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Tinman Function Is Essential for Vertebrate Heart Development: Elimination of Cardiac Differentiation by Dominant Inhibitory Mutants of the *tinman*-Related Genes, *XNkx2-3* and *XNkx2-5*

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In *Drosophila,* the *tinman* gene is absolutely required for development of the dorsal vessel, the insect equivalent of the heart. In vertebrates, the *tinman* gene is represented by a small family of *tinman*-related sequences, some of which are expressed during embryonic heart development. At present however, the precise importance of this gene family for vertebrate heart development is unclear. Using the *Xenopus* embryo, we have employed a dominant inhibitory strategy to interfere with the function of the endogenous *tinman*-related genes. In these experiments, suppression of *tinman* gene function can result in the complete elimination of myocardial gene expression and the absence of cell movements associated with embryonic heart development. This inhibition can be rescued by expression of wild-type *tinman* sequences. These experiments indicate that function of *tinman* family genes is essential for development of the vertebrate heart.

Key Words: **cardiac development; homeobox; homeodomain; NK2; *tinman; Xenopus.***

INTRODUCTION

In *Drosophila*, the homeobox gene *tinman* is required for the specification of cardiogenic cells and the development of the dorsal vessel (Kim and Nirenburg, 1989; Azpiazu and Frasch, 1993; Bodmer, 1993). Mutation of *tinman* leads to a complete absence of dorsal vessel formation. At the molecular level, expression of myosin in the heart is absent and so are the precardiac markers, *zfh-1* and *eve* (Azpiazu and Frasch, 1993; Bodmer, 1993). This observation indicates that *tinman* function is required early in the heart development pathway. Rescue experiments have been carried out using the wild-type *tinman* gene driven by a heat shock promoter. In these experiments, *tinman* expression partially rescues the mutant phenotype, but widespread expression of *tinman* does not result in ectopic expression of cardiac markers (Bodmer, 1993). These experiments show that Tinman itself is not sufficient to initiate the regulatory pathway leading to heart formation and very likely requires the cooperation of additional regulatory proteins.

A vertebrate gene related to *tinman*, *Nkx2-5/csx* (Komuro and Izumo, 1993; Lints *et al.*, 1993), was first identified in mouse and has since been characterized in a number of other species, including *Xenopus* (Tonissen *et al.*, 1994), chicken (Schultheiss *et al.*, 1995), and zebrafish (Lee *et al.*, 1996). In humans, mutations in the Nkx2-5 homolog are known to be responsible for a certain class of congenital heart defects (Schott *et al.*, 1998). *Nkx2-5* is expressed in the precardiac tissues and in the differentiated myocardium of all species so far examined and therefore appears to represent a universal marker for early heart development. Ablation of *Nkx2-5* gene function in the mouse results in embryonic lethality at about day 10.5, due to heart defects (Lyons *et al.*, 1995). These defects include a failure of the heart tube to undergo correct looping morphogenesis, reduced trabeculation and an abnormally thin ventricular myocardium (Lyons *et al*, 1995). At the molecular level, expression of a number of genes is reduced or eliminated in the *Nkx2-5* mutant mouse, including *MLC-2v* (Lyons *et al.*, 1995), *CARP* (Zou, *et al.*, 1997), *eHand* (Biben and Harvey, 1997), and *ANF* (Durocher *et al.*, 1996). However, despite the reduced expression of this subset of cardiac genes in *Nkx2-5* mutant mice, beating myocardial tissue is present and development of the heart is superficially normal until the heart tube stage. This observation indicates that the early stages of heart development are not strictly dependent on Nkx2-5 function and that, unlike the *Drosophila tinman* gene, *Nkx2-5* alone is not required for heart specification and differentiation.

An alternative explanation for the relatively late-stage

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cardiac defects observed in the *Nkx2-5* mutant mouse is the presence of redundant genes that can partially compensate for the absence of Nkx2-5 function early in development. Indeed, a search for genes related to *Nkx2-5* has revealed the presence of a small family of additional *tinman*-related genes that are expressed in the developing vertebrate heart. These include *XNkx2-3* and *XNkx2-9* in *Xenopus* (Cleaver *et al.*, 1996; Evans, *et al.*, 1995; Newman *et al.*, 1998), *cNkx2-3* and *cNkx2-8* in chicken (Buchberger *et al.*, 1996; Boettger *et al.*, 1997; Brand *et al.*, 1997; Reecy *et al.*, 1997), *nkx2-3* and *nkx2-7* in zebrafish (Lee *et al.*, 1996), and *Nkx2-6* in mouse (Nikolova *et al.*, 1997; Biben *et al.*, 1998). Somewhat surprisingly, the mouse orthologue of *Nkx2-3* is not expressed in the developing mouse heart (Pabst *et al.*, 1997). So far, the *Nkx2-6, Nkx2-7, Nkx2-8,* and *Nkx2-9* genes have only been isolated from a single species and so it is unclear whether these sequences are present in other organisms and whether the embryonic expression patterns are conserved.

