Development 125, 4439-4449 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV4050

Vertebrate *tinman* homologues *XNkx2-3* and *XNkx2-5* are required for heart formation in a functionally redundant manner

Yuchang Fu[1], Wei Yan[1], Tim J. Mohun[2] and Sylvia M. Evans[1,*]

[1]Department of Medicine, University of California at San Diego, La Jolla, CA 92093-0613C, USA
[2]Laboratory of Developmental Biochemistry, National Institute for Medical Research, Mill Hill, London NW7 1AA, UK
*Author for correspondence (e-mail syevans@ucsd.edu)

Accepted 10 September; published on WWW 20 October 1998

SUMMARY

***Tinman* is a *Drosophila* homeodomain protein that is required for formation of both visceral and cardiac mesoderm, including formation of the dorsal vessel, a heart-like organ. Although several vertebrate *tinman* homologues have been characterized, their requirement in earliest stages of heart formation has been an open question, perhaps complicated by potential functional redundancy of *tinman* homologues. We have utilized a novel approach to investigate functional redundancy within a gene family, by coinjecting DNA encoding dominantly acting repressor derivatives specific for each family member into developing *Xenopus* embryos. Our results provide the first evidence that vertebrate *tinman* homologues are required for earliest stages of heart formation, and that they are required in a functionally redundant manner. Coinjection of dominant repressor constructs for both *XNkx2-3* and *XNkx2-5* is synergistic, resulting in a much higher frequency of mutant phenotypes than that obtained with injection of either dominant repressor construct alone. Rescue of mutant phenotypes can be effected by coinjection of either wild-type *tinman* homologue. The most extreme mutant phenotype is a complete absence of expression of *XNkx2-5* in cardiogenic mesoderm, an absence of markers of differentiated myocardium, and absence of morphologically distinguishable heart on the EnNkxHD-injected side of the embryo. This phenotype represents the most severe cardiac phenotype of any vertebrate mutant yet described, and underscores the importance of the *tinman* family for heart development. These results provide the first *in vivo* evidence that *XNkx2-3* and *XNkx2-5* are required as transcriptional activators for the earliest stages of heart formation. Furthermore, our results suggest an intriguing mechanism by which functional redundancy operates within a gene family during development. Our experiments have been performed utilizing a recently developed transgenic strategy, and attest to the efficacy of this strategy for enabling transgene expression in limited cell populations within the developing *Xenopus* embryo.**

Key words: Homeobox genes, Heart development, Microinjection, *Xenopus laevis XNkx2-3, XNkx2-5*

INTRODUCTION

Tinman is an NK-homeodomain protein in *Drosophila* which is required for differentiation of visceral and cardiac mesoderm, and formation of dorsal vessel (a heart-like organ) (Bodmer, 1993; Azpiazu and Frasch, 1993). Several *tinman* homologues have subsequently been cloned from vertebrate systems, and are expressed in patterns consistent with the idea that they are involved in formation of visceral and/or cardiac mesoderm (reviewed by Harvey, 1996; Newman and Krieg, 1998). The criteria for defining tinman homologues are based both on their expression in patterns analogous to those of tinman, (in developing heart, pharynx, and/or visceral mesoderm), and the presence of two domains exhibiting a high degree of homology to the tinman protein (the homeobox and the 'tin' or TN domain which is a decapeptide in the N-terminal region). Vertebrate homologues have two additional conserved domains not found within tinman itself: the NK-2 domain just C-terminal to the homeodomain, and a five amino acid GIRAW sequence at the C terminus (Newman and Krieg, 1998).

Despite the conservation of expression of this Nk-homeodomain family in visceral and cardiogenic mesoderm in a range of organisms from *Drosophila* to man, each species appears to express an overlapping but distinct repertoire of *tinman* homologues, and to express them in a species-specific manner.

A mouse knockout of one of these homologues, *mNkx2-5*, results in embryonic lethality at approximately E9.5, probably as a result of abortive cardiac development (Lyons et al., 1995). In contrast to a *tinman* mutant in *Drosophila* which results in no formation of the dorsal vessel, *mNkx2-5* mutants develop a differentiated heart tube which expresses most cardiac markers examined, but stops developing prior to undergoing looping morphogenesis. The reasons for this less severe phenotype relative to that of *tinman* mutants in *Drosophila* are not yet known. One possibility is that *tinman* homologues may be required for earlier stages of heart formation, but that there is

functional redundancy of two or more homologues for this early requirement. This idea is supported by the observation that expression of vertebrate homologues which are expressed very early in cardiogenic mesoderm is often overlapping with that of at least one other *tinman* homologue (Harvey, 1996; Newman and Krieg, 1998).

