been defined – the *DMef-2* gene, which encodes a MADS-box transcription factor essential for expression of myofilament genes during muscle differentiation (Bour et al., 1995; Lilly et al., 1995; Ranganayakulu et al., 1995). Activation of *DMef-2* by tin in cardioblasts is mediated by two high-affinity tin-binding sites in a cardiac-specific enhancer located upstream of the gene (Gajewski et al., 1997). Thus, tin has an early role in mesodermal patterning and a later role in heart myogenesis.

In vertebrates, several *NK-2* class homeobox genes related to *tin* have been identified (Buchberger et al., 1996; Harvey, 1996; Lee et al., 1996; Boettger et al., 1997; Brand et al., 1997; Reecy et al., 1997). A 'cardiac' subgroup of these (*Nkx2-3*, *Nkx2-5*, *Nkx2-6*, *Nkx2-7*, *Nkx2-8*) is expressed in heart and/or visceral muscle progenitors (depending on the species) and may have a *tin*-like function. However, their evolutionary relationship to *tin* is uncertain, since all vertebrate NK-2 proteins are more similar (within their homeodomains) to *Drosophila* members vnd or bap, than to tin itself. Furthermore, the tin protein is atypical for the class, lacking a conserved motif (the NK2-specific domain; NK2SD) found C-terminal to the homeodomain in all *Drosophila* members (except tin) and in all vertebrate members.

Thus far, *Nkx2-5* is the only member of the 'cardiac' group of vertebrate *NK-2* genes known to be expressed in heart progenitors of all experimental models examined (Harvey, 1996), suggesting a conserved role in heart commitment. Other expression features support such a function. Chicken *Nkx2-5* is activated as an early response to cardiac induction by BMP factors, while, in *Xenopus*, *Nkx2-5* expression encompasses the cardiac morphogenetic field, a highly regulative region from which definitive cardiac muscle cells are selected by positive and negative interactions. However, the *tin* mutant phenotype (loss of cardiac and midgut lineages) contrasts that of *Nkx2-5* in mice. In *Nkx2-5* mutants, the myocardial lineage is established apparently normally and a linear beating heart tube forms, although its downstream myogenesis and morphogenesis is perturbed (Lyons et al., 1995). This milder phenotype could result from genetic redundancy if other *NK-2* genes were expressed in early heart progenitors. Alternatively, *tin* and *Nkx2-5* may not be functionally equivalent, their modes of deployment in cardiogenesis having diverged.

To test directly whether mouse *Nkx2-5* possesses an early cardiogenic function equivalent to *tin*, and to assess whether such functions have been conserved, we introduced *Nkx2-5* and various mutant derivatives into the *Drosophila* germline and tested for their ability to rescue the *tin* mutant phenotype. Our results show that *Nkx2-5* can substitute for *tin* in specification of visceral mesoderm, but cannot rescue heart. Non-cardiac *NK-2* genes rescue neither. We have delineated the region of tin responsible for its cardiogenic function to within 52-amino acids at its N terminus. When transferred to Nkx2-5, this region conferred strong cardiogenic activity in transgenic flies. These findings demonstrate that the cardiac and visceral mesodermal functions of *NK-2* genes can be uncoupled in the *Drosophila* assay. They further highlight the close functional kinship between *tin* and *Nkx2-5* in their shared ability to bind and activate genes required for specification of visceral muscle within nascent mesoderm. However, the mode of deployment of *NK-2* genes in cardiogenesis in insects and vertebrates appears to have diverged. The data support a model in which cardiogenesis in these species is built upon a common ancestral program for specification of visceral muscle.

MATERIALS AND METHODS

Drosophila stocks

The tin^{EC40} mutant allele has a nonsense mutation in the recognition helix of the homeodomain and by all criteria is a null allele (Bodmer, 1993). bap^{208} is a severe hypomorph (Azpiazu and Frasch, 1993).

GAL-4 lines

The *twist GAL4* line was described (Baylies and Bate, 1996).The *DMef2-GAL4* line was made as follows. A 9.4 kb partially digested *Eco*RI fragment from a *DMef-2* genomic clone, containing the enhancer and promoter elements necessary for *DMef-2* expression in somatic, visceral and cardiac muscle lineages (Ranganayakulu et al., 1996), was blunt-ended and cloned into pGATN (Brand and Perrimon,

1993). Subsequently, a *Not*I-*Xba*I fragment, which includes the *D-Mef2* promoter and GAL4 sequences was cloned into the transformation vector, pCaSpeR-4 (Thummel and Pirrotta, 1992). Flies transformed with this GAL4 construct drove expression of a reporter gene first in all mesodermal cells of late stage 7 embryos and then in cardial cells of the heart, visceral and somatic muscle cells until the end of embryogenesis. A compound stock with *P(twist-GAL4)* on the second and *P(DMef2-GAL4)* along with the tin^{EC40} mutation on the third chromosome was constructed by recombination and maintained over a *TM3-Pw*$^+$*-lacZ* balancer. This stock was used in all the rescue experiments.

UAS lines

Wild-type and mutant derivatives of *Nkx2-5* and *tin* cDNAs were cloned into the pUAST vector (Brand and Perrimon, 1993). Deletion and hybrid constructs of *tin* and *Nkx2-5* cDNAs were made by PCR and cloned using appropriate restriction site overhangs. NkxΔC is a deletion of amino acids (aa) 202-318; NkxΔNK is a deletion of aa 202-232; N,HD-Nkx/C-tin is a gene fusion between aa 1-181 of Nkx2-5 and aa 346-416 of tin. tinΔC is a deletion of aa 364-416 of tin. tinΔHD is a deletion of aa 300-360 of tin. In tin(HDs) aa 304-345 of tin were replaced with aa 142-183 of Nkx2-5. N,HD-tin/C-Nkx is a fusion of aa 1-345 of tin and aa 184-318 of Nkx2-5. N-tin/HD,C-Nkx is a fusion of aa 1-304of tin and aa 142-318 of Nkx2-5. tin 1-220/HD,C-Nkx was made by fusing aa 1-220 of tin with aa 137-318 of Nkx2-5. tin 1-134/HD,C-Nkx is a gene fusion using aa 1-134 of tin with aa 137-318 of Nkx2-5. The homeodomains of chimeric proteins have a conservative change at position 1 [R→K in tin(HDs) and N-tin/HD,C-Nkx] and three alterations within aa 55-60 [SKRGD→CKRQR in N,HD-tin/C-Nkx and CKRQR→SKRGD in tin(HDs)]. All constructs were sequenced for accuracy. Stable transgenic lines were made by injecting various constructs into strain yw^{67c23} by P-element-mediated germline transformation and w$^+$ selection (Rubin and Spradling, 1982). For each construct, at least four lines with dark eye color were further tested for expression levels using tin and Nkx2-5 antibodies. All proteins accumulated in the nucleus.