Vertebrate Tinman-related proteins display the highest levels of sequence conservation in the homeodomain, the TN domain, and the NK2-SD domain (Harvey, 1996). The presence of these conserved protein domains suggests that the different proteins may possess similar structural and regulatory properties and it is plausible that different members of the Tinman protein family possess some degree of functional redundancy in the cardiogenic pathway. This possibility is supported by the observation that, when either XNkx2-3 or XNkx2-5 is overexpressed in the precardiac mesoderm of *Xenopus* embryos, the two sequences display apparently identical activities in increasing the total number of myocardial cells (Cleaver *et al.*, 1996).

Cell culture studies suggest that the Nkx2-5 protein may act together with other transcription factors to regulate the expression of cardiac genes. For example, the ability of Nkx2-5 to activate transcription from the cardiac α-actin promoter is strongly increased by the presence of the MADS domain protein, serum response factor (SRF) (Chen and Schwartz, 1996). Even higher levels of transcriptional activation are achieved from this promoter when Nkx2-5, SRF, and the zinc finger protein GATA-4 are all present in the same cell (Sepulvada *et al.*, 1998). Additional studies indicate that Nkx2-5 and GATA-4 act together to regulate transcription from the atrial natriuretic factor (ANF) promoter and that the two proteins are able to physically interact (Durocher *et al.*, 1997; Sepulvada *et al.*, 1998; Lee *et al.*, 1998). Furthermore, *in vitro* expression experiments indicate that transcriptional activation by a different Tinman family protein, chicken Nkx2-8, is synergized by the mouse GATA-4 protein (Reecy *et al.*, 1997) and it seems likely that this activation is also mediated via protein–protein interactions. Overall, these experiments suggest that efficient transcriptional activation of target genes by vertebrate Tinman proteins involves formation of a physical complex with additional transcription factors such as GATA-4 and/or SRF.

Throughout the early stages of *Xenopus* heart development, at least three *tinman*-related genes, *XNkx2-3*, *XNkx2-5*, and *XNkx2-9*, are expressed in the same tissues and could potentially serve redundant functions in the cardiogenic pathway. We have employed a dominant inhibitory strategy to block the functional activity of multiple members of the Tinman-related protein family during *Xenopus* heart development. The experiments described in this report demonstrate that expression of dominant inhibitory mutants of either XNkx2-3 or XNkx2-5 in the frog embryo strongly inhibits the expression of myocardial differentiation markers. In some cases, all detectable transcriptional and morphological markers of myocardial development are eliminated. These results indicate that Tinman function is essential for development of the vertebrate heart.

MATERIALS AND METHODS

Plasmid Constructions and mRNA Synthesis

Dominant inhibitory forms of XNkx2-3, XNkx2-5, and XHex were created by substituting a proline for a highly conserved leucine at position 40 of each homeodomain. These mutations were generated via inverse PCR mutagenesis. The mutant *XNkx2-3LP* was generated from *pT7TS-XNkx2-3* (Cleaver *et al.*, 1996) using oligo 2-3LP-1, CCCACCTCTACCCAAGT; and 2-3LP-2, TTTCAGGCTGTTTGCCA. The mutant *XNkx2-5LP* was generated from *pT7TS-XNkx2-5* (Cleaver *et al.*, 1996) using oligo 2-5LP-1, CCCACCTCCACACAGGT; and 2-5LP-2, CTTCAGGACATTGGCTA. The mutant *XHexLP* was generated from *pCS2-XHex* using oligo HexDN-1, AGCGAGAGACAGGTCAA; and HexDN-2, GGGCTGCAGCATTTTAG (Newman *et al.*, 1997). The presence of the desired mutations was confirmed by DNA sequencing. Capped mRNAs were synthesized from *Eco*RI linearized templates using the mMessage mMachine *in vitro* transcription kit (Ambion). Transcripts were purified by passage over a G-50 Sephadex column and translated *in vitro* using reticulocyte lysate (Ambion) to ensure full-length translation products.

Microinjections

Using standard techniques (Kay, 1991), varying amounts of synthetic, capped mRNAs were microinjected into *Xenopus* embryos in a volume of 4.6 nl using a Drummond Nanoject variable automatic injector. The experimental mRNA was lineage-traced by coinjecting 250 pg of GFP mRNA in distilled water containing 5 mg/ml neutral tetramethylrhodamine dextran, M_r 70,000 (RDA). Each injection was targeted to the dorsal–vegetal blastomeres of an eight-cell *Xenopus* embryo, since these cells will later contribute to the heart (Dale and Slack, 1987). To control for effects of nonspecific injection damage, water containing only lineage tracers was also injected. After injection, embryos were cultured at 13°C in 100% Steinberg's buffer containing 3% Ficoll for 12 h and then in 20% Steinberg's buffer until reaching the desired stage of development for assay. The embryos were sorted according to the position of the lineage tracers using fluorescent microscopy, fixed in MEMPFA, and stored in methanol until assayed. For myocardial differentiation assays, only embryos with lineage tracer in the heart region were scored.

