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* germ line, 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 chambers 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 aortic-pulmonary 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 tinECI mutant allele has a nonsense mutation in the recognition helix of the homeodomain and by all criteria is a null allele (Bodmer, 1993). bap208 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 EcoRI 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).
1993). Subsequently, a NofI-XbaI 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 cardiac 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 titEC40 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 overlaps. NkxAC is a deletion of amino acids (aa) 202-318; NkxΔNK is a deletion of aa 202-232; N;HD-NkxC-tin is a gene fusion between aa 1-181 of Nkx2-5 and aa 346-416 of tin. tinAC is a deletion of aa 364-416 of tin. tinΔH 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 1-304 of tin and aa 142-318 of Nkx2-5. N-tin/HD,C-Nkx is a fusion of aa 1-304 of tin and aa 142-318 of Nkx2-5. Tin-220/HD,C-Nkx was made by fusing aa 1-220 of tin with aa 137-318 of Nkx2-5. Nkx is a fusion of aa 1-304 of tin and aa 184-318 of Nkx2-5. Nkx/HD,C-Nkx is a fusion of aa 1-304 of tin and 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].

**Immunocytochemistry and in situ hybridization**

Immunocytochemistry 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 hap probe and double staining of embryos with antibody and hap 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 pEFl.oFGLAgpgpuRO (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 (GAL4147) in the pMvec (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′ downstream from the promoter. The p3xHA reporter plasmid was introduced into the fly genome by P-element-mediated germline transformation and w+; bap 208 P(D-Mef2-GAL4)/TM3-Pw+–lacZ. 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 cardiac cells of the heart.

**DMeif2 enhancer lines**

A DNA fragment 3.36 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 transfectants 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); tinEC40/P(DMef2-GAL4)/TM3-Pw+–lacZ and a stock with the genotype P(twist-GAL4); tinEC40/P(DMef2-GAL4)/TM3-Pw+–lacZ. Full-length Nkx2-5 and tin cDNAs were introduced into the expression vector pEFl.oFGLAgpgpuRO (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 (GAL4147) in the pMvec (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 p81Luc plasmid backbone (Nordeen, 1988). The GAL4-dependent reporters pG5Tluc and pG5E1BACat 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 1.5x105 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. The transactivation domain of Nkx2-5, transient cotransfections were carried out by calcium phosphate precipitation using pG5E1BACat 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.
(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 targeting 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...
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, Ntx2-5 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 cardiac cells 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 cardiac 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); Fasiclin 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).

**Ntx2-5 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 Ntx2-5 could rescue visceral mesoderm, as judged by staining for Fasiclin 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 Ntx2-5 and bap (67%) is comparable to that seen between Ntx2-5 and tin (65%) (Lints et al., 1993), it was possible that Ntx2-5 could rescue visceral mesoderm in tin mutants by compensating for loss of bap expression. To address this issue, Ntx2-5 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 Fasiclin III staining and a morphological midgut with its characteristic constrictions. However, Ntx2-5 rescued none of these features (data not shown). Thus, Ntx2-5 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 Ntx2-5 transgenes activated bap expression in visceral mesoderm (Fig. 3G,J), indicating that Ntx2-5, like tin, can induce the expression of a key transcriptional regulator of the visceral muscle program.

Although Ntx2-5 could activate Fasiclin 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 immunohistochemistry results, we found that Ntx2-5 was unable to activate the DMEF-2 enhancer–lacZ reporter in the visceral lineage (Fig. 3K,L). As expected, no heart progenitors were

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**Fig. 3.** Ntx2-5 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 tinEC40 mutant embryo (D). G and J are tinEC40 mutant embryos rescued with UAS-tin and UAS-Ntx2-5, 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 tinEC40 mutant embryo, expression is completely abolished except in the presumptive hindgut area (E). H and K are tinEC40 mutant embryos rescued with UAS-tin and UAS-Ntx2-5, respectively. Tin activates lacZ activity in the visceral mesoderm and also in founder cells. Ntx2-5 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 cardiac cells of wild-type (C) and mutant (F) embryos. UAS-tin transgene rescues lacZ expression in the cardiac cells (I) and UAS-Ntx2-5 does not (L). vm, visceral mesoderm; fc, somatic muscle founder cells; cc, cardial cells.
Based on homeodomain comparisons, it can be concluded that the activities 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, this idea was proven. Nkx2-5 inhibits cardiogenesis through a dominant-negative activity.

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

<table>
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<tr>
<th>Constructs</th>
<th>Visceral markers</th>
<th>Cardiac markers</th>
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<tr>
<td></td>
<td>Fasc III</td>
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<td>tinman</td>
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<td>Nkx2-5</td>
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<td>tin (HDs)</td>
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<tr>
<td>bagpipe</td>
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*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.

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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 *tiff1*) 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.
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 transactivation 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 DMel-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 10T1/2 fibroblasts (Fig. 7B,C). For tin, a strong transactivation domain was revealed by 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 DMel-2-positive pericardial and cardiac 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; DMel-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. 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 in C3H10T1/2 fibroblasts for transcriptional activity with a GAL4-dependent luciferase reporter. Values shown are the mean and standard deviation of three independent experiments.
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
Regulation of the D-Mef2 enhancer by NK-2 do so in the fly assay. Thus, it is possible that addition to participating in its differentiation. In general terms, in 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 Dmeif2 gene via a cardiac-specific enhancer containing two high-affinity tin-binding sites (Gajewski et al., 1997). However, Nkx2-5 cannot 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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