Genetic crosses

For experimental crosses, the GAL4 line *(w; P(twist-GAL4); tin^{EC40} P(DMef2-GAL4)/TM3-Pw$^+$-lacZ)* was crossed with a test UAS line *(w; P(UAS-cDNA); tin^{EC40}/TM3-Pw$^+$-lacZ).* A quarter of the progeny embryos were of the genotype *w; P(twist-GAL4)/P(UAS-cDNA); tin^{EC40} P(D-Mef2-GAL4)/tin^{EC40}*. Balancer chromosomes carried either a *P(ftz-lacZ) or P(Ubx-lacZ)* insertion allowing the identification of heterozygous embryos from homozygous embryos. The *P(UAS-Nkx2-5)*-containing transgenic line was crossed into bap^{208} background to generate *w; P(UAS-Nkx2-5); bap^{208}/TM3-Pw$^+$-lacZ*. The *D-Mef2-GAL4* line was recombined with *bap^{208}/TM3-Pw$^+$-lacZ* to generate *w; bap^{208}P(D-Mef2-GAL4)/TM3-Pw$^+$-lacZ*. Embryos of the genotype *w; P(UAS-Nkx2-5)/+; bap^{208}/bap^{208}P(D-Mef2-GAL4)* were generated by intercrossing the GAL4 and UAS parental lines. Nkx2-5 was expressed in the wild-type embryos by crossing *w; P(twist-GAL4); P(D-Mef2-GAL4)* flies with *w; P(UAS-Nkx2-5)*. All progeny embryos carry one copy of *twist-GAL4; D-Mef2-GAL4* and one copy of *UAS-Nkx2-5*. Increasing the gene dosage of *Nkx2-5* was accomplished by crossing *w; P(UAS-Nkx2-5); P(UAS-Nkx2-5)* flies with the above GAL4 line. Resulting embryos carried two copies of *UAS-Nkx2-5*.

DMef-2 enhancer lines

A DNA fragment −3.56 to −5.962 kb upstream from the *D-Mef2* transcription start site (Lilly et al., 1995) was cloned into the CHAB transformation vector (Thummel and Pirrotta, 1992) and stable transformants were generated by germline transformation. This enhancer *P(DMef2-lacZ)*, drove *lacZ* expression from early stage 11 onwards. This enhancer fragment has two tin-binding sites which are required for *lacZ* expression in somatic muscle precursors, visceral mesoderm and heart precursors of stage 11 embryos. A line with a *P(DMef2-lacZ)* insertion on the X chromosome was crossed into *w; P(twist-GAL4); tin^{EC40}P(DMef2-GAL4)/TM3-Pw$^+$-lacZ* and a stock with the genotype *P(DMef2-lacZ); P(twist-GAL4); tin^{EC40}P(DMef2-GAL4)/TM3-Pw$^+$-lacZ* was established. To determine the effect of *Nkx2-5* and *tin* transgenes on the activity of *P(DMef2-lacZ)* in *tin* mutant background, flies with the genotype *w; P(UAS-cDNA); tin^{EC40}/TM3-Pw$^+$-lacZ* and *P(DMef2-lacZ); P(twist-GAL4); tin^{EC40}P(DMef2-GAL4)/TM3-Pw$^+$-lacZ* were intercrossed to generate *P(DMef2-lacZ)/+; P(twist-GAL4)/P(UAS-cDNA); tin^{EC40}/tin^{EC40}P(DMef2-GAL4).*

Immunochemistry and in situ hybridization

Immunochemistry was performed as described (Patel et al., 1987). Antibody dilutions were as follows: anti-tin (provided by R. Bodmer) 1:500, anti-Nkx2-5 1:300, anti-Fasciclin III (obtained from Developmental Studies Hybridoma Bank, University of Iowa) 1:40, anti-zfh1 (provided by Z. Lai) 1:1000, anti-eve (provided by M. Frasch) 1:2000, anti-D-Mef2 (provided by B. Paterson) 1:2000, anti-TTF-1 (provided by R. Di Lauro) 1:50, anti-β-galactosidase 1:1000. In situ hybridization of embryos with bap probe and double staining of embryos with antibody and bap probe was as described (Azpiazu and Frasch, 1993).

Expression and reporter plasmids

Full-length Nkx2-5 and tin cDNAs were introduced into the expression vector pEF1αFLAGpgkPURO (Huang et al., 1997); resultant proteins contained the FLAG (MDYKDDDDK) peptide at the N terminus. The expression plasmids containing full-length or various subfragments of tin and Nkx2-5 were generated by cloning immediately 3′ of the GAL4 DNA-binding domain ($GAL4^{1-147}$) in the pMvector (Sadowski et al., 1992). All clones were sequenced for accuracy. The p3xHA reporter plasmid has three tandem copies of the high-affinity Nkx2-5-binding site (3xHA: CTCAAGTGG) (Chen and Schwartz, 1995; Mohun, 1997) subcloned immediately 5′ of a minimal TATA-containing promoter in the pt81luc plasmid backbone (Nordeen, 1988). The GAL4-dependent reporters pG5Tluc and pG5E1BCAT were described previously (Chang and Gralla, 1994; Sadowski et al., 1992).

Cell culture and transient transfections

C3H10T1/2 cells were maintained in Dulbecco's modified medium supplemented with 10% fetal calf serum. For transient transfection assays 1.5×10^5 cells were plated in 6-well plates 24 hours before transfection. For determining the trans activation domain of Nkx2-5, cells were transfected using Lipofectamine (GIBCO BRL) in accordance with manufacturer instructions. A total of 1 μg of DNA was transfected: 0.7 μg expression vector, 0.2 μg of pG5Tluc reporter vector and 0.1 μg of an internal control vector pSV-β-galactosidase (Promega Corp). Cells were harvested 36 hours after transfection and assayed for luciferase and β-galactosidase activity. To determine the transcription activation domain of tin, transient cotransfections were carried out by calcium phosphate precipitation using pG5E1BCAT reporter . Transfections were carried out in 60 mm plates with 8 μg of reporter DNA and 4 μg of expression plasmid DNA. The vector pSV-β-galactosidase was used as a reference plasmid for determining transfection efficiencies. Cells were harvested 36 hours after transfection and assayed for CAT and β-galactosidase activity (Sambrook et al., 1989). Results represent the mean of three independent transfection assays, normalized to β-galactosidase activity.

RESULTS

In addition to an NK-2 class homeodomain, both tin and Nkx2-5 carry a conserved N-terminal sequence of 12 amino acids

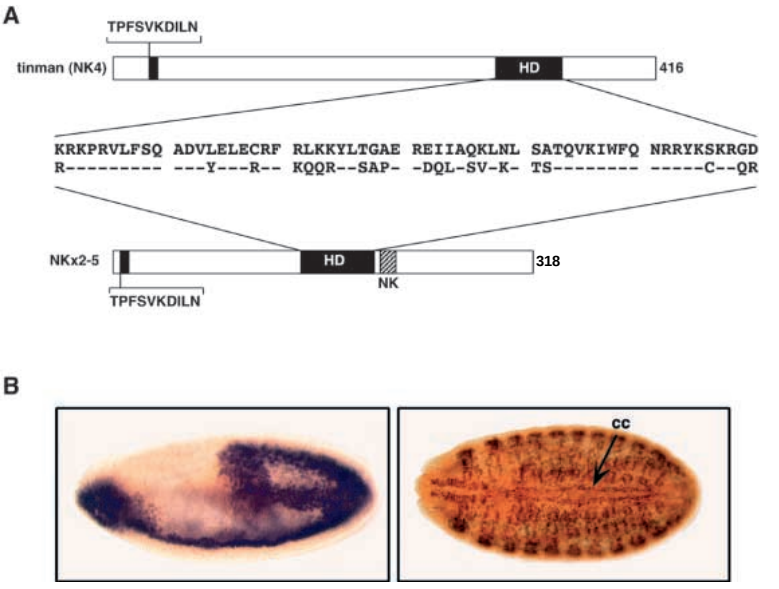


Fig. 1. (A) Schematic diagrams of Tin and Nkx2-5 proteins. Structural motifs in tinman and Nkx2-5 are shown. Comparison of the sequences of the homeodomains are shown in the center. Residues in Nkx2-5 that are identical to those in tin are indicated by a dash. The 10-amino acid TN domain is present near the N terminus of each protein. HD, homeodomain; NK, NK2SD.
(B) Expression of Nkx2-5 protein in transgenic embryos. Embryos with the genotype *twist-GAL4/UAS-Nkx2-5; DMef-2-GAL4/+* were immunostained with Nkx2-5 antibody as described in methods. Embryos were oriented with anterior to the left. The embryo in left panel is at stage 9 and the one in right is at 16. Note the persistence of transgene expression in the somatic musculature. cc, cardial cells.