In Situ Hybridization and Histological Analysis

Using antisense digoxigenin-labeled probes, embryos were assayed for the expression of marker genes by whole-mount *in situ* hybridization using standard conditions (Harland, 1991), except that CHAPS was omitted from all buffers. Myocardial differentiation was assayed using *Xenopus cardiac troponin I* (*TnIc*) (Drysdale *et al.,* 1994), *Xenopus* myosin light chain 2, (*XMLC2*) (Chambers *et al.,* 1994) and *α-cardiac actin* (Mohun *et al.,* 1984). For sectioning, embryos were dehydrated in ethanol, permeabilized in xylene (2 × 10 min), incubated in xylene/Paraplast (10 min), and incubated in Paraplast overnight at 60°C. Embryos were then embedded in Paraplast and sectioned at thicknesses of 10 and 12 μm. The sections were dried, dewaxed in xylene, mounted with Permount, and photographed.

RESULTS

Dominant Inhibitory Mutants of XNkx2-3 and XNkx2-5 Block Heart Differentiation

A number of studies suggest that homeodomain proteins can interact with each other and also with different classes of transcription regulatory proteins. These protein–protein interactions increase the DNA-binding affinity of the complex for its target site and increase the efficiency of target gene activation (Goutte and Johnson, 1994; Grueneberg *et al.,* 1995; Kamper *et al.,* 1995; Lu *et. al.,* 1995). To block the functional activity of the multiple Tinman-related proteins present during *Xenopus* heart development, we have employed a dominant inhibitory strategy that has previously been used to interfere with the biological functions of two other *Xenopus* homeodomain proteins, Mix.1 (Mead *et al.,* 1996) and Xvent-2 (Onichtchouk *et al.,* 1998). We have constructed mutant forms of Nkx2-5 and Nkx2-3 (called Nkx2-5LP and Nkx2-3LP, respectively) containing a proline residue in place of the highly conserved leucine residue between helix II and helix III of the homeodomain. This mutation disrupts the ability of the homeodomain protein to bind DNA (Le Roux *et al.,* 1993; Mead *et al.,* 1996), while maintaining the ability to form protein–protein interactions (Mead *et al.,* 1996). Overexpression of the Nkx2-3LP and Nkx2-5LP mutants will titrate essential partner proteins, away from endogenous Tinman proteins, into complexes with reduced DNA binding ability, thereby interfering with the normal function of the Tinman proteins.

In *Xenopus,* as with other organisms, the heart develops from paired cardiac primordia. Using whole-mount *in situ* hybridization, expression of myocardial differentiation markers such as *TnIc* can first be detected at stage 27 as two patches of staining on either side of the ventral midline (Fig. 1A). If embryo overexpression experiments are assayed when the symmetrical precardiac tissues are present, the uninjected side of the embryo serves as an internal control for the injected, experimental side of the embryo. An example is shown in Fig. 1B, where overexpression of wild-type *Nkx2-5* mRNA in the embryo has resulted in an increase in *TnIc* expression on the injected side (Cleaver *et al.,* 1996). We have used the same assay procedure to determine the consequences of overexpression of mutant Tinman proteins on heart development. Approximately 250 pg of synthetic capped mRNA encoding the dominant inhibitory proteins XNkx2-3LP and XNkx2-5LP was injected into a single, dorsal–vegetal blastomere of the eight-cell *Xenopus* embryo. The embryos were then assayed for *TnIc* expression at the early tailbud stage. Figures 1C and 1D present the results of overexpression of XNkx2-3LP and XNkx2-5LP, respectively. In each case, the injected side of the embryo shows a strong reduction in *TnIc* expression. A lateral view of a different Nkx2-3LP overexpressing embryo exhibiting greatly reduced expression of *TnIc* is presented in Fig. 1E. The results of multiple injection experiments using different preparations of synthetic mRNA and different batches of embryos are compiled in Table 1. In embryos overexpressing XNkx2-5LP, 45% showed a severe reduction in the level of marker expression on the side of injection and a further 20% showed a significant, but less dramatic reduction from wild-type levels. Similarly, in embryos expressing XNkx2-3LP, 37% exhibited a severe phenotype, while another 4% showed a moderate phenotype. Water-injected control embryos showed a very low incidence (6%) of the moderate phenotype indicating that the defects in myocardial development are not due to damage resulting from the injection procedure. Sectioning through the heart region of experimental embryos at a later stage of development revealed that some embryos with no obvious external phenotype exhibited thinner myocardial tissue on the injected side (data not shown). Therefore, the proportion of embryos scored by external phenotype in Table 1 represents a conservative estimate of the incidence of the mutant phenotype.

The internal structure of embryos expressing dominant inhibitory constructions was examined by histological sectioning of *in situ* stained embryos. As expected, water-injected control embryos show approximately equal expression of *TnIc* on both sides of the ventral midline (Fig. 1F), while sections through XNkx2-5LP-expressing embryos show the absence of detectable *TnIc* marker expression on the experimental side of the embryo (Fig. 1G). If embryos were allowed to develop longer, until after the cardiogenic patches had fused at the midline, the external asymmetry was less apparent and in many cases the hearts appeared to develop normally. However, a large number of embryos showed the presence of a smaller heart with apparently normal morphology, located at the midline (data not shown). This heart tissue is probably derived from the precardiac tissues formerly located on the uninjected side of the embryo. We conclude from these single-sided injection experiments that both XNkx2-3LP and XNkx2-5LP can severely inhibit myocardial differentiation, as assayed by *TnIc* marker expression. The phenotypes produced by the two dominant inhibitory constructions are indistinguishable and the constructions are approximately equally efficient in generating the mutant effect.

To confirm that the observed inhibition of myocardial differentiation is not limited to the *TnIc* marker sequence,

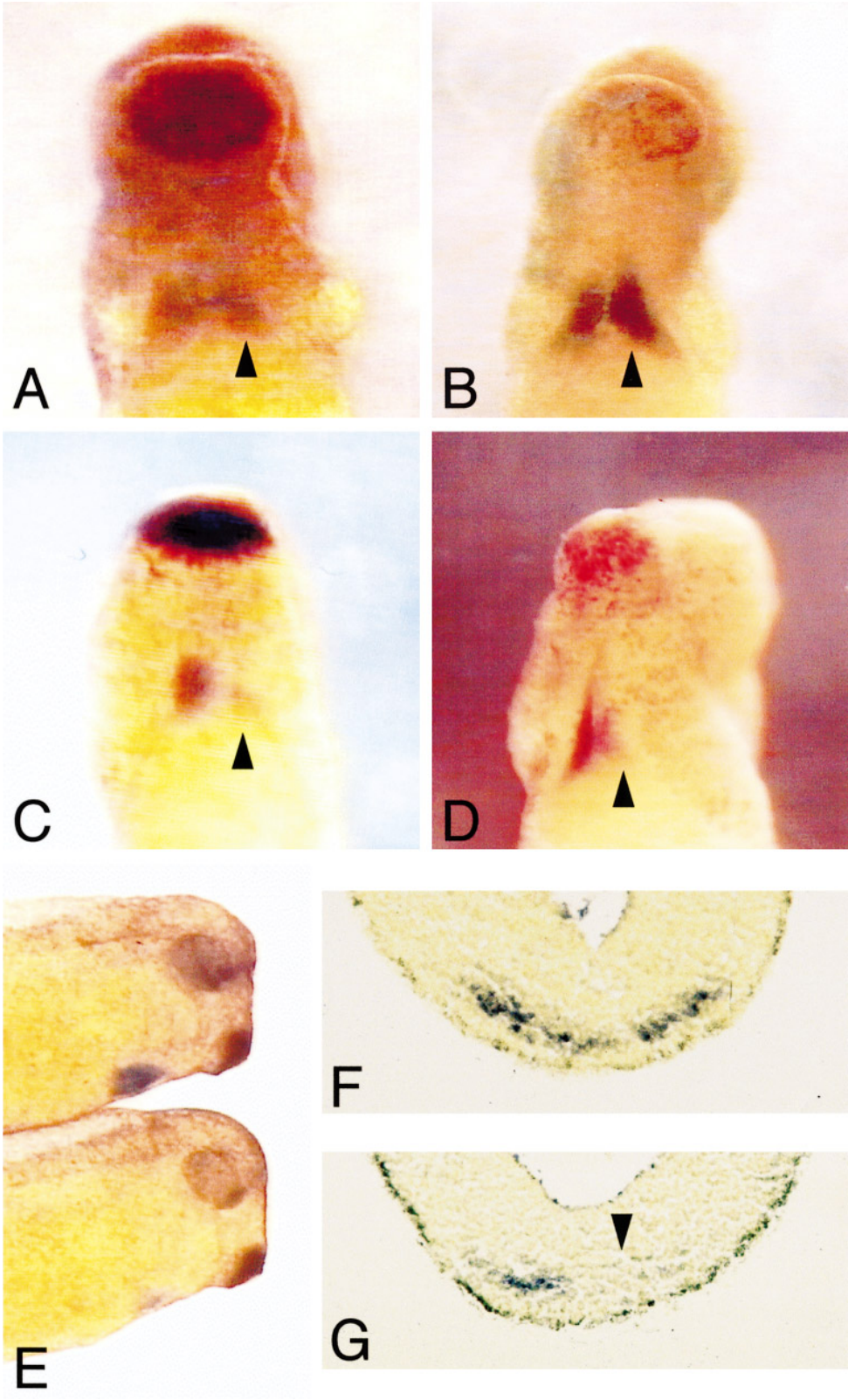

FIG. 1. Single-sided expression of dominant inhibitory *XNkx2-3* and *XNkx2-5* inhibits myocardial gene expression. In all cases the arrowhead indicates the injected side of the embryo. All embryos were assayed for expression of the myocardial marker, *TnIc*, using whole-mount *in situ* hybridization. (A) Stage 28, water-injected control embryo, showing normal expression of *TnIc*. At this early stage,

TABLE 1
Summary of Dominant Inhibitory Experiments

mRNA	Strong phenotype	Weak phenotype	Normal	Total
		Single-sided injections		
XNkx2-3LP	40 (37%)	4 (4%)	63 (59%)	107
XNkx2-5LP	29 (45%)	13 (20%)	23 (35%)	65
XHexLP	0	0	13 (100%)	13
Water	0	5 (6%)	84 (94%)	89
Uninjected	2 (3%)	4 (5%)	69 (92%)	75

mRNA	No heart	Severely reduced	Reduced	Normal	Total
			Double-sided injections		
XNkx2-5LP	13 (18%)	12 (17%)	14 (20%)	32 (45%)	71
XHexLP	0	0	2 (2%)	106 (98%)	108
Water	0	1 (2%)	2 (4%)	46 (94%)	49
Uninjected	0	0	0	121 (100%)	121

Note. In the single-sided injection experiments, embryos showing absence or extremely reduced expression of *cardiac troponin I* (*TnIc*) on the injected side of the two bilateral cardiogenic regions were scored as having a strong phenotype. Those exhibiting significant asymmetry were scored as having a weak phenotype. In the double-sided injection experiments, embryos were scored for levels of *TnIc* staining relative to uninjected control embryos. In addition to the mRNA sequences stated in the table, all injections contained 250 pg of mRNA encoding green fluorescent protein plus 5 mg/ml RDA–dextran as lineage tracers.

we have repeated the assay using probes for the heart-specific myosin light chain 2, *XMLC2* (Chambers *et al.*, 1994) and the general muscle marker *α-cardiac actin* (Mohun *et al.*, 1984). *In situ* hybridization reveals that embryos injected with *XNkx2-5LP* mRNA show inhibition of both *XMLC*2 and *α-cardiac actin* expression in the developing heart (data not shown). This indicates that the expression of at least three independent markers of myocardial differentiation is inhibited by Tinman-related dominant inhibitory mutants. This strongly suggests that the Tinman dominant inhibitory constructions are influencing the myocardial development pathway in general, not merely the expression of a specific differentiation marker sequence.

By carrying out double-sided injections of a dominant inhibitory construction, it should be possible to block Tinman function in the precardiac regions on both sides of the embryo. Since the Nkx2-5LP and Nkx2-3LP constructions yielded identical phenotypes in the single-side injection experiments, we have carried out the double-sided injections using only Nkx2-5LP. Messenger RNA encoding the XNkx2-5LP dominant inhibitory mutant was injected into both dorsal–vegetal blastomeres of the eight-cell embryo (125 pg per blastomere). Embryos were assayed at stages 30–32, when the primitive heart tube has normally formed and just before the onset of cardiac looping. The results of a double-sided injection experiment are presented in Fig. 2. In this case, overexpression of XNkx2-5LP on both sides of the embryo has resulted in a complete loss of detectable myocardial differentiation, as assayed using the *TnIc* marker. Note that the overall morphology of the embryo is normal, with no detectable disruption to other anterior structures, such as the eye or cement gland. This suggests that heart differentiation is specifically inhibited, since the overall development of the organism remains normal. The results of a series of double-sided injection experiments are compiled in Table

TnIc expression marks bilaterally symmetrical precardiac tissues, located on each side of the ventral midline. (B) Stage 28 embryo injected with 250 pg of wild-type *Nkx2-5* mRNA showing increased expression of *TnIc* on the injected side. (C) Stage 28 embryo, after single-sided injection of 250 pg of *Nkx2-3LP* mRNA. Expression of *TnIc* is dramatically reduced on the injected side (indicated by the arrowhead). (D) Stage 29 embryo, after single-sided injection of 250 pg of *Nkx2-5LP* mRNA. Expression of *TnIc* is dramatically reduced on the injected side (arrowhead). (E) Lateral view of control and experimental stage 30 embryos. The water-injected control embryo is shown on top. The bottom embryo was injected with 250 pg of *Nkx2-3LP* mRNA and shows severe inhibition of *TnIc* expression in the cardiac region. Note that the overall morphology of the experimental embryo is indistinguishable from the water-injected control. (F) Histological section through the heart region of a stage 30, water-injected control embryo assayed for *TnIc* expression. Two regions of *TnIc* expression are visible on either side of the ventral midline. (G) Section through the heart region of a stage 30 embryo injected with 250 pg of *Nkx2-3LP*. Note the absence of *TnIc* expression on the injected side (arrowhead).

FIG. 2. Double-sided injection of a dominant inhibitory *tinman* construction can completely eliminate myocardial differentiation. Lateral view of control and experimental stage 30 embryos. The top embryo was injected with water and shows normal expression of *TnIc* in the developing heart tube. The bottom embryo was injected on each side with 125 pg of *Nkx2-5LP* mRNA. No expression of the *TnIc* myocardial marker is visible. Note that the overall morphology of both the control and experimental embryos is normal.

1. Complete loss of detectable *TnIc* expression was observed in 18% of the embryos, while an additional 37% showed obviously reduced expression levels relative to controls.

Sections though the heart region of an uninjected stage 32 embryo (Fig. 3A) illustrate normal heart tube morphology, with the *TnIc*-expressing myocardium fully delaminated from the surrounding tissues and enclosing an endocardial tube. Embryos showing severe inhibition of TnIc expression were also sectioned (Fig. 3B). In addition to the absence of TnIc expression, these embryos show dramatically altered morphology in the region of the developing heart, with no visible delamination of the myocardial layer and no indication of pericardial coelom or endocardial tube formation. Sections through other regions of the Nkx2-5LP-overexpressing embryos showed no differences from wild-type morphology. These observations suggest that inhibition of Tinman function not only blocks expression of myocardial differentiation markers, but also interferes with the cellular movements associated with heart morphogenesis.

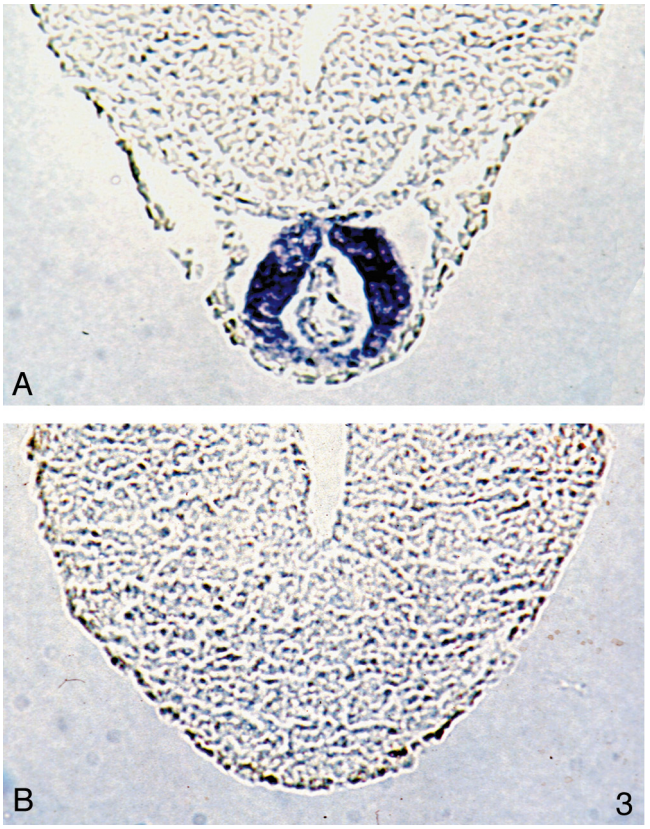

FIG. 3. Double-sided injection of a dominant inhibitory *tinman* construction eliminates the cellular movements associated with heart formation. Histological sections through the heart region of control and experimental stage 30 embryos assayed for *TnIc* expression. (A) Control embryo showing expression of *TnIc* in the developing heart tube. The myocardial layer has completely separated from the overlying endoderm and the underlying somatic mesoderm layers. The myocardial tube surrounds endothelial cells that will later form the endocardium. (B) Experimental embryo injected with 125 pg of *Nkx2-5LP* mRNA on each side. No detectable expression of the myocardial marker *TnIc* is visible. In addition, there is no visible delamination of the tissue layers and no evidence of heart tube formation.

TABLE 2
Rescue of the Dominant Inhibitory Phenotype

mRNA	Asymmetric phenotype	Normal	Total	Probability
XNkx2-3LP	17 (45%)	21 (55%)	38	$P < 0.0001$
XNkx2-3LP plus *XNkx2-5*	3 (8%)	33 (92%)	36	$P = 0.42$
Uninjected	2 (4%)	46 (96%)	48	

Note. Embryos were injected either with 250 pg of *XNkx2-3LP* mRNA or 250 pg *XNkx2-3LP* mRNA plus 100 pg of wild-type *XNkx2-5* mRNA. All embryos were assayed for expression of *TnIc* using whole-mount *in situ* hybridization. Asymmetic phenotype was scored when the *TnIc* expression on the injected side was reduced relative to the uninjected control side. The probability that the observed frequency of phenotype is significantly different from the uninjected control embryos was determined using contingency table analysis. Probability values less than 0.05 are considered significant, while values close to 0.5 are consistent with random variation.

The Effects of Dominant Inhibitory Mutant Expression Are Heart Specific

We have carried out additional experiments to determine whether the effects of the dominant inhibitory Tinman-related proteins are cardiac-specific. By altering the point of injection in embryonic blastomeres, mutant protein expression was targeted to the head or somite region. As in previous experiments, the location of the injected material was followed using coinjected GFP and rhodamine lineage tracers. When expressed in the head, the dominant inhibitory mutants caused no visible alteration to the shape of the head or to the development of sensitive tissues such as the eyes (data not shown). Similarly, embryos that received transcripts in developing somites were assayed with the general muscle marker *α-cardiac actin,* but expression in the somites was normal (data not shown). We conclude from these experiments that XNkx2-3LP and XNkx2-5LP are influencing the myocardial development pathway specifically and are not causing nonspecific developmental defects at the level of embryonic patterning or tissue differentiation.

The heart differentiation pathway may be unusually sensitive to overexpression of mutant homeodomain proteins in general, not to Tinman-related mutants in particular. To determine whether other mutant homeodomain proteins can also interfere with cardiac development, we have overexpressed XHexLP, a dominant inhibitory form of XHex containing the same point mutation in the homeodomain. *XHex* is first expressed at gastrulation in the dorsal mesendoderm and later in the developing liver, the developing vascular system, and the endocardium of the heart, but never in the myocardium (Newman *et al.,* 1997). If the inhibition of cardiac differentiation is specific to Tinman family mutant proteins, then overexpression of XHexLP should have no effect on heart development. As described above, bilateral injections of 125 pg of *XNkx2-5LP* mRNA per blastomere are sufficient to inhibit myocardial differentiation. On the other hand, a double-sided injection of 500 pg of *XHexLP* mRNA per blastomere (a total of 1 ng) has no detectable effect on the expression of *TnIc.* The results of XHexLP expression in the heart region are included in Table 1. In contrast to the absence of a cardiac phenotype, expression of the same amount of *XHexLP* mRNA in the flanking region of the embryo dramatically alters the pattern of vascular development (data not shown), thereby demonstrating that the XHexLP protein is functional.

Rescue of the Mutant Phenotype

Based on the results of previous experiments (Cleaver *et al.,* 1996; Reecy *et al.,* 1997), it seems likely that different members of the Tinman family of proteins are capable of acting redundantly during heart development. Indeed, it is possible that the activity of just a single Tinman family protein in precardiac tissues is sufficient to regulate the expression of cardiac differentiation markers. If this is the case in *Xenopus,* we predict that expression of wild-type XNkx2-5 will be sufficient to rescue myocardial gene expression in embryos overexpressing the XNkx2-3LP dominant inhibitory protein. To address this question, we have taken a large batch of eight-cell *Xenopus* embryos and injected one group with 250 pg of mRNA encoding XNkx2-3LP and another group with 250 pg of *XNkx2-3LP* mRNA plus 100 pg of wild-type *XNkx2-5* mRNA. One hundred picograms of rescuing mRNA was used in these experiments because pilot experiments showed that coinjection of larger amounts of wild-type *XNkx2-5* mRNA caused nonspecific toxicity during gastrulation, as previously reported (Vize *et al.,* 1991; Cleaver *et al.,* 1996). The results of the rescue experiments are presented in Table 2. These data show that the injection of mRNA encoding Nkx2-3LP caused an obvious decrease in *TnIc* expression in 45% of embryos, a proportion consistent with previous experiments (see Table 1). However, inclusion of 100 pg of wild-type *XNkx2-5* mRNA caused a dramatic rescue of the dominant inhibitory phenotype, with only 8% of embryos showing inhibition. This number is not significantly different from the 4% of uninjected embryos that exhibit some abnormalities in cardiac marker expression. We conclude from these experiments that function of a wild-type Tinman sequence can rescue the dominant inhibitory phenotype.

DISCUSSION

Based on the essential role of the *tinman* gene for heart development in *Drosophila,* the vertebrate *tinman*-related gene family is also likely to be important for heart development in higher organisms. The importance of one *tinman*-related gene, *Nkx2-5,* has been confirmed by gene ablation experiments in mouse (Lyons *et al.,* 1995). In mouse embryos lacking Nkx2-5 function, a beating heart tube forms, but the ventricular walls are thin and the heart fails to loop correctly. The apparently normal formation of the embryonic heart up to the linear tube stage in the *Nkx2-5*$^{-/-}$ embryos could potentially be explained by the activity of redundant genes (Lyons *et al.*, 1995). Thus far, the only other *tinman* family gene known to be expressed in the developing mouse heart is *Nkx2-6.* Although *Nkx2-6* is expressed primarily in developing foregut endoderm, pharyngeal arches, and neural tissues, transcripts are also present at low levels in the developing heart prior to differentiation (Biben *et al.*, 1998). While it is possible that *Nkx2-6* function is responsible for rescuing early cardiac development in *Nkx2-5* mutant embryos, this activity remains to be demonstrated. At present therefore, the importance of *tinman*-related genes for the regulation of the early stages vertebrate heart development remains unclear.

In *Xenopus,* three distinct *tinman* family genes, *Nkx2-5, Nkx2-3,* and *Nkx2-9,* are expressed during early heart development (Tonissen *et al.*, 1994; Evans *et al.*, 1995; Cleaver *et al.*, 1996; Newman and Krieg, 1998) and the overlapping expression patterns of these genes offer the possibility of redundant function. To investigate the importance of *tinman* gene function for early heart development in *Xenopus,* we have employed a dominant inhibitory strategy designed to interfere with the activity of multiple members of the Tinman-related protein family. Our experiments demonstrate that overexpression of mutant forms of either Nkx2-5 or Nkx2-3 is sufficient to eliminate expression of myocardial differentiation markers (Figs. 1 and 2). This has been confirmed using three independent markers, indicating that the effect is not specific to a particular gene pathway. In *Xenopus,* expression of *Nkx2-5* and *Nkx2-3* commences at the gastrula stage and continues in cardiogenic tissues throughout subsequent development. In our experiments, expression of the dominant inhibitory mutants from injected mRNA will first occur before gastrulation and so the mutant proteins are likely to inhibit normal Tinman function from the very earliest stages of heart development. In *Drosophila,* Tinman is known to regulate expression of the *D-MEF2* gene (Gajewski *et al.*, 1997) and it is possible that Tinman function is also required for expression of the *MEF2* genes during vertebrate myocardial development. The *D-MEF2* gene is essential for expression of a wide range of muscle differentiation markers (Lilly *et al.*, 1995), and so the absence of detectable myocardial marker expression in *Xenopus* embryos expressing Nkx2-5LP or Nkx2-3LP could be due to elimination of *MEF2* expression. Future experiments will attempt to address this question. Alternatively, some cardiac genes may be direct targets of Nkx2-5 regulation (Durocher *et al.*, 1997) and loss of expression of these genes could be a specific consequence of the inhibition of Nkx2-5 activity. Finally, sectioning through embryos overexpressing Nkx2-5LP shows a complete absence of the morphological structures normally associated with heart formation (Fig. 3). While we cannot exclude the possibility that some cell movements and tissue delamination involved in heart development have occurred, it appears that at least some aspects of the morphogenetic program fall under control of the *tinman* gene family. If these morphogenetic movements are essential for subsequent gene activity, then expression of a wide range of cardiac marker sequences could be indirectly inhibited. Overall, our results suggest that *tinman* family genes play an essential role in vertebrate heart development, both in the regulation of myocardial gene expression and in the morphological program associated with heart formation.

The dominant inhibitory strategy that we have used to interfere with *tinman* gene function is based on previous studies investigating the function of the *Mix.1* (Mead *et al.*, 1996) and *XVent2* genes (Onichtchouk *et al.*, 1998) and assumes that the homeodomain protein is part of a multimeric transcription complex. Overexpression of the dominant inhibitory protein will sequester critical cofactor proteins and thus prevent endogenous Tinman proteins from forming active transcription complexes. Although the precise molecular details of Tinman interactions have not been determined, several lines of evidence indicate that protein–protein interactions are required for Tinman activity, possibly with GATA family members or SRF (Chen and Schwartz, 1996; Durocher *et al.*, 1997; Sepulveda *et al.*, 1998; Lee *et al.*, 1998). Alternatively, homeodomain proteins can form homodimers or heterodimers with other homeodomain proteins via the homeodomain itself and sometimes through other protein-binding domains (Goutte and Johnson, 1994; Kamper *et al.*, 1995; Mead *et al.*, 1996) and so it is possible that the different Tinman family proteins are also able to physically interact.

It could be argued that the inhibition of myocardial marker expression that we observe does not necessarily imply a direct role for the Tinman proteins themselves, but rather for some other protein (for example, GATA-4) that is sequestered by the overexpressed Tinman mutant proteins. This seems unlikely however, since overexpression of wild-type Tinman proteins results in an increase in the number of cells expressing myocardial markers (Fig. 1B and Cleaver *et al.*, 1996; Chen and Fishman, 1996) and not an inhibition of myocardial differentiation. Since the mutant Tinman proteins only differ from the wild-type at a single residue, they are expected to bind the same partner proteins. The fact that overexpression of wild-type Tinman proteins produces opposite effects to mutant Tinman proteins supports a direct role for Tinman in the pathway leading to myocardial development.

The three Tinman-related proteins, Nkx2-3, Nkx2-5, and Nkx2-9, expressed in cardiogenic tissues during *Xenopus* development contain conserved structural domains (Tonissen *et al.,* 1994; Evans *et al.,* 1995; Cleaver *et al.,* 1996; Newman *et al.,* 1998). It is reasonable therefore to assume that these sequences will show some degree of functional redundancy. This expectation is supported by studies in which wild-type Nkx2-5 and Nkx2-3 are overexpressed in the *Xenopus* embryo. The phenotype of overexpression of either Nkx2-5 or Nkx2-3 is indistinguishable, with each causing an increase in the number of cells present in the myocardial layer of the embryonic heart (Cleaver *et al.,* 1996). Furthermore, coexpression of both sequences in the embryo results in an additive, not synergistic, increase in myocardial cell number. In this report, we demonstrate that mutant forms of Nkx2-3 and Nkx2-5 produce apparently identical effects in inhibiting embryonic heart development. Since these mutants are likely to work via a dominant inhibitory mechanism, this suggests that these two Tinman proteins interact with the same protein partners, once again supporting redundant activities. In addition, we show that coinjection of wild-type *Nkx2-5* mRNA is sufficient to rescue the suppression of myocardial differentiation caused by the Nkx2-3LP dominant inhibitory mutant (Table 2). This latter result can be interpreted in two ways—first, that Nkx2-5 and Nkx2-3 possess at least partially redundant regulatory activities and that the presence of either is sufficient to activate myocardial marker expression. Second, the result can be interpreted to mean that Nkx2-5 is the single major regulator of myocardial differentiation in the embryo and the activity of other Tinman family proteins is not relevant. While this second interpretation is consistent with our experiments, it is contradicted by the mouse gene ablation experiments which demonstrate that the majority of myocardial genes are expressed at normal levels in embryos lacking Nkx2-5 activity. In the mouse therefore, Nkx2-5 alone cannot be the major regulator of cardiac development. In view of our results, it seems likely that, independent of *Nkx2-5* activity, *Nkx2-6* or another *tinman* family gene is capable of directing early heart tube morphogenesis and the expression of most differentiation markers during mouse cardiogenesis. It is important to remember however that several myocardial genes are *not* expressed in the *Nkx2-5*$^{-/-}$ embryo (Lyons *et al.,* 1995; Biben and Harvey, 1997; Zou *et al.,* 1997), and so the putative Tinman family protein(s) cannot possess precisely the same regulatory properties as Nkx2-5. Taken together, the mouse gene ablation experiments and the dominant inhibitory experiments carried out in *Xenopus* suggest that vertebrate *tinman* family genes are essential for myocardial development. It seems probable that the combined activities of the vertebrate *tinman* genes serve a regulatory function equivalent to that of the single *tinman* gene in *Drosophila.*

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