In *Xenopus*, three *tinman* homologues have been described to date, *Nkx2-3*, *Nkx2-5*, and *Nkx2-9* (Tonissen et al., 1994; Evans et al., 1995; Newman and Krieg, 1998). Expression of mRNAs for both *XNkx2-3* and *XNkx2-5* is upregulated at a time when cardiac specification is known to be occurring. Both genes are first expressed in gastrulae, and from neurula stages onward are expressed in cardiogenic mesoderm and pharyngeal endoderm in an overlapping manner. *XNkx2-9* is first detected at late neurula stages, in presumptive cardiogenic and endodermal tissues, and in contrast to the other two homologues, disappears from the heart completely near the time of differentiation, suggesting that perhaps it is playing a role in keeping cardiogenic cells in an undifferentiated state (Newman and Krieg, 1998).

Ectopic expression of either wild-type *XNkx2-3* or *XNkx2-5*, by mRNA injection into developing embryos, results in an enlarged heart phenotype (Fu and Izumo, 1995; Cleaver et al., 1996). Similar results have been obtained after ectopic expression of zebrafish *Nkx2-5* (Chen and Fishman, 1996). Whether the enlarged heart results from a recruitment of otherwise-fated cells to the cardiac lineage, or whether normal cardiogenic precursors undergo an additional round of replication before differentiation is not yet clear. However these results suggest that both *Nkx2-3* and *Nkx2-5* can affect cardiac cell fate at early stages of development.

To investigate the requirement for either *XNk2-3* or *XNk2-5* in cardiac specification and determination, and to investigate the possible functional redundancy between these two *tinman* homologues, we have attempted to create dominantly acting engrailed-fusion repressor constructs (Han and Manley, 1993; Badiani et al., 1994) which would be specific for each homologue.

Our results from ectopically expressing these En-Nkx fusion proteins either singly or together in developing *Xenopus* embryos indicate that each fusion protein can act in a specific manner, that *tinman* homologues *XNkx2-3* and *XNkx2-5* are required for the earliest stages of heart formation and cardiac cell differentiation, and that they are functionally redundant in this regard. In addition, our results suggest an intriguing mechanism for functional redundancy within a gene family.

MATERIALS AND METHODS

Plasmid constructs

The design of the dominant repressor constructs is diagrammed in Fig. 1. Expression constructs containing the engrailed repressor domain (En) were made by inserting an *Eco*RI-*Stu*I linkered polymerase chain reaction (PCR)-amplified En repressor domain encoding amino acids 2-298 of the engrailed protein from *Drosophila* (Han and Manley, 1993) into an *Eco*RI, *Stu*I digested pCS2$^+$nls vector (Turner and Weintraub, 1994). *Xho*I-*Xba*I linkered homeodomain-containing fragments from either *XNkx2-3* or *XNkx2-5* were PCR-amplified from full length *XNkx2-3*a (Evans et al., 1995), or *XCsx2* (courtesy of Seigo Izumo) respectively, and inserted into *Xho*I, *Xba*I-digested pCS2$^+$nls vector, with or without the En domain to generate En-HDNkx constructs or HDNkx control constructs. All fragments generated by PCR were sequenced to ensure that no errors had occurred during amplification.

Adeno-associated virus inverted terminal repeats (AAV-ITRs) were added to each En-containing expression cassette by digesting CS2nlsEn, CS2nlsEnHDX2.3, or CS2nlsEnHDX2.5 with *Ssp*I and *Sst*II to remove the pBluescript vector fragment, and blunt-end ligating the expression cassette to an *Xba*I pBluescript vector fragment from plasmid p2L (Fu et al., 1998), which contains one copy of the left AAV-ITR and one copy of the right AAV-ITR. The resulting configuration is diagrammed in Fig. 1.

Transient transfection analyses

Primary neonatal rat ventricular myocytes were isolated by a Percoll gradient technique and cultured as previously described at a density of 10^6 cells per 60 mm gelatin-coated dish (Zhu et al., 1991). After overnight incubation, the culture medium was changed to 4% horse serum for 3-5 hours prior to transfection. Transfections were performed by a modification of the calcium phosophate precipitation method (Chen and Okayama, 1987). 6 μg of DNA was added per dish, and the total amount of vector DNA concentration was kept constant. Approximately 48 hours following transfection, cells were harvested by lysis in 0.5% Triton buffer, and assayed for luciferase and β-galactosidase activities (Zou et al., 1997). Each sample was performed in triplicate for a single experiment, and each experiment was repeated a minimum of three times.

The Nkx-luciferase reporter contains three copies of a consensus Nk-homeodomain (Nkx) binding site upstream of a minimal 81 bp TK promoter in the luciferase vector pT81 (Nordeen, 1988), and has been described elsewhere (Zou et al., 1997). The vector pON249, a CMV-driven β-galactosidase reporter (Cherrington and Mocarski, 1989) was used to normalize transfection efficiency.

Embryo microinjection

Xenopus laevis were purchased from Xenopus I and Nasco companies. Ovulation was induced by injecting females with 300 U of human chorionic gonadotropin each. Fertilized eggs were dejellied with 2% cysteine-HCl (pH 7.8) immediately following cortical rotation. Plasmid DNAs were linearized prior to injection, and injected with 5 μg/μl neutral tetramethylrhodamine dextran (RDA; M_r 70,000) as a lineage tracer (Cleaver et al., 1996) in 1× MMR solution (Peng, 1991) adding 3% Ficoll 400 and sodium penicillan and streptomycin sulfate, each at 10 μg/ml. One cell of two cell stage embryos were injected. After several cell cycles (about 3-4 hours), the embryos were transferred to 0.1X MMR solution for further development at 15°C. Developmental stages of injected embryos were determined by comparison with non-injected control embryos (Nieuwkoop and Faber, 1956). Only morphologically normal embryos were scored, and each experiment was performed a minimum of three times.

DNAs encoding EnHDNkx and control constructs were linearized with *Sst*II prior to injection. For lineage tracer experiments, 100 pg of mRNA encoding β-galactosidase were coinjected with plasmid DNAs. The β-galactosidase was generated by linearizing plasmid pCS2nβgal (courtesy of Dave Turner) with *Pst*I, and transcribing with SP6 RNA polymerase.

Whole-mount in situ hybridization and histology

Riboprobes were prepared by in vitro transcription of linearized DNA templates in the presence of digoxigenin-11-UTP (Boehringer Mannheim), or fluor-UTP (Boehringer Mannheim). The cDNA plasmid encoding *XMLC2* has been described previously (Evans et al., 1995), and was linearized with *Bam*HI and transcribed with T7 RNA polymerase to generate an antisense riboprobe. The cDNA plasmid encoding *XcTnI* was obtained by a PCR reaction containing degenerate oligonucleotide primers complementary to conserved regions of chick and mouse *cTnI* and cDNA from adult *Xenopus* heart.

The resulting cDNA cloned into vector pCRII encoded sequences in agreement with those reported for *XcTnI* (Drysdale et al., 1994). pCRcTnI was linearized with *Eco*RI and transcribed with T7 RNA polymerase to generate an antisense riboprobe for *XcTnI*. Riboprobes for *XNkx2-5* were obtained by linearizing plasmid *XCsx2* (courtesy of Seigo Izumo) with *Xba*I, and transcribing with T7 RNA polymerase. This results in a probe containing non-coding and coding sequences upstream and exclusive of the homeodomain, which specifically recognizes endogenous *XNkx2-5* mRNA, but not mRNA from the injected HDX2.5 constructs. Probes were purified by an RNAse-free G-50 Sephadex spin column (Boehringer Mannheim) prior to use, and were not hydrolyzed.

Embryos were fixed and processed as described by Harland (1991), staining with BCIP/NBT or, for dual staining experiments, BCIP alone followed by staining for BCIP/NBT. The staining reaction was allowed to proceed for between 4 hours to overnight. Stained embryos were fixed and dehydrated prior to photographing.

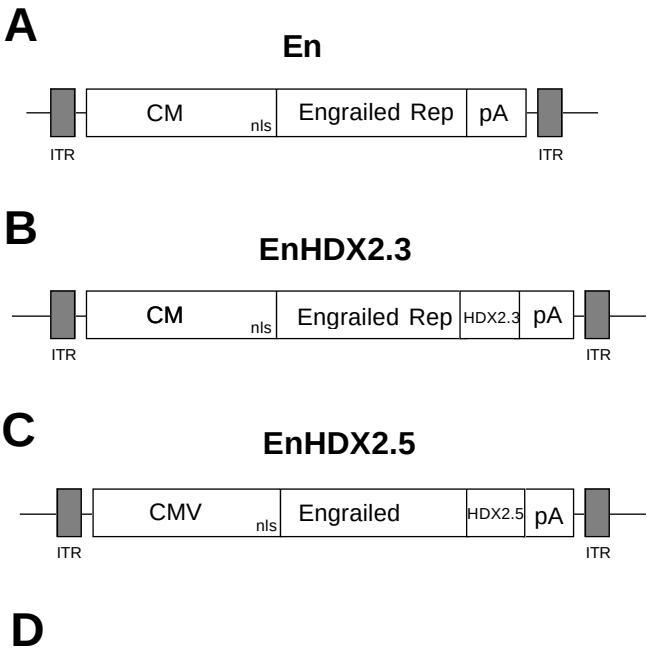
Some specimens were sectioned after staining, and were fixed overnight, dehydrated in ethanol, permeabilized briefly in histoclear, followed by 2× 20 minute changes in 1:1 xylene:paraffin wax at 60ºC and embedded in paraffin wax. Sections (10 μm) were cut, dried, dewaxed by passing through histoclear 2× 10 minutes, mounted in Permount and photographed (Kelly et al., 1991).

RESULTS

Dominant repressor constructs of XNkx2-5 and XNkx2-3

To investigate the requirement for *XNkx2-3* and *XNkx2-5* in early cardiogenesis, we constructed dominantly acting repressor proteins by fusing the active repressor domain from the *Drosophila* engrailed protein (En; Han and Manley, 1993) to the DNA-binding homeodomain regions from either *XNkx2-5* (HD2.5) or *XNkx2-3* (HD2.3). To ensure that the proteins would act as repressors, we utilized isolated Nkx homeodomain regions to avoid inclusion of potential transcriptional activation domains (T. J. M. and S. E. M., unpublished data). The cDNAs encoding the fusions were cloned into the expression vector CS2nls, containing a nuclear localization signal from SV40 large T antigen just upstream of a polylinker site (Turner and Weintraub, 1994, and Fig. 1). Control constructs were made with either the engrailed domain alone, or the homedomain of each *tinman* homologue alone. Several amino acids upstream and downstream of the homeodomain were included to increase the potential specific recognition of DNA targets (Fig. 1D). Previous work by other investigators has demonstrated that highly homologous homeodomains, while capable of recognizing the same DNA target, can also recognize distinct DNA targets (Cleary et al., 1993; Simon et al., 1997; see Discussion).

To confirm that the constructs could act to dominantly repress transcription from Nk-homeodomain (Nkx) target sites, we performed cotransfections in neonatal rat ventricular myocytes. The reporter plasmid for these assays, NkxLuc, contained three copies of a consensus Nk2 binding site (Chen and Schwartz, 1996; Zou et al., 1997; T. J. M. and S. E. M., unpublished data) upstream of a minimal TK promoter-luciferase reporter, pT81 (Nordeen, 1988). Wild-type *XNkx2-3* and *XNkx2-5* were cotransfected with increasing amounts of their respective EnHD counterparts, keeping the total amount of vector DNA constant, to investigate whether the En fusion constructs could in fact act in a dominant repressor fashion.



D

xNkx-2.3: EDPDRPKQRSRRKPRVLFSQAQVFELERRFKQQ
xNkx-2.5: -------E----------K-------------------------Y----------

RYLSAPEREHLANSLKLTSTQVKIWFQNRRYKCK
K----------D------V-----------------------------------

RQRQDKSLE 100%
----------QT---- 89%

Fig. 1. Structure of dominant repressor *tinman* constructs. (A-C) Expression modules. Diagrammes of the expression cassettes utilized for the dominant repressor construct. Note the flanking ITRs (inverted terminal repeats of adeno-associated virus) (Fu et al., 1998). Details of plasmid construction are given in Materials and methods. CMV, cytomegalovirus promoter from pCS2$^+$ (Turner and Weintraub, 1994); nls, nuclear localization signal from simian virus 40 (SV40); Engrailed Rep, amino acids 2-298 of the engrailed protein, containing an active repressor domain (Han and Manley, 1993); HDX2.3/HDX2.5, homeodomain-containing fragments from *XNkx2-3* and *XNkx2-5*, respectively (see Fig. 1D); pA, polyadenylation signal from SV40. (D) Putative amino acid sequences of homeodomain-containing fragments for *XNkx2-3* and *XNkx2-5* repressor derivatives. As can be seen from this representation, the homeodomain-containing regions of *XNkx2-3* and *XNkx2-5* which were used in repressor plasmid construction differ at eight amino acids. The actual homedomains are indicated by the double underline.

The pT81 parent exhibits no background activity when cotransfected with either wild-type *tinman* homologue (data not shown). The data shown in Fig. 2 are normalized for transfection efficiency by β-galactosidase activity as evidenced by a constant amount of a cotransfected CMV-β-galactosidase reporter (Cherrington and Mocarski, 1989).

As shown in Fig. 2, our results indicated that increasing amounts of EnