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Overexpression of the *tinman*-related genes *XNkx-2.5* and *XNkx-2.3* in *Xenopus* embryos results in myocardial hyperplasia

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SUMMARY

Drosophila tinman **is an NK-class homeobox gene required for formation of the dorsal vessel, the insect equivalent of the vertebrate heart. Vertebrate sequences related to *tinman*, such as mouse *Nkx-2.5*, chicken *cNkx-2.5*, *Xenopus XNkx-2.5* and *XNkx-2.3* are expressed in cardiac precursors and in tissues involved in induction of cardiac mesoderm. Mice which lack a functional *Nkx-2.5* gene die due to cardiac defects. To determine the role of *tinman*-related sequences in heart development, we have overexpressed both *XNkx-2.3* and *XNkx-2.5* in *Xenopus laevis* embryos. The resulting embryos are morphologically normal except that they have enlarged hearts. The enlarged heart phenotype is due to a thickening of the myocardium caused by an increase in the overall number of myocardial cells (hyperplasia). Neither ectopic nor precocious expression of cardiac differentiation markers is detectable in overexpressing embryos. These results suggest that both *XNkx-2.3* and *XNkx-2.5* are functional homologues of *tinman*, responsible for maintenance of the heart field.**

Key words: cardiac development, homeobox, organogenesis, heart field, *Xenopus*, *tinman*, *XNkx-2.5*

INTRODUCTION

Classical embryological studies of amphibian heart development have provided the fundamental descriptions of the inductive interactions and the cellular movements associated with cardiac organogenesis (Keller, 1976; Sater and Jacobson, 1989, 1990a). Recent molecular studies have revealed that, although many of the same proteins are expressed in both cardiac and skeletal muscle, none of the well-characterized myogenic transcription factors are expressed at the right time or at sufficient levels to direct cardiac differentiation (Olson, 1993; Sassoon et al., 1989; Sassoon, 1993; Sartorelli and Kedes, 1992). It seems likely, therefore, that other transcription factors are required for the regulation of cardiac muscle cell fate.

Tissue-specific homeodomain transcription factors involved in organogenesis have been identified (McGinnis and Krumlauf, 1992). Examples include *LFB1* in liver tissue (Frain et al., 1989), *Pax 6/eyeless* in the eye (Quiring et al., 1995; Halder et al., 1995), *GFH1/Pit* in the pituitary gland (Bodner et al., 1988; Ingraham et al., 1988), *Nkx-2.1/TTF-1* in the thyroid (Lazarro et al., 1991; Guazzi et al., 1990, 1994) and *Hox 11* in the spleen (Roberts et al., 1994; Dear et al., 1995). There is also convincing evidence that members of the NK-class of homeodomain proteins are key players in the establishment of myogenic lineages during development (Kim and Nirenberg, 1989; Komuro and Izumo, 1993; Lints et al., 1993; Tonissen et al., 1994). In *Drosophila*, the NK homeobox gene, *tinman*, is required for mesoderm formation and is essential for differentiation of the visceral muscles and the dorsal vessel, the insect equivalent of the heart (Bodmer, 1993). Embryos that lack *tinman* function do not develop any dorsal vessel or gut muscle progenitor cells. The murine *tinman*-related gene, *Nkx 2.5*, is expressed in early cardiomyocyte precursors long before the initiation of the myogenic program (Lints et al., 1993; Komuro and Izumo, 1993). The frog and chicken equivalents of this gene, *XNkx-2.5* (Tonissen et al., 1994) and *cNkx-2.5* (Shultheiss et al., 1995), respectively, are also expressed in cardiogenic precursor cells from very early stages of development. Therefore, the expression pattern of this vertebrate *tinman*-related gene is consistent with a role in cardiogenesis. When the function of the mouse *Nkx-2.5* gene is interrupted using gene targeting techniques, the resulting embryos display a number of heart defects including thinning of the ventricular myocardium and a failure to undergo looping morphogenesis (Lyons et al., 1995). Significantly, in contrast to the *tinman* mutants, the embryonic heart still forms and most cardiac differentiation markers are expressed normally. Of the markers assayed, only the expression of myosin light chain 2V is absent. Although these results indicate that *Nkx 2.5* is involved in the regulation of at least one distinct pathway in the cardiac myogenic program, it is perhaps surprising that elimination of *Nkx-2.5* in cardiac progenitors does not result in more severe consequences for cardiac development. At present there is no evidence for expression of functionally redundant *tinman*-related genes in the developing mouse heart

and so this raises the question of whether *Nkx-2.5* indeed plays a role equivalent to *tinman*.

At present, therefore, the precise role of *tinman*-related genes in vertebrate cardiac development is unclear. They may be master regulators capable of initiating the myocardial differentiation pathway. Alternatively, these genes may be involved in partitioning the mesoderm in the early embryo, and maintaining it in a state capable of responding to other cardiac differentiation signals, analogous to the role of *tinman* in *Drosophila*. In an attempt to learn more about the function of these genes, we have used the *Xenopus* embryo system to overexpress *XNkx-2.5*, and the recently reported *tinman*-related gene, *XNkx-2.3* (Evans et al., 1995), during early cardiac development. Our results show that overexpression of either *XNkx-2.3* or *XNkx-2.5* results in a significant increase in size of the embryonic heart. In no case do we observe expression of myocardial markers at an ectopic location. These results are consistent with *XNkx-2.3* and *XNkx-2.5* playing a role in the maintenance or specification of mesodermal cell fates.

MATERIALS AND METHODS

Isolation of cDNA clones containing *XNkx-2.3* sequences

Approximately 10^6 plaques from a *Xenopus laevis* adult heart cDNA library (Ji et al., 1993) were screened at low stringency using probe derived from the *XNkx-2.5* homeobox sequence (bases 444-562; Tonissen et al., 1994). Almost 100 duplicate positives of varying intensity were detected and seven with reduced intensity were purified. Preliminary sequencing of these clones using a degenerate homeobox helix 3 primer (5′-AACCADATYTTNACYTG-3′) indicated that four clones contained the same homeobox sequence, which is closely related to mouse *Nkx-2.3* (Price et al., 1992). Several closely related homeobox sequences, named *XNkx-2.3*a, b1 and b2, have been independently isolated by Evans et al. (1995). Our isolate is most similar to *XNkx-2.3*a, but shows a number of nucleotide differences leading to a total of 4 amino acid changes. The sequence has been submitted to the GenBank Database (accession number U30318).

Embryo generation and microinjection

Xenopus laevis embryos were generated using standard techniques and staged according to Nieuwkoop and Faber (1994). Synthetic mRNAs encoding XNkx-2.5, XNkx-2.3, or Mix.2 (Vize, 1996) were microinjected into the left dorsal-vegetal blastomere of 8-cell embryos using a Drummond Nanoject variable automatic injector. Injected embryos were cultured at 13°C in 3% Ficoll/100% Steinberg's buffer for 12 hours and then in 20% Steinberg's buffer until heart differentiation had occurred. In the standard protocol, 125 pg of mRNA was injected in 4.6 nl of water containing 5 mg/ml lysine-fixable fluorescein dextran (FDA), M_r 70,000 or 5 mg/ml neutral tetramethylrhodamine dextran (RDA), M_r 70,000. Successful identification of the dorsal blastomeres depends on the pigmentation of the particular batch of embryos, but, based on lineage tracing, the correct blastomere was injected about 80% of the time. Regardless of the location of the lineage tracer, all embryos were subjected to whole mount in situ hybridization analysis using myocardial specific probes.

In vitro transcription

Capped mRNAs were synthesized in vitro using *Bam*HI linearized pT7TS-XNkx-2.5, pT7TS-XNkx-2.3 and pMix.2 (Vize, 1996) plasmid templates and the Message Machine in vitro transcription kit (Ambion). The pT7TS plasmid contains the 5′ and 3′ UTRs of *Xenopus* β-globin mRNA, isolated from a modified version of the translation vector pSP64T (Krieg and Melton, 1984) cloned into pGEM4Z (Promega). pT7TS-XNkx-2.5 contains the 1,230 bp *Kpn*I to *Bam*HI fragment of the full-length *XNkx-2.5* cDNA cloned into the *Eco*RV site of pT7TS. pT7TS-XNkx-2.3 contains a 1,031 bp PCR product generated by amplification of an *XNkx-2.3* cDNA plasmid template using primers flanking the *XNkx-2.3* coding region (5′ACCATGATGTTACCTAGC3′ and 5′GCAGAAGCATTCGTCCA3′). The coding region fragment was inserted into the *Eco*RV site of pT7TS. Messenger RNA was quantitated by measuring percentage incorporation of labeled nucleotide and then stored as an ethanol precipitate. All mRNAs were translated in vitro to confirm the presence of complete protein coding regions.

In situ hybridization and histology

Whole-mount in situ hybridization using antisense digoxigenin-labeled *Xenopus* cardiac troponin Ic (*XTnIc*) probe (Drysdale et al., 1994) or *Xenopus* myosin light chain 2 (*XMLC2*) probe (Chambers et al., 1994) was performed using standard conditions (Harland, 1991) except that CHAPS was omitted from all buffers. For sectioning, embryos were subsequently dehydrated in ethanol, permeabilized in xylene (2× 10 minutes), incubated in xylene/Paraplast (1× 10 minutes), incubated in Paraplast (2× 10 minutes, 1× overnight) at 60°C, then embedded in Paraplast. Sections (10 μm) were dried, dewaxed in xylene, mounted with Permount and photographed using Nomarski optics. Sections used for nuclear staining were dewaxed in xylene and rehydrated in 2× SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0). A 1:300 dilution of SYTOX Green (Molecular Probes) was applied to the sections (1× 5 minutes) and then rinsed in 2× SSC (3× 5 minutes). Sections were mounted in Prolong Antifade medium (Molecular Probes) and viewed using fluorescence microscopy at 502 nm.

Analysis of phenotype

After whole mount in situ hybridization using *XTnIc* probes, injected embryos were visually assayed for heart morphology by two independent observers in a blind assay. Agreement between the two observers was necessary for scoring. Cross-sectional area of the myocardial tissue in injected embryos was determined directly from microphotographs. Cell numbers in the myocardial tissue were assayed by counting the number of nuclei visible after staining with SYTOX Green (Molecular Probes). The nuclei visible in different sections were counted by three independent observers and the numbers averaged.

RNase protection analysis of myocardial specific transcripts

The heart region was excised from stage 37 embryos that had been injected with either water or *XNkx-2.5* mRNA (20 embryos for each experiment). Total RNA was isolated from the excised heart region, and from the remaining carcass, by homogenization in 4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarkosyl, and 0.1 M β-mercaptoethanol, followed by addition of 1/10 volume 2 M sodium acetate, pH 4.0, phenol/chloroform extraction and ethanol precipitation. Transcripts present in the heart and carcass fractions were detected by RNase protection using probes specific for *XTnIc*, *XMLC2* (Chambers et al., 1994) or the ubiquitous *Max* transcript, *XMax2* (Tonissen and Krieg, 1994), which serves as a control for recovery of RNA. The *XTnIc* probe was synthesized from pTrop.p plasmid template containing a 375 bp *Pst*I fragment from the cardiac troponin cDNA. The *MLC2* and *XMax2* probes protect fragments of 309 nt and 151 nt, respectively.

RESULTS

Developmental expression of *XNkx-2.3* and *XNkx-2.5*

XNkx-2.3 and *XNkx-2.5* transcripts first appear in the frog embryo during gastrulation (Evans et al., 1995; K. D.

Patterson, unpublished observations). RNase protection analysis of RNA isolated from gastrula stage embryos, shows that *XNkx-2.5* transcripts are exclusively located in the dorsal quarter of the embryo. This region includes the precardiac mesoderm, dorsal mesoderm and underlying endoderm (data not shown). We have compared the *XNkx-2.3* and *XNkx-2.5* expression patterns throughout embryogenesis using whole mount in situ hybridization (Harland, 1991). Both *XNkx-2.3* and *XNkx-2.5* transcripts may be detected as early as stage 13 in a crescent of tissue located just ventral to the developing neural plate (data not shown). This crescent contains the bilaterally symmetric pre-cardiac mesoderm as well as the underlying anterior endoderm. While the endodermal expression of *XNkx-2.5* gradually decreases during neurulation, *XNkx-2.5* and *XNkx-2.3* transcripts are maintained in the precardiac region (Fig. 1A,B). As development proceeds, *XNkx-2.3* transcripts are expressed in anterior endoderm, underlying the cement gland, and in the cardiac mesoderm (Fig. 1C). Later in development, *XNkx-2.3* is expressed in differentiated myocardial tissue and in the pharyngeal region (Fig. 1D). *XNkx-2.3* transcripts are slightly more broadly distributed in the pharyngeal region than *XNkx-2.5* but, over time, both become restricted to several narrow stripes in the pharyngeal region (Fig. 1D). In contrast to the results of Evans et al. (1995), no *XNkx-2.3* staining is observed in the head, and both *XNkx-2.3* and *XNkx-2.5* transcripts are present at high levels in the heart at all stages examined (Fig. 1C,D). Based on the staining pattern observed in the in situ assay and on RNase protection analysis (data not shown) transcripts of the two genes are present in similar locations at approximately equal amounts throughout development.

Overexpression of *XNkx-2.3* and *XNkx-2.5* in the *Xenopus* embryo

Embryonic expression of both *XNkx-2.5* and *XNkx-2.3* commences in precardiac mesoderm during gastrulation, very soon after cardiac tissue is known to be specified (Sater and Jacobson, 1989). These homeodomain sequences are therefore present at the right time and in the right place to play a role in the pathway leading to heart muscle differentiation. To investigate this possibility further, we have used microinjection techniques to overexpress *XNkx-2.3* and *XNkx-2.5* sequences in the early *Xenopus* embryo. In initial experiments to establish the injection protocol, different amounts of mRNA, ranging from 60 pg to 6 ng, were injected into single cells of embryos at the one, two or 8 cell stage. Injection of large amounts of mRNA into one or two-cell embryos produced severe axial abnormalities, defects in neurulation and a high rate of lethality. Similar effects were observed following injection of large amounts of mRNA encoding *Mix.2* a homeobox sequence which is not expressed endogenously in the heart (Vize, 1996) and which serves as a control for non-specific effects resulting from homeobox protein overexpression (Vize et al., 1991). After analyzing many hundreds of embryos, injected with different amounts of transcript in different blastomeres, we settled on a standard protocol, in which 125 pg of either *XNkx-2.3* or *XNkx-2.5* mRNA was injected into a dorsal, vegetal blastomere of the 8-cell embryo, in conjunction with a fluorescently labeled lineage marker. This protocol concentrates the injected mRNA in the precardiac mesoderm during subsequent development (Dale and Slack, 1987), and, most significantly, the injected embryos appeared normal in overall morphology. Examples of embryos injected in the dorsal/vegetal and ventral/vegetal blastomeres are shown in Fig. 2A and 2B, respectively.

Probes for two myocardial-specific differentiation markers, *XTnIc* and *XMLC2*, were used to detect heart structures in the embryo. Results of a whole mount in situ hybridization assay showing the hearts of embryos injected with water, or mRNAs encoding Mix.2 control, XNkx-2.3 or XNkx-2.5 are presented in Fig. 2. The hearts in the *XNkx-2.3* (Fig. 2E,G,I) and *XNkx-2.5* overexpressing embryos (Fig. 2F,H,J) are located at the normal position but are distinctly larger than those in control embryos (Fig. 2C,D,K). In most cases, the heart tube undergoes normal looping morphogenesis. Later in development, *XNkx-2.3* and *XNkx-2.5* injected embryos generate a functional beating heart, blood-flow is unimpeded and they remain healthy at least until they become feeding tadpoles. Therefore, overexpression of *XNkx-2.3* and *XNkx-2.5* causes an increase in heart size, without compromising cardiac function. The results from many independent injection experiments are summarized in Table 1. For those embryos where the lineage tracer was located in the heart region, approximately 60% displayed enlarged heart structures. Embryos injected with either *Mix.2* mRNA or water never showed enlargement of the *XTnIc* or *XMLC2* staining tissue, indicating that the large heart phenotype is not a common artifact arising from injection trauma or a non-specific consequence of homeobox-protein overexpression. We have also analyzed embryos in which injected mRNAs were concentrated in regions other than the heart such as the tail, gut, somites, or pharynx, as indicated by the lineage tracer. In all cases these embryos displayed normal heart morphology and size. In no instance did we observe ectopic hearts, failure of myocardial mesoderm migration or fusion, or any detectable expression of *XTnIc* or *XMLC2* transcripts outside of myocardial tissues (Fig. 2I,J,K,L and data not shown).

Analysis of the early stages of myocardial differentiation

Expression of myocardial differentiation transcripts in normal embryos is very weakly detectable at about stage 24 by in situ hybridization analysis. By stage 26, two small, symmetrical patches of tissue, expressing *XTnIc* are clearly visible on either side of the ventral midline (Fig. 3A). When embryos overexpressing *XNkx-2.3/2.5* mRNA are examined at the same stage, we usually observe a much larger region of tissue expressing myocardial differentiation markers. In some cases the regions of increased expression are symmetrical (Fig. 3B) and in some

Table 1. Frequency of enlarged heart phenotype observed in injected embryos

	Water injected	*Mix.2* injected	*XNkx-2.5* injected	*XNkx-2.3* injected
Total no. embryos	203	70	218	262
Embryos with phenotype	0	0	122	173
Frequency of phenotype	0%	0%	56%	66%

All embryos were co-injected with mRNA and lineage tracer. Only embryos with lineage tracer in the heart region were included in this survey. Heart tissue was visualized by in situ hybridization using *XTnlc* probe.

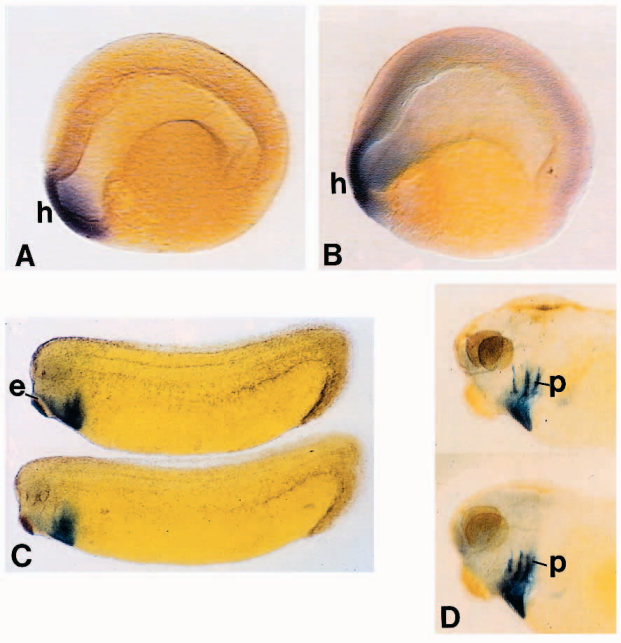

Fig. 1. *XNkx-2.3* and *XNkx-2.5* are expressed in similar regions of the developing embryo. In situ hybridization with antisense *XNkx-2.3* and *XNkx-2.5* probes demonstrates that both of these sequences are expressed in early heart progenitors (h) and are maintained in cardiac tissue throughout development. *XNkx-2.3* and *XNkx-2.5* are also expressed in similar, but distinct regions of anterior endoderm (e) and pharynx (p). (A) Stage 18 *XNkx-2.5* stained embryo. (B) Stage 18 *XNkx-2.3* stained embryo. (C) Stage 28 embryos. (D) Stage 37 embryos. In C and D the *XNkx-2.3* stained embryo is on top.

cases asymmetrical (Fig. 3C). If asymmetrical, however, the larger patch of expression is always present on the injected side of the embryo. This variable expression pattern appears to be due to variable amounts of cell mixing between the injected and uninjected sides of the embryo. By observing the distribution of the lineage tracer, it is possible to find examples where very little migration across the ventral midline has occurred (Fig. 3E) but it is much more common to observe a significant degree of cell-mixing (Fig. 3D). There is no distinguishable difference in the extent of ventral cell-mixing between water and *XNkx-2.3/2.5* injected embryos. A variable degree of cell migration in ventral regions of the embryo has previously been reported by Dale and Slack (1987). Embryos in which the area of differentiation is much greater on the injected side of the embryo have presumably experienced little cell migration across the ventral midline.

Overexpression of *XNkx-2.3* or *XNkx-2.5* results in increased myocardial cell number

To examine the large heart phenotype in more detail, embryos stained for *XTnIc* transcripts were examined in histological sections. *XNkx-2.3* or *XNkx-2.5* injected embryos were sectioned at stages 31 to 33, when the linear heart tube is beginning to close, but looping has not commenced. Analysis at this stage considerably simplifies interpretation of the myocardial structures. As shown in Fig. 4 (Fig. 4A,B,E,F) transverse sections through *XNkx-2.3/2.5* injected embryos revealed a thickening of the myocardial tissue relative to

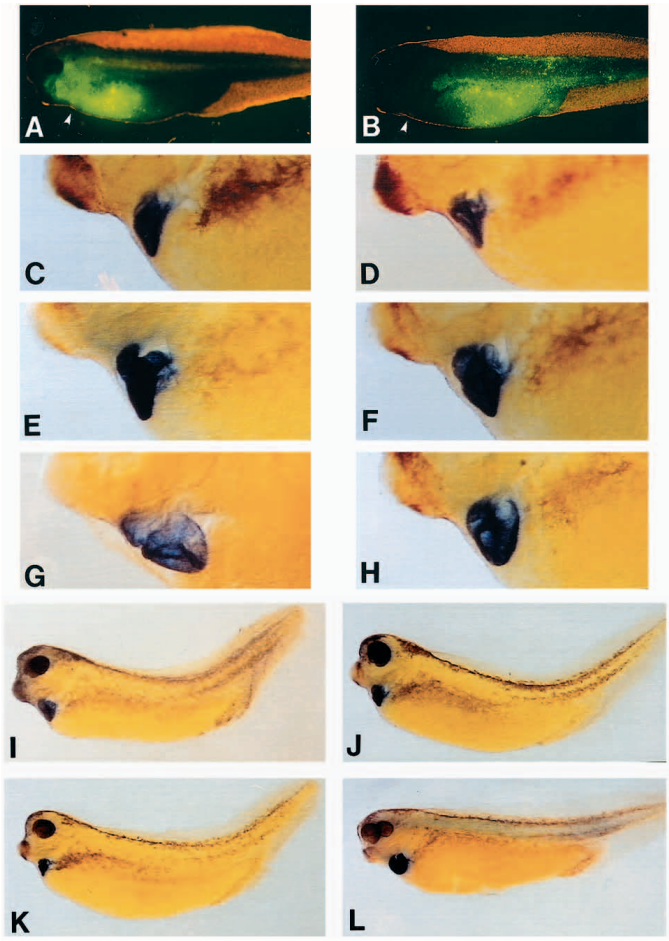

Fig. 2. Overexpression of *XNkx-2.3* and *XNkx-2.5* in heart progenitors results in enlarged cardiac structures. Embryos injected with lineage tracer plus water, control *Mix.2* RNA, *XNkx-2.3* RNA or *XNkx-2.5* RNA are shown. (A) Fluorescence micrograph of an embryo injected with fluoresceinated dextran in water into the dorsal vegetal blastomere at the 8-cell stage. The heart region is indicated by an arrowhead. (B) Embryo injected as in A into the ventral vegetal blastomere. These embryos are labeled primarily in the posterior gut and ventral mesoderm. (C-K) In situ hybridization depicting cardiac troponin I expression (blue stain) in stage 37 embryos that were injected into the dorsal vegetal blastomere as in A. (C,K) Water injected (D) *Mix.2* injected (E,G,I) *XNkx-2.3* injected (F,H,J) *XNkx-2.5* injected (L) *XNkx-2.5* injected embryo stained by in situ hybridization with *XMLC2* probe. The heart is enlarged in *XNkx-2.3* and *XNkx-2.5* injected embryos (E,F,G,H,I,J,L), but not in water or *Mix.2* injected embryos (C,D,K). This is not a non-specific effect of overexpressing homeobox sequences since *Mix.2* injected embryos have normal size or small hearts (D). No ectopic cardiac differentiation is detected by in situ hybridization with either *XTnIc* or *XMLC2* probe (I,J,L). Embryos in C-H were photographed at ×70 magnification and embryos in I-L were photographed at ×20 magnification. The brown color in all embryos is natural pigmentation.

controls. In most cases, the thickening of the myocardial layer is even, and is not noticeably greater on the injected side of the embryo. Examples of symmetrical thickening and moderately asymmetrical thickening are illustrated in Fig. 4E,F and A,B, respectively. As discussed earlier, the absence of clear asymmetry is almost certainly due to cell mixing across the ventral midline (see Fig. 3D). In order to count the number of

cells in the myocardial layer, sections were co-stained with SYTOX Green which causes the nuclei to fluoresce under blue light (Fig. 4C,D,G,H). Using the combination of in situ hybridization staining of the myocardial cell layer and the nuclear staining, we are able to measure both the cross-sectional area of the myocardial tissue and the number of cells in the layer. The results from comparisons of a number of carefully matched sections are summarized in Table 2. These comparisons indicate that the myocardial layer of *XNkx-2.3/2.5* injected embryos has approximately 1.9 times the cross-sectional area of the water injected control embryos and that the number of cells in the myocardium has increased about 1.6 times. We conclude from this experiment that the increased heart size results from an increased number of myocardial cells and not from either a swelling of the heart tissue (edema) or from increased size of the myocardial cells (hypertrophy).

Overexpression of *XNkx-2.3 or XNkx-2.5* does not lead to ectopic expression of myocardial markers

Our standard injection protocol results in the distribution of high levels of synthetic mRNA to approximately one eighth of the cells in the developing embryo, which includes many tissues in addition to the heart (Fig. 2A). In addition, hundreds of embryos have been injected in a ventral/vegetal blastomere, which concentrates transcripts in posterior endodermal and mesodermal tissues, including the somites (Fig. 2B). In no case, have we observed ectopic expression of myocardial markers. While whole mount in situ hybridization is very sensitive for detection of target transcripts concentrated in a small number of cells, it might not detect low level ectopic expression of myocardial markers over an extended region of the embryo. To test this possibility, RNase protection analysis was carried out

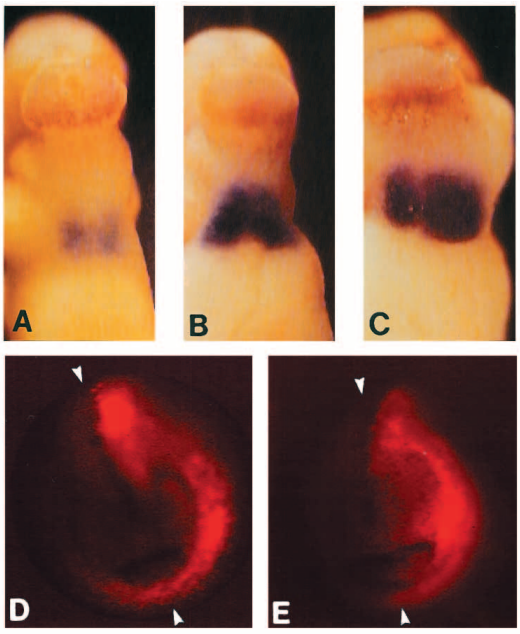

Fig. 3. Increased regions of marker gene expression at early stages of cardiac differentiation. In situ hybridization of stage 26 embryos to detect cardiac troponin I expression indicates that cardiac differentiation begins in two symmetrical regions within the heart field (A) and that these regions are enlarged in *XNkx-2.5* and *XNkx-2.3* injected embryos (B,C). (A) Water injected embryo. (B) *XNkx-2.3* injected embryo with symmetrically large troponin expressing regions. (C) *XNkx-2.3* injected embryo with asymmetrically large troponin expressing region. The left side which received the injection is larger. Fluorescence microscopy of stage 25 embryos injected into the left dorsal vegetal blastomere at the 8-cell stage demonstrates extensive cell mixing between the left and right sides in the heart region. This cell mixing may result in significant overexpression of injected RNAs on both the left and right sides leading to expansion of the heart field on both sides. (D) Anterior view of stage 25 embryo injected with tetramethylrhodamine-conjugated dextran showing mixing on the ventral side. (E) Anterior view of stage 25 embryo injected as in D but with much less cell mixing. In each case, the plane of the dorsal/ventral axis is indicated by opposing arrowheads.

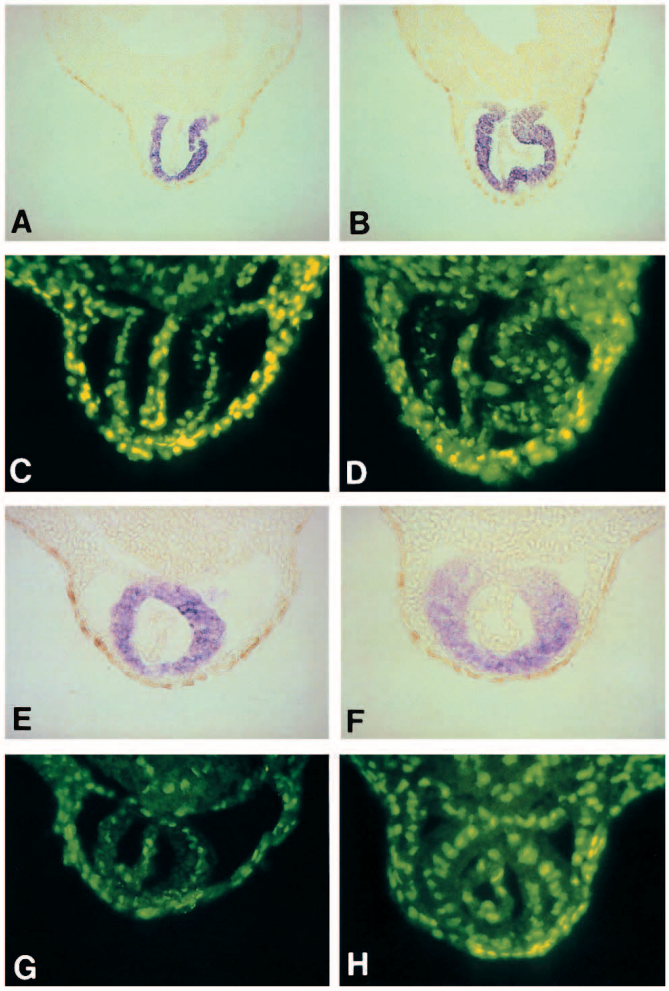

Fig. 4. Paraffin sections demonstrate an increase in myocardial volume and in the number of myocardial cells in *XNkx-2.3* and *XNkx-2.5* injected embryos. In situ hybridization to detect cardiac troponin I transcripts (the purple color in A,B,E,F) was completed before sectioning. Nuclei were visualized in C,D,G, and H with the fluorescent nuclear stain SYTOX Green (Molecular Probes). (A) Cross section through the heart region of a water injected embryo at stage 33. (B) Section equivalent to that in A from a *XNkx-2.5* injected embryo. (C) Section adjacent to that in A. (D) Section adjacent to that in B. (E) Cross section through the heart region of a water injected embryo at stage 31. (F) Section equivalent to E from a *XNkx-2.3*-injected embryo. (G) Same section as in E. (H) Same section as in F. A-B were photographed at ×200. C-H were photographed at ×400.

Table 2. Comparison of myocardial area (transverse section) and myocardial cell number

	Water injected	*XNkx-2.5* and *XNk-2.3* injected	No. of samples
Area ratio ± s.e.m.	1	1.87±0.09	35 pairs
Cell number ratio ± s.e.m.	1	1.57±0.14	15 pairs

Prior to sectioning, embryos were precisely matched for their developmental stage. Sections from equivalent positions along the anterior-posterior axis of the developing heart tube were chosen for comparison.

using RNA samples isolated from the cardiac and non-cardiac regions of embryos injected with *XNkx-2.5* mRNA (Fig. 5A). The probes used in these experiments were *XTnIc* and *XMLC2*, both of which appear to be truly restricted to the myocardial lineage during early development (Drysdale et al., 1994; Chambers et al., 1994). Probe for the ubiquitous *Max* sequence (Tonissen and Krieg, 1994) was included to control for amounts of RNA in the dissected fractions. As shown in Fig. 5B, all detectable *XTnIc* and *XMLC2* transcripts are detected in the cardiac region of the embryo, even though the non-cardiac samples contain approximately 8-fold more total RNA. We conclude that overexpression of *XNkx-2.5* does not lead to detectable ectopic expression of myocardial markers. In this experiment, the amount of *XTnIc* and *XMLC2* transcript detected in *XNkx-2.5* embryos is not quantifiably greater than that in water injected controls. In one experiment we detected a two-fold difference in *XTnIc* transcript levels in the *XNkx-2.5* injected embryos, but in other experiments no difference was detectable between the experimental and control samples. Somewhat surprisingly, this suggests that no increase in marker transcript levels occurs in *XNkx-2.3/2.5* overexpressing embryos, or that the increase is too small to measure reliably.

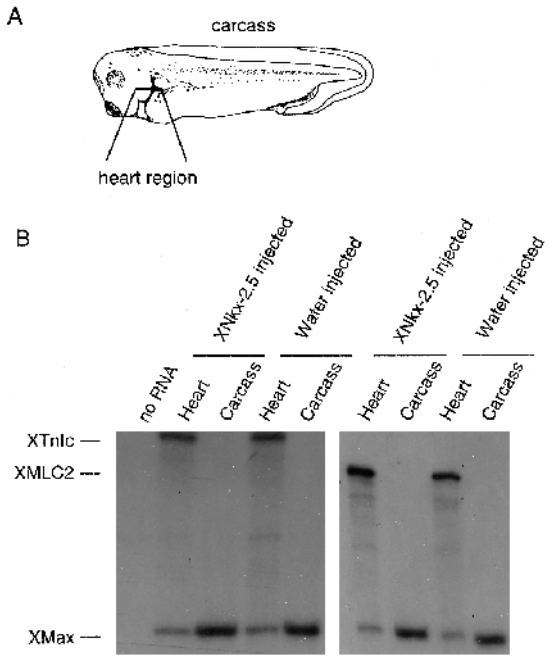

Fig. 5. Ectopic expression of *XNkx-2.5* does not result in ectopic cardiac differentiation. (A) Heart regions of water and *XNkx-2.5* injected embryos were microdissected at stage 37. This figure is modified from Nieuwkoop and Faber, 1994. (B) RNA from the hearts and carcasses was assayed for cardiac troponin I and myosin light chain-2 transcripts by RNase protection. *XMax* transcripts were detected as a control for the amount of RNA assayed. Neither *XTnIc* nor *XMLC2* are detectable outside of the heart region in water or *XNkx-2.5* injected embryos.

DISCUSSION

The identification of *XNkx-2.3* (Evans et al., 1995, and this paper) demonstrates that at least two *tinman*-related homeobox sequences are expressed in *Xenopus* cardiac muscle. *XNkx-2.3* and *XNkx-2.5* exhibit very similar temporal and spatial patterns of expression during *Xenopus* early development (Tonissen et al., 1994; Evans et al., 1995). Both transcripts are first detected during gastrulation, soon after cardiac tissues have been specified (Sater and Jacobson, 1989), and also in anterior endoderm which is involved in the induction of mesoderm to form heart (Nascone and Mercola, 1995). Levels of both transcripts peak at the late neurula stage of development (stage 22-24) prior to the appearance of transcripts encoding cardiac differentiation markers (Evans et al., 1995, and data not shown). By in situ hybridization, the expression patterns of *XNkx-2.3* and *XNkx-2.5* are essentially identical and it is likely that many of the cells in the precardiac mesoderm express both sequences. These expression profiles indicate that *XNkx-2.3* and *XNkx-2.5* are expressed at the appropriate time and location to play a role in regulation of myocardial differentiation, either directly or as part of a regulatory cascade.

Overexpression of *XNkx-2.3* or *XNkx-2.5* leads to enlargement of the embryonic heart

In an effort to determine the role of the *XNkx-2.3/2.5* sequences in heart development, we have carried out a series of overexpression experiments in which mRNAs encoding *XNkx-2.3* or *XNkx-2.5* have been injected into developing *Xenopus* embryos. Using the standardized protocol, approximately 60% of all embryos receiving either *XNkx-2.3* or *XNkx-2.5* sequences developed larger than normal hearts (Table 1) and the phenotype resulting from overexpression of the two sequences is apparently identical. No enlarged hearts were ever observed in embryos injected with water or with *Mix.2* mRNA. The amount of mRNA injected in these overexpression experiments is quite modest, representing only about 1% of the total mRNA in the injected blastomere. At these doses, the embryos develop normally and the only discernible consequence of overexpression is the large heart structures. Varying the parameters of the injection protocol lead to two main observations. First, there are no obvious synergistic effects resulting from overexpression of both *XNkx-2.3* and *XNkx-2.5*. Comparisons of embryos injected with a mixture of *XNkx-2.3* and *XNkx-2.5* mRNA (125 pg of each) and embryos injected with 250 pg of a single sequence, showed no detectable differences in either size of the heart or in the frequency with which the phenotype was obtained (data not shown). Second, there is no obvious dose response resulting from injection of larger amounts of *XNkx-2.3* or *XNkx-2.5* mRNA. In preliminary experiments, we determined that injection of large amounts of mRNA (500 pg and above) caused a very high proportion of embryos to develop abnormally or to die. This effect was also observed in embryos injected with *Mix.2* mRNA and is believed to be a

non-specific effect of overexpressing homeodomain sequences (Vize et al., 1991). In these experiments, however, the surviving embryos did not exhibit larger hearts than those injected with 125 pg of mRNA. This observation may be explained if the XNkx-2.3 and XNkx-2.5 proteins, or any gene products downstream, need to interact with additional factors in order to be biologically active. It is generally accepted that homeobox proteins are capable of forming heterodimers (Grueneberg et al., 1992) and it is possible that XNkx2.3 and XNkx-2.5 require a partner protein to function in the embryo. If the partner protein is only expressed in developing cardiac tissues, then the effects of *XNkx-2.3/2.5* overexpression will be limited to the region of the embryo that will normally form heart, as we observe in our experiments.

Analysis of the large heart phenotype

The large hearts observed by in situ hybridization could be created by several different mechanisms, including swelling of the myocardium (edema), an increased number of myocardial cells (hyperplasia) or increased size of the myocardial cells (hypertrophy). Sectioning of *XNkx-2.3* and *XNkx-2.5* injected embryos clearly reveals that the cross-sectional area of the myocardial cell layer is increased and that the number of cells in the myocardium is also increased, relative to water injected controls (Fig. 4). As summarized in Table 2, the proportional increase in area, and cell number, is about 1.9-fold and 1.6-fold, respectively. While the increase in cell number can account for the majority of the increase in myocardial cross sectional area, it remains possible that the cells in the *XNkx-2.3/2.5* injected embryos are also slightly larger. There are two general processes that could lead to an increased number of cells in the heart: increased proliferation of those cells already fated to become myocardium, or recruitment of additional embryonic cells to the formation of myocardial tissue. At present, we cannot distinguish between these different mechanisms. Another consequence of overexpression is a disorganization of the myocardial tissue. In control embryos the nuclei are often aligned near the middle of the myocardial cell layer (Fig. 4C,G) and the myocardial cells have a regular columnar appearance. In *XNkx-2.3/2.5* overexpressing embryos, however, the myocardial cells are often disorganized and the regular alignment of the nuclei has been lost (Fig. 4D,H). While the myocardial walls of water or *Mix.2* injected embryos were generally one cell layer thick (Fig. 4C,G), the myocardium of *XNkx-2.3* or *XNkx-2.5* injected embryos was increased to two to three cell layers (Fig. 4D,H).

Fu and Izumo (1995) have previously reported larger hearts and precocious expression of myocardial differentiation markers in embryos injected with *XNkx-2.5* mRNA. In these experiments, PCR analysis was used to detect expression of *MHCα* mRNA in early neurula embryos (stage 14), well ahead of its normal activation at about stage 26 (Logan and Mohun, 1993). In our experiments, very low levels of cardiac troponin I expression have been detected as early as stage 24 in water and *XNkx-2.3/2.5*-injected embryos, coincident with the normal activation of cardiac troponin I transcription as assayed by RNase protection (unpublished data). In no case have we observed expression of myocardial differentiation markers prior to stage 24 and so our results suggest that *XNkx-2.3/2.5* overexpression has little, if any, effect on the timing of myocardial differentiation.

The function of *XNkx-2.3* and *XNkx-2.5* in cardiogenesis

The phenotypes resulting from overexpression of *XNkx-2.3* and *XNkx-2.5* are indistinguishable at all levels examined. These two genes, therefore, appear to function in the same pathway and it is possible that they play largely or completely redundant roles during early development. What is the mechanism by which overexpression of *XNkx-2.3* or *XNkx-2.5* leads to enlarged hearts? It is unlikely that the *XNkx-2.3* and *XNkx-2.5* genes are master regulators of myocardial development capable of initiating the myocardial differentiation pathway. A master regulator would be expected to direct ectopic cardiac differentiation markers when expressed in ectopic locations in the embryo. Our injection protocol results in overexpression of *XNkx-2.3/2.5* in many tissues in the embryo in addition to the developing myocardium and always overlaps into the developing somitic tissue (see Fig. 2A,B). It seems reasonable to argue that it would be easier to convert cells normally fated to be mesoderm (e.g. somites) into cardiac mesoderm than to convert endoderm into mesoderm (cardiac or otherwise). In no case, however, is ectopic expression of myocardial differentiation markers detected, either in somitic tissues or in any other region of the embryo. This suggests that expression of these homeobox sequences is not sufficient to initiate or maintain the complete myocardial development pathway.

In *Xenopus*, the precardiac mesoderm, the group of cells specified to form heart, is larger than the group of cells that will eventually express cardiac differentiation markers (Sater and Jacobson, 1990b). In all likelihood, *XNkx-2.3/2.5* overexpression leads to an increase in the amount of tissue comprising the precardiac mesoderm, either by stabilizing the existing precursors against inhibitory signals arising from nearby tissues (Sater and Jacobson, 1990b) or by recruiting additional cells to the specified state. In the unmanipulated embryo, *XNkx-2.3* and *XNkx-2.5* expression may maintain specified cardiac precursors (the heart field) in a state in which they are responsive to additional cardiac differentiation signals. If, at the time of differentiation, the pool of cardiac precursor cells in injected embryos is larger than in normal embryos, then more cells will express myocardial genes and the resulting heart will be larger. This proposed function for *XNkx-2.3/2.5* closely parallels the likely role of *tinman* in the *Drosophila* embryo (Bodmer et al., 1990; Bodmer, 1993; Azpiazu and Frasch, 1993), where expression appears to maintain somatic mesoderm in a state capable of differentiating into a number of specialized tissues, including the insect heart. Consistent with this interpretation, ectopic expression of *tinman* sequences in mutant *Drosophila* embryos does not lead to ectopic expression of heart markers, but does allow a small amount of cardiac tissue to differentiate at the normal location (Bodmer, 1993). In summary, our results show that overexpression of *XNkx-2.3* and *XNkx-2.5* sequences in the embryo leads to myocardial hyperplasia. These two genes, which are similar in sequence and in expression pattern, appear to function in the same pathway and may play redundant roles in early heart development. The overexpression phenotype supports the contention that *XNkx-2.3* and *XNkx-2.5* act as functional homologues of *tinman* in the vertebrate embryo.

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