(the TN domain), the core of which can be found in a range of other transcription factors (Fig. 1A; Lints et al., 1993; Smith and Jaynes, 1996). Outside of these domains, however, the two proteins show significant differences. Nkx2-5 is more typical of the class, possessing a longer region C-terminal to the homeodomain, which contains a conserved and class-specific motif termed the NK-2-specific domain (NK2SD) (see Harvey, 1996). Tin, on the contrary, has a shorter C terminus lacking the NK2SD, as well as a much longer N terminus (Fig. 1A).

The in vivo rescue assay

To express *Nkx2-5* in *tin* mutant embryos, we used the GAL4-UAS conditional targetting system (see Materials and Methods). In our strategy, a fly line expressing multiple copies of the gene for the yeast transcriptional activator GAL4, controlled by both *twist* and *DMef-2* promoters, was crossed to a second line carrying target genes (*tin*, *Nkx2-5* or their mutant derivatives), controlled by a GAL4-dependent upstream activating sequence (UAS). Such crosses resulted in activation of the target gene in exactly those cells expressing GAL4. Together, the *twist* and *DMef-2* promoters drive GAL4 expression in all cells of the embryonic mesoderm from stage 7 onwards, and subsequently in the cardiac, visceral and somatic musculature. Antibody staining of progeny embryos from transgenic crosses revealed that Nkx2-5 and most derivative proteins analysed (see below) could be stably expressed in presumptive mesodermal cells before gastrulation and, subsequently, in dorsal mesoderm and heart, at levels comparable to that of endogenous tin in wild-type embryos (Fig. 1B). Transgenes were also expressed in

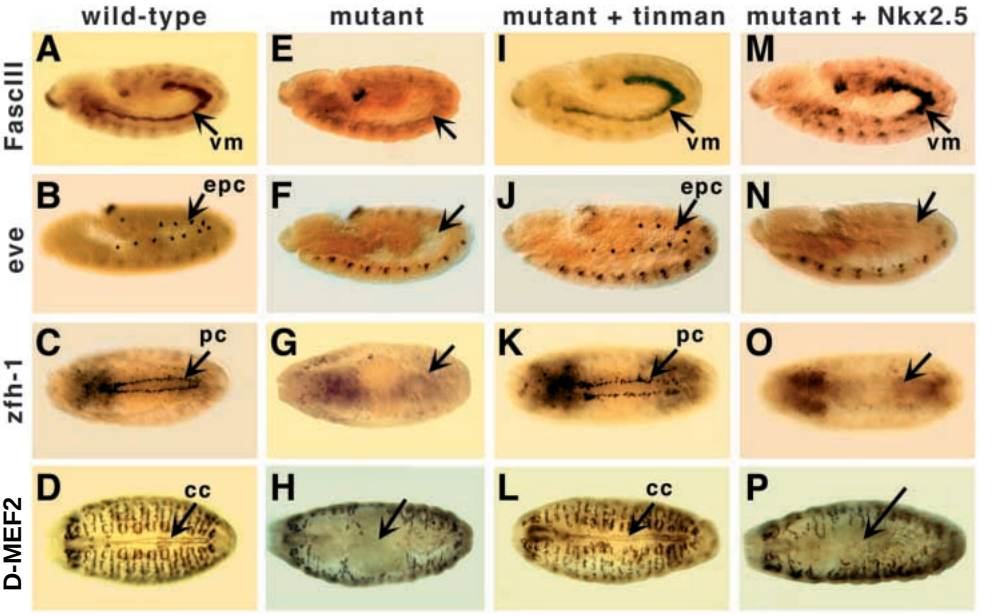


Fig. 2. Expression of muscle markers in tin mutant embryos rescued with *tin* and *Nkx2-5* transgenes. (A-D) Wild-type embryos; (E-H) tin^{EC40} embryos. Note the complete absence of Fasciclin III staining in the visceral mesoderm, *eve*-positive pericardial and dorsal somatic muscle founder cells, zfh-1-positive pericardial and cardial cells and *D-Mef2*-positive cardial cells. (I-L) tin^{EC40} embryos rescued with *UAS-tin*. Note the rescue of visceral mesoderm, different cell types of the dorsal vessel and dorsal somatic muscle founders. (M-P) tin^{EC40} embryos rescued with *UAS-Nkx2-5*. Note the rescue of only the visceral mesoderm. vm, visceral mesoderm; epc, eve-positive cells. Each eve-positive cluster has 3 or 4 cells, one of which is the founder cell of somatic muscle number 1 and the others form the pericardial cells (Lawrence et al., 1995); pc, pericardial cells; cc, cardial cells. Fasciclin III and eve staining are shown at stage 11 and zfh-1 and D-MEF2 at stage 16.

ventral mesoderm and the definitive somatic and visceral muscle lineages where endogenous tin is not expressed.

Since tin is essential for formation of heart, visceral mesoderm and dorsal somatic muscles, *Nkx2-5* was assessed for its ability to rescue the formation of these structures using a variety of markers for component cell types (see Fig. 2A-D): D-MEF2, a MADS-box transcription factor expressed in cardial cells of the heart as well as somatic and visceral muscles (Bour et al., 1995; Lilly et al., 1995); zfh-1, a zinc finger/homeodomain transcription factor expressed in pericardial and cardial cells (Lai et al., 1991); evenskipped (eve), a homeodomain factor expressed in a subset of pericardial cells and also founder cells of the dorsal somatic muscles (Frasch et al., 1987; Lawrence et al., 1995); Fasciclin III, a cell surface antigen expressed in visceral mesoderm (Patel et al., 1987) and *bap*, an *NK-2* class homeobox gene acting downstream of *tin* in visceral muscle (Azpiazu and Frasch, 1993).

Nkx2-5 rescues visceral mesoderm but not heart in *tin* mutant embryos

As noted above, heart and visceral mesoderm, and a subset of somatic muscle founder cells fail to form in *tin* mutant embryos (Fig. 2E-H). Enforced expression of *tin* in *tin* mutants using the GAL4-UAS system restored most of the dorsal vessel progenitors, as well as visceral mesoderm and somatic founder cells (Fig. 2I-L). In contrast, while *Nkx2-5* could rescue visceral mesoderm, as judged by staining for Fasciclin III (Fig. 2M), we found no trace of heart rescue using three cardiac lineage markers (Fig. 2N-P), or rescue of somatic muscle founder cells (Fig. 2N).

Previous studies have shown that *bap*, also an *NK-2* class homeobox gene, acts downstream of *tin* in specification of visceral mesoderm (Azpiazu and Frasch, 1993). Although not proven, *bap* may be a direct target gene of tin in visceral development. In *tin* mutants, *bap* expression is dramatically reduced (compare Fig. 3A and D) and, in *bap* mutants, midgut musculature is not formed (Azpiazu and Frasch, 1993). Since the degree of homeodomain homology between Nkx2-5 and bap (67%) is comparable to that seen between Nkx2-5 and tin (65%) (Lints et al., 1993), it was possible that Nkx2-5 could rescue visceral mesoderm in *tin* mutants by compensating for loss of *bap* expression. To address this issue, *Nkx2-5* and *bap* were compared for their ability to rescue visceral mesoderm in a *bap* mutant strain that lacks a morphological midgut (Azpiazu and Frasch, 1993; see Materials and Methods). In this mutant strain, *bap* rescued both Fasciclin III staining and a morphological midgut with its characteristic constrictions. However, *Nkx2-5* rescued none of those features (data not shown). Thus, *Nkx2-5* cannot functionally compensate for loss of *bap*, and its ability to rescue visceral mesoderm in *tin* mutants is most likely due to a *tin*-like activity. Accordingly, *tin* mutants carrying either *tin* or *Nkx2-5* transgenes activated *bap* expression in visceral mesoderm (Fig. 3G,J), indicating that *Nkx2-5*, like *tin*, can induce the expression of a key transcriptional regulator of the visceral muscle program.

Although *Nkx2-5* could activate Fasciclin III and *bap* expression in visceral mesoderm in *tin* mutants, antibody staining indicated that D-MEF2, a regulator of muscle differentiation in that lineage, was not induced (data not shown). Expression of a *DMef-2* enhancer-*lacZ* transgene was subsequently used to assay for *DMef-2* expression (see Materials and Methods). The *DMef-2* 5′ enhancer utilized carries two consensus tin DNA-binding sites that are essential for directing *lacZ* expression in visceral mesoderm, heart and a subset of somatic muscle precursors (Fig. 3B,C; B. Zhao, R. Cripps, and E. N. O., unpublished results). Confirming the immunochemistry results, we found that *Nkx2-5* was unable to activate the *DMef-2* enhancer-*lacZ* reporter in the visceral lineage (Fig. 3K,L). As expected, no heart progenitors were

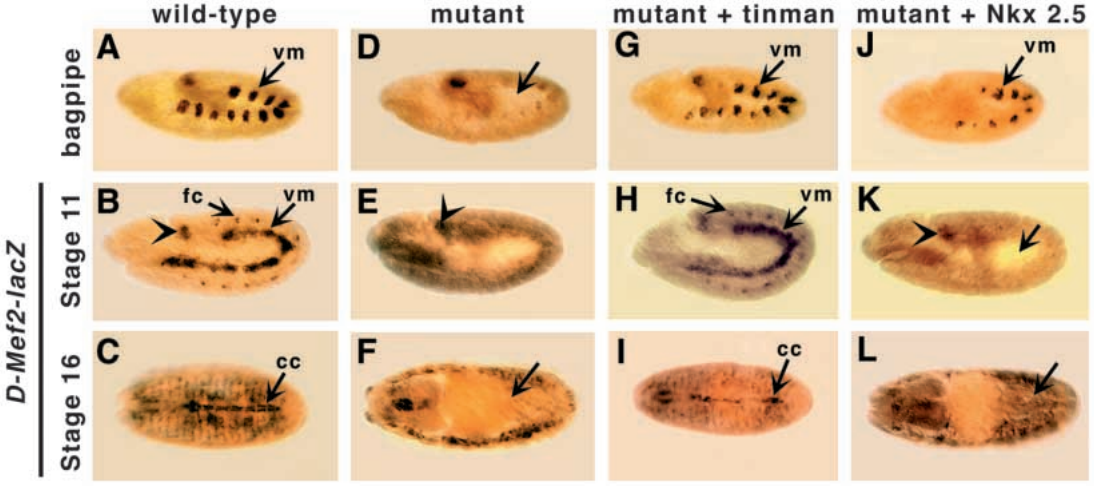


Fig. 3. Nkx2-5 specifies visceral mesoderm but cannot activate the *DMef-2* visceral mesoderm enhancer. (A,D,G,J) Detection of *bap* expression by in situ hybridization. In the wild-type embryo (A), there are 11 *bap*-expressing patches. A subset of these cells becomes specified as visceral mesoderm (Azpiazu and Frasch, 1993). *bap* expression is dramatically reduced in a tin^{EC40} mutant embryo (D). G and J are tin^{EC40} mutant embryos rescued with *UAS-tin* and *UAS-Nkx2-5*, respectively. Note the significant increase in the levels of *bap* expression over mutant. (B,E,H,K) *DMef-2*-enhancer-*lacZ* expression. In the wild-type embryo (B), expression is detected in the visceral mesoderm (vm) and also in the founder cells (fc) of the ventral somatic mesoderm. Expression is also seen in the presumptive hindgut area (arrowhead in all panels). In the tin^{EC40} mutant embryo, expression is completely abolished except in the presumptive hindgut area (E). H and K are tin^{EC40} mutant embryos rescued with *UAS-tin* and *UAS-Nkx2-5*, respectively. Tin activates *lacZ* activity in the visceral mesoderm and also in founder cells. Nkx2-5 activates *lacZ* activity only marginally in a few founder cells but not in the visceral mesoderm. (C,F,I,L) Expression of *DMef-2-lacZ* in cardial cells of wild-type (C) and mutant (F) embryos. *UAS-tin* transgene rescues *lacZ* expression in the cardial cells (I) and *UAS-Nkx2-5* does not (L). vm, visceral mesoderm; fc, somatic muscle founder cells; cc, cardial cells.

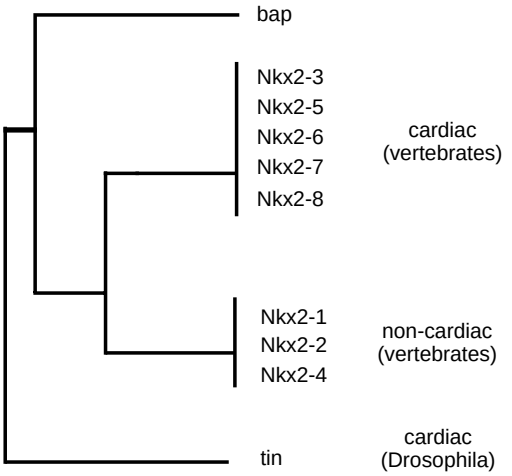


Fig. 4. Simplified dendrogrammatic representation of sequence relationships of NK class homeogenes**.** Figure was modified and adapted from Harvey (1996). Classification of vertebrate members into cardiac and non-cardiac groups was based on their expression in the cardiac lineage. Nkx2-3 is cardiac in frog and chicken (Evans et al., 1995; Buchberger et al., 1996); Nkx2-5 is cardiac in frog, chicken, fish and mice (Lints et al., 1993; Evans et al., 1995; Chen and Fishman, 1995; Buchberger et al., 1996); Nkx2-6 is cardiac in mice (Biben et al., 1998); Nkx2-7 is cardiac in fish (Lee et al., 1996); Nkx2-8 is cardiac in chicken (Reecy et al., 1997). bap is also expressed in a few cells of the dorsal vessel of *Drosophila* embryos, but does not appear to be required there early (Azpiazu and Frasch, 1993)..

Table 1. Rescue of *tin* mutant phenotypes with wild-type and mutant derivatives of *tin* and *Nkx2.5*

	Visceral markers		Cardiac markers		
Constructs	Fasc III	DMef2-lacZ	eve	Zfh-1	D-MEF2
tinman	+++	+++	+++	+++	+++
Nkx2.5	++	–	–	–	–
tin (HDs)	+++	+++	+++	+++	+++
tin ΔHD	–	–	–	–	–
Nkx ΔNK	++	+	±	–	–
Nkx ΔC*	–	–	–	–	–
N,HD-Nkx/C-tin	±	–	–	–	–
N,HD-tin/C-Nkx	+++	+++	+++	+++	+++
N-tin/HD,C-Nkx	+++	+++	+++	+++	+++
tin ΔC	+++	+++	+++	+	+
tin 1-220/HD,C-Nkx	+++	+++	+++	+++	+++
tin 1-134/HD,C-Nkx	+++	+++	+++	+++	+++
tin Δ43-123	+++	+++	++	++	++
tin 1-53/Nkx 54-319	+++	+++	++	++	++
bagpipe	–	ND	–	ND	ND
TTF-1(Nkx2.1)	–	ND	–	ND	ND

*Construct is unstable.

+++, 70-100% of wild type; ++, 40-70% of wild type; +, 20-40% of wild type; ±, less than 20% of wild type; –, no rescue; ND, not determined.

Percentage rescue was arbitrarily calculated relative to expression in wild-type embryos. In stage 11 embryos, Fasciclin III is expressed as a continuous band in the visceral mesoderm underlying parasegments 2-12 and eve is expressed in 11 pairs of clusters in the dorsal mesoderm. In stage 16 embryos, D-MEF2 is expressed in ~6 pairs of cardial cells and Zfh1 is expressed in ~4-5 pairs of pericardial cells in T2-A6 segments. The expression of Zfh-1 in the cardial cells was not taken into consideration in this assay.

evident using this marker, although a few somatic muscle precursors showing weak expression were revealed. The results suggest that *Nkx2-5* can only partially substitute for *tin* in visceral mesoderm development: it can activate *bap* and Fasciclin III, but not *DMef-2*.

The homeodomains of tin and Nkx2-5 are interchangeable

Based on homeodomain comparisons, *NK-2* genes and their encoded homeoproteins fall into distinct phylogenetic sister groups (Fig. 4). Members of different groups recognize the same high-affinity DNA-binding site in vitro (consensus: 5′ T(C/T)AAGTG) (Mohun, 1997; Chen and Schwartz, 1995). Those genes most closely related to *Nkx2-5*, referred to here as the ‘cardiac’ group, are expressed in heart and/or visceral muscle progenitors in various vertebrate species. In contrast, none of the genes in the distinct sister group containing *Nkx2-1* (also called *TTF-1, T/ebp* and *titf1)* are expressed in those lineages.

Two points relating to these findings were examined. First, we determined whether the different activities of tin and Nkx2-5 in rescue experiments were due to differences in their inherent DNA-binding specificity. A chimeric protein (tin(HDs)) was created in which the homeodomain of tin was swapped with that of Nkx2-5 (see Fig. 8A). Expression of this chimeric protein in *tin* mutant embryos rescued heart development to the same extent as wild-type tin (Table 1). A tin mutant lacking the homeodomain (tin ΔHD) was totally inactive (Table 1).

We next examined whether visceral mesoderm could be rescued by members of ‘non-cardiac’ sister groups. We expressed the rat *Nkx2-1* gene, transcribed in developing thyroid, lung and brain (Price et al., 1992), as well as *Drosophila bap*, in *tin* mutants (see Fig. 4). No evidence for visceral mesodermal rescue was detected with either gene, as judged by Fasciclin III staining (Table 1). The results suggest that the ability of Nkx2-5 to rescue visceral mesoderm in *Drosophila* is specific to the cardiac group of vertebrate *NK-2* genes. In this stringent assay, the homeodomains of tin and Nkx2-5 are interchangeable, indicating that their distinct activities must be conferred by sequences outside of the homeodomain.

Nkx2-5 inhibits cardiogenesis through a dominant-negative activity

Since Nkx2-5 has an identical DNA-binding specificity to tin, yet lacks cardiogenic activity in the rescue assay, we reasoned that it might be able to function as a dominant-negative inhibitor of *Drosophila* cardiogenesis by competing with endogenous tin for binding sites within its target genes. To test this idea, we expressed *Nkx2-5* in wild-type embryos using the GAL4-UAS system and analyzed for marker gene expression. While transgenic embryos showed no defects in Fasciclin III expression in visceral mesoderm, heart formation assayed by expression of eve and zfh-1 was severely compromised (Fig. 5). When the dosage of the Nkx2-5 transgene was increased (see Materials and Methods), formation of cardial and pericardial cells was all but eliminated. These results support the conclusion drawn above that the activities of tin and Nkx2-5 in the rescue assay do not relate to differences in DNA-binding specificity. Presumably, a unique domain of tin, missing in Nkx2-5, confers its ability to rescue heart.

Deletion of an inhibitory domain within Nkx2-5 rescues some eve-positive cells

Previous studies have demonstrated that sequences within the C terminus of Nkx2-5 strongly inhibit transcriptional activity in transient transfection assays (Chen et al., 1995). Deletion of the conserved NK2SD leads to a modest increase in transcriptional activity, further enhanced by removal of all sequences C-terminal to the homeodomain. Whether the NK2SD masks the activity of a transactivation domain or influences DNA-binding affinity is not yet known.

To determine whether the C terminus of Nkx2-5 masks a cardiogenic function in *Drosophila*, two C-terminal deletion mutants similar to those examined in vitro were tested for their ability to rescue heart and visceral mesoderm in *tin* mutants (Fig. 8A). One mutant lacking essentially all amino acids C-terminal to the homeodomain (NkxΔC) was unstable and could not be assayed. In contrast, a mutant lacking only the NK2SD (NkxΔ NK) was stable and produced three new effects when compared to wild-type Nkx2-5: (1) it rescued visceral mesoderm to a greater extent, as judged by Fasciclin III staining; (2) it now activated the *DMef-2* enhancer-*lacZ* reporter in visceral mesoderm and (3) it rescued 2 or 3 clusters of eve-positive cells in a few (~10%) mutant embryos (Fig. 6; Table 1). Thus, deletion of the NK2SD improves both the extent and integrity of the rescued visceral lineage. A negative regulatory function for this domain can therefore be demonstrated both in vitro (Chen et al., 1995) and in vivo. In rescuing a few eve-positive cells, the deletion mutant may also unmask a weak cardiogenic activity for Nkx2-5 (see Discussion).

Analysis of transactivating functions of tin and Nkx2-5

The rescue of a few eve-positive cells by mutant Nkx ΔNK (see above), raised the possibility that the differences between tin and Nkx2-5 in the rescue assay might relate to their relative potencies as transcriptional transactivators. To examine this issue, we set out to map the transactivation domains of the two proteins. Preliminary transient transfection assays in cultured mouse fibroblasts utilizing a multimerized high-affinity tin/Nkx2-5-binding site (Mohun, 1997) linked to a minimal promoter and reporter gene, indicated that tin was approximately 10-fold more active than wild-type Nkx2-5 (Fig 7A). On the contrary, as noted above, Nkx2-5 activity is masked by negative regulatory sequences within its C terminus (Chen et al., 1995). To resolve this uncertainty, the transactivation domains of tin and Nkx2-5 were mapped in finer detail by fusing subregions of the proteins to a GAL4 DNA-binding domain and assaying with a GAL4-dependent reporter gene after transient transfection into mouse C3H $10T^1/_2$ fibroblasts (Fig. 7B,C). For tin, a strong transactivation domain was revealed between amino acids 54-134, comparable in strength to the potent transactivation domain of the viral protein VP16 (data not shown).

Previous studies have delimited an N-terminal transactivation domain within Nkx2-5 to amino acids 42-121 (Chen et al., 1995). In the GAL4 assay, we found that Nkx2-5 carries two domains capable of transactivation, located at or near its N and C termini. The C-terminal domain appears to include the last 17 amino acids, since deletion of this region eliminated activity. Two transactivation domains have also been described in similar positions within Nkx2-1 (De Felice et al., 1995). The potency of each Nkx2-5 domain was approximately 10% of that of the tin transactivation domain (Fig. 7C).

To determine whether the potent transactivation domain of tin is required for cardiogenic function, an internal deletion mutant of tin (tin Δ43-123) which removed amino acids 43-123 was tested for activity in the rescue assay. Surprisingly, mutant embryos expressing this construct still convincingly rescued heart and visceral mesoderm, although more weakly than wild-type tin (Fig. 8A; Table 1). We conclude that the transactivation domain of tin is not essential for its cardiogenic activity, and therefore that the differences between tin and Nkx2-5 do not relate to differences in potency of their respective transactivation domains. The cardiogenic activity of tin must be conferred primarily by a region outside of the transactivation domain. Since the 43-123 amino acid deletion altered the invariant core of the conserved TN-domain (DILN to DLYG), it is likely that this domain is also non-essential for cardiogenic function.

The N terminus of tin carries the cardiogenic domain

We next turned our attention to locating the specific domain within tin that confers cardiogenic activity. Two chimeric proteins were created in which the C termini of tin and Nkx2-5 were exchanged. The mutant N,HD-tin/C-Nkx, carrying the tin N terminus, homeodomain and Nkx2-5 C terminus, retained the ability to rescue heart formation, whereas the reciprocal mutant N,HD-Nkx/C-tin was inactive (Fig. 8A; Table 1). These results demonstrate that the cardiogenic regulatory domain of tin lies within its N terminus. An additional chimera (N-tin/HD, C-Nkx), identical to N,HD-tin/C-Nkx except that it contained the Nkx2-5 homeodomain, also functioned as effectively as tin to rescue heart formation (Table 1), confirming that the cardiogenic activity of tin is not specified by its homeodomain.

To assess whether the cardiogenic function of tin was wholly confined to the N terminus, the mutant tin ΔC, lacking most amino acids C-terminal to the homeodomain, was examined. This mutant was able to rescue eve-positive pericardial cells strongly, but zfh-1 and *DMef-2*-positive pericardial and cardial cells only weakly (Fig. 8A; Table 1). Thus, while the N terminus of tin carries the strong cardiogenic domain, its efficacy for certain downstream cardiogenic functions appears to be modulated by the C terminus. However, as noted above, substitution of the C terminus of Nkx2-5 with that of tin (mutant N,HD-Nkx/C-tin) did not confer upon Nkx2-5 the ability to rescue heart (Table 1).

The N-terminal cardiogenic domain of tin can be transferred to Nkx2-5

We further mapped the cardiogenic domain of tin by making a series of N-terminal deletion mutants. Constructs containing N-terminal amino acids 1-220 or 1-134 of tin fused to the homeodomain and C terminus of Nkx2-5 (mutants tin 1-220/HD,C-Nkx and tin 1-134/HD,C-Nkx) were both able to strongly rescue heart formation in *tin* mutant embryos (Fig. 8A; Table 1). These results suggested that the region of tin spanning amino acids 1-134 contained the cardiogenic activity. Since deletion of amino acids 43-123 within this region had no deleterious effect (see above), it appeared that the essential

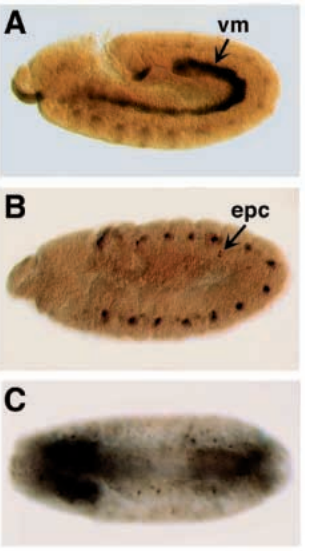


Fig. 5. Nkx2-5 inhibits cardiogenesis in wild-type *Drosophila* embryos. Stage 11 embryos were analyzed for (A) Fasciclin III and (B) eve expression. (C) A stage 16 embryo was stained with Zfh-1 antibody. All embryos shown carry one copy of *twist-GAL; DMef-2-GAL* and *UAS-Nkx2-5*. Note the loss of most of eve and Zfh-1 expression (compare levels of expression with embryos shown in Fig. 2B,C.

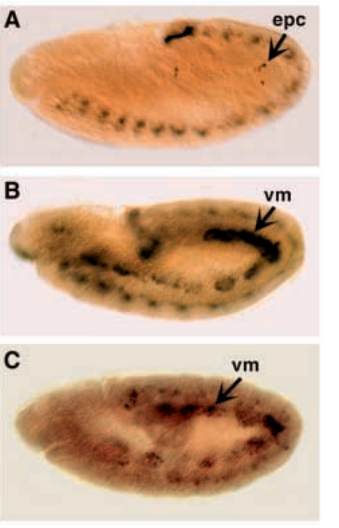


Fig. 6. Acquisition of weak cardiogenic activity by Nkx2-5 following deletion of the C-terminal inhibitory NK2SD. *tin*^{EC40} mutant embryos were rescued with NkxΔNK2 construct (see Fig. 8). Stage 11 embryos were analyzed for eve (A), Fasciclin III (B) and*DMef-2* enhancer *lacZ* (C) expression. Note the weak rescue of few eve-positive cells in the dorsal mesoderm (compare with Fig. 2N) and also activation of *DMef-2* enhancer *lacZ* in the visceral mesoderm (compare with Fig. 3K).

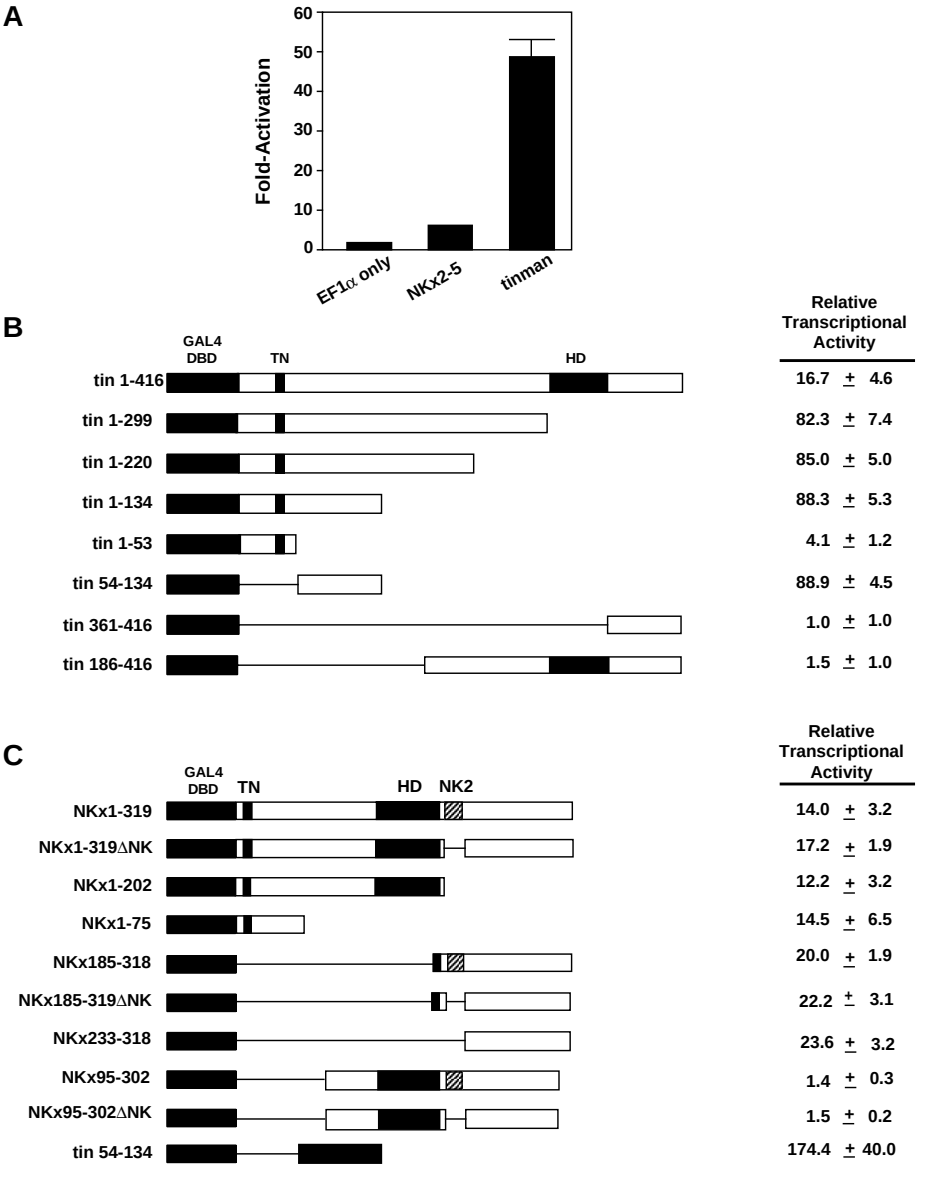
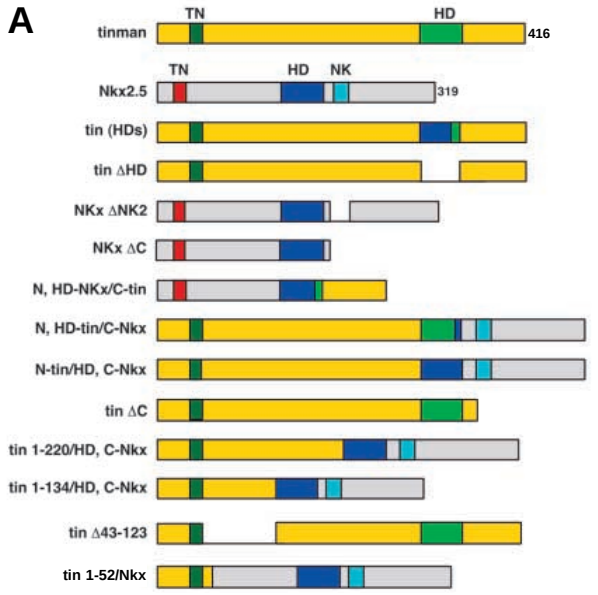


Fig. 7. Transcriptional activity of *tin* and *Nkx2-5*. (A) C3H10T1/2 fibroblasts were transiently transfected with a luciferase reporter gene linked to three tandem copies of the high-affinity Nkx2-5-binding site, along with expression vectors encoding Nkx2-5 or tinman under control of the EF1α promoter as described in Methods. Transcriptional activities are expressed relative to the level of reporter gene expression in the presence of the EF1α expression vector lacking a cDNA insert. (B) Mapping of transcription activation domain of tin. GAL4 and GAL4/tin chimeric proteins were assayed in C3H10T1/2 fibroblasts for transcriptional activity by cotransfection of expression constructs with a GAL4-dependent CAT reporter. Values are expressed relative to the level of CAT activity in cells transfected with the CAT reporter construct and empty expression vector. (C) Identification of transactivation domains in Nkx2-5. GAL4 and GAL4/Nkx2-5 chimeric proteins were assayed for transcriptional activity with a GAL4-dependent luciferase reporter in C3H10T1/2 fibroblasts. Data is presented as fold activation above the activity seen with the GAL4DBD alone with luciferase reporter. Values shown are the mean and standard deviation of three independent experiments.



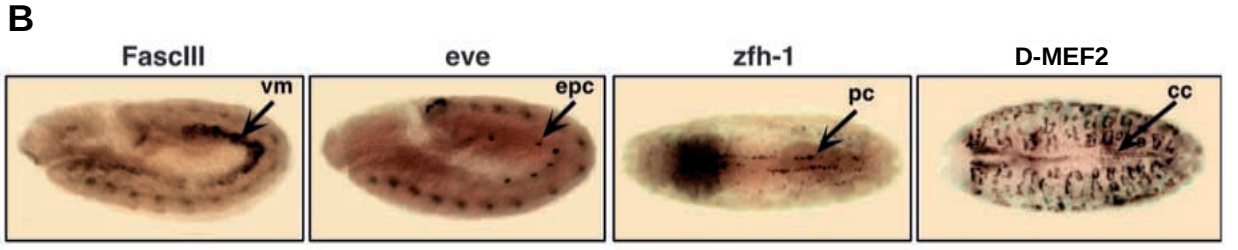


Fig 8. (A) Mutational analysis of tin and Nkx2-5. Schematic representation of tin and Nkx 2-5 deletion and fusion constructs. The sizes of wild-type constructs are indicated with amino acid numbers. In tin (HDs), amino acids 304-345 of tin were substituted with amino acids 142-183 of Nkx2-5 and the resulting homeodomain (HD) has a conservative R→K change at position 1 and three alterations within amino acids 55-60 (CKRQR→SKRGD). The homeodomain in construct N,HD-tin/C-Nkx has three alterations within amino acids 55-60 (SKRGD→CKRQR). The homeodomain in construct N-tin/HD, C-Nkx has a conservative R→K change at position 1. Lengths are approximately to scale. HD, homeodomain; TN, a conserved decapeptide domain at the amino terminus; NK, a 17 amino acid domain present in several NK-class proteins. (B) A domain at the N terminus of tin (amino acids 1-52) confers on Nkx2-5 the ability to rescue heart formation in tin mutant embryos. *tin*^{EC40} embryos were rescued with *UAS-tin1-52/Nkx*. vm, visceral mesoderm; epc, eve-positive cells; pc, pericardial cells; cc, cardial cells. Markers are shown above each panel.

region was contained either within amino acids 1-42 or 124-134. We therefore fused N-terminal amino acids 1-52 of tin to amino acids 53-319 of Nkx2-5 (Fig. 8A) and tested for rescue. This N-terminal tin domain conferred full cardiogenic activity on Nkx2-5 (mutant tin 1-52/Nkx; Fig. 8B).

DISCUSSION

Our results provide key insights into the molecular mechanisms of mesoderm specification by *NK-2* homeobox genes. First, the rescue assay has allowed us to discriminate between the activities of *NK-2* genes in cardiac and visceral development. They show that, while *tin* rescued both cardiac and visceral lineages in the *tin* mutant background, *Nkx2-5* rescued only visceral. Both types of rescue are specific, since non-cardiac *NK-2* genes rescued neither. The lack of rescue with Nkx2-5 was not due to its inability to activate the *Dmef-2* enhancer, used along with the *twist* enhancer to drive GAL4 expression, since heart rescue with tin could be achieved with the *twist* enhancer only (data not shown). Our findings are in agreement with similar experiments using the heat-shock overexpression system, showing that different cardiac *NK-2* homeogenes rescue only visceral development in *tin* mutants (R. Bodmer, personal communication). These data demonstrate a close functional kinship between tin and the cardiac group of vertebrate NK-2 proteins – both can specifically recognize and activate target genes involved in visceral mesodermal specification. However, within the bounds of this assay, they diverge with respect to their roles in cardiogenesis.

Specification of visceral mesoderm by *tin* and *Nkx2-5*

In the *Drosophila* embryo, *tin* is essential for specification of dorsal mesoderm from which the cardiac, visceral and dorsal somatic muscle lineages arise. Both heart and gut mesoderm have a segmental origin, each structure arising from metamerically organized cell clusters arranged in dorsal mesoderm in an alternating pattern (Azpiazu and Frasch, 1993; Azpiazu et al., 1996; Jagla et al., 1997). Several signaling molecules and mesoderm-specific transcription factors have been implicated in subdivision of dorsal mesoderm into these alternating cardiac and visceral segments (Azpiazu et al., 1996; Lawrence et al., 1995; Riechmann et al., 1997; Wu et al., 1995), although how they influence the function of tin is not known.

In the visceral lineage, a genetic relationship between *tin* and *bap* has been established (Azpiazu and Frasch, 1993) and, indeed, *bap* may be a direct target gene of the tin protein. In the rescue experiments reported here, *Nkx2-5* was able to mimic the activity of *tin* in activating *bap* and specifying the visceral program in *tin* mutants. Enforced expression of *Nkx2-5* in *bap* mutants demonstrated that *Nkx2-5* could not substitute for *bap* itself. *Nkx2-5* also induced Fasciclin III in visceral mesoderm, and *DMef-2*, as long as the NK2SD was deleted. An important

conclusion emerges from these results. The fact that Nkx2-5 can activate two key transcription factors for visceral development, as well as an adhesion molecule, highlights the close functional kinship between *Nkx2-5* and *tin*, remarkable since insects and mammals have been separated by more than 500 million years of evolution. Despite its atypical structure, *tin* can now be allied with the cardiac group of *NK-2* genes, and clearly set apart from sister groups containing *Nkx2-1* and *bap*, which cannot rescue visceral development. The ability of *Nkx2-5* to specify the visceral program in *Drosophila* may relate directly to its activity in vertebrates: while *Nkx2-5* itself is expressed only in a small region of visceral mesoderm at the inferior aspect of the stomach and in spleen, its close relative *Nkx2-3* is expressed extensively throughout developing midgut and hindgut mesenchyme (Pabst et al., 1997). These genes do not appear to be expressed in nascent mesoderm, and so their relative roles in specification versus differentiation in the visceral lineage requires further clarification. It is worth noting, however, that in *tin* mutants dorsal mesoderm does not form, and so the ability of Nkx2-5 to rescue visceral mesoderm implies that it retains the capacity to specify this lineage within naive mesoderm, in addition to participating in its differentiation. In general terms, shared genetic pathways may be adapted to different contexts. Thus, it is possible that *Nk-2* genes no longer play a specification role in mice, even though they retain the ability to do so in the fly assay.

Regulation of the *D-Mef2* enhancer by NK-2 homeodomain factors

Previous studies have shown that tin directly activates expression of the *Dmef2* gene via a cardiac-specific enhancer containing two high-affinity tin-binding sites (Gajewski et al., 1997). However, Nkx2-5 can not activate this enhancer in embryos and can only activate it weakly in tissue culture cells (G. R. and E. N. O., unpublished results). More recent evidence indicates that the same enhancer is used along with additional flanking sequences for expression in visceral mesoderm and a subset of ventral somatic muscle founder cells (R. Schulz, personal communication; B. Zhao, R. Cripps and E. N. O., unpublished results). However, bap, also an NK-2 protein expressed in visceral mesoderm, can not activate this enhancer in vitro (unpublished results). Thus, the enhancer can discriminate between related NK-2 homeodomain proteins. These findings hint that activation of target genes by NK-2 proteins is mechanistically heterogeneous, perhaps through associations with different accessory factors.

The N-terminal cardiogenic domain of tin

Studies on other homeodomain proteins have demonstrated that binding to particular sets of target genes is directed by the homeodomain alone, but that selectivity of activation depends on regions outside the homeodomain (Kuziora and McGinnis, 1989; Gibson et al., 1990; Mann and Hogness, 1990; Morrissey et al., 1991). This also appears to be true for tin and Nkx2-5: tin protein carrying an Nkx2-5 homeodomain can still rescue heart. Furthermore, Nkx2-5 acts as a dominant-negative repressor of cardiogenesis in *Drosophila*, presumably by competing for tin-binding sites in its target genes. Our findings suggest that Nkx2-5 and tin can bind common target genes required for the cardiac and visceral programs, but that transcriptional selectivity of tin for the cardiac program is dependent upon sequences outside of its homeodomain. Mutational analysis has shown that the sequences that confer cardiac induction are contained within a distinct region at the N terminus of tin excluding the transactivation domain. This sequence presumably makes contacts with accessory factors that allow tin to select only cardiac targets, analogous to those modifying the selectivity of other homeodomain proteins (Stern et al., 1989; Ananthan et al., 1993; Popperl et al., 1995; Copeland et al., 1996). Alternatively, this region of tin may be post-translationally modified in the cardiac lineage, in a manner that affects its transcriptional function or ability to bind cofactors. Further characterisation of the N-terminal tin domain should extend our knowledge of *Drosophila* cardiogenesis and tin function.

NK-2 homeobox genes and evolution of cardiac programs

Nkx2-5 rescues visceral mesoderm, but not heart, in the *Drosophila* rescue assay. This would appear to indicate basic mechanistic differences between the cardiogenic programs of flies and mammals. However, we must consider the possibility that similar programs have diverged just enough to be non-complementary in the trans-species assay. A hint that this is the case may come from the ability of the Nkx2-5 mutant, Nkx ΔNK, to rescue a few eve-positive pericardial cells (1-3 clusters in 10% of embryos). Since this mutant also enhanced visceral rescue, it may be a more potent transcriptional activator, sufficiently so to overcome the evolutionary drift and activate the cardiac program in a few cases. In contrast, our finding of a unique region at the N terminus of tin, not encompassing the transactivation domain, and which can confer cardiogenic activity to Nkx2-5, does not support similar mechanisms in mammals and flies. We could find no homology between the tin N-terminal domain and Nkx2-5, and our mutational analysis suggests that the adjacent shared TN-Domain, the core of which is present in a host of other transcription factors (Smith and Jaynes, 1996), is not part of the cardiogenic activity. Thus, our data favour a model of divergent cardiogenic mechanisms in flies and mammals. While the phenomenon of weak rescue with mutant Nkx ΔNK remains to be explained, it could conceivably occur because regulation of Nkx2-5 activity has become deranged. The induction of larger hearts and hypertrophic cardiomyocytes in frog and fish embryos by ectopic expression of *Nkx2-5* (Fu and Izumo, 1995; Chen and Fishman, 1996; Cleaver et al., 1996), and the defective myogenesis and morphogenesis seen in the hearts of *Nkx2-5* knockout mice (Lyons et al., 1995), suggest that *Nkx2-5* has indeed acquired a variety of cardiogenic functions unique to vertebrate systems each of which may be finely regulated. The work presented here provides the basis for a more intricate dissection of the role of the tin N-terminal domain and of how the various cardiogenic functions of Nkx2-5 are imparted.

Phylogenetic comparisons suggest that the hearts of vertebrates and invertebrates evolved as independent adaptations of pulsatory muscular vessels, themselves derived from visceral musculature (Harvey, 1996). The results of the rescue experiments also suggest a link between visceral mesoderm and heart, and allow us to formulate a more precise evolutionary hypothesis: that the genetic circuitry underlying cardiac development in mammals and insects has been built upon a common and more ancient program for specification of

visceral muscle, one which utilized *NK-2* homeogenes. The ability of zebrafish *nkx2-5* to rescue the function of pharyngeal muscles in nematodes lacking the *NK-2* gene *ceh-22* (Haun et al., 1998) may also reflect the common ancestral role for *NK-2* genes in visceral development. In this context, it is interesting that *ceh-22* clearly lies within the 'non-cardiac' clade by phylogenetic analysis (Harvey, 1996), the group that appears to lack rescue function in the fly assay. Thus, the nematode rescue assay reveals a myogenic function for both cardiac and non-cardiac genes.

In summary, we have demonstrated that the genetic functions of NK-2 homeoproteins tin and Nkx2-5 in cardiogenesis are not freely interchangeable in a trans-species assay. Nevertheless, their shared ability to specify visceral mesoderm in *Drosophila* suggests a close evolutionary relationship between the two proteins, and a common ancestral mechanism upon which the heart programs of vertebrates and invertebrates were independently built. Certain features of mammalian cardiogenesis, those relating to the more ancient visceral program, will be accessible through genetic analysis in *Drosophila*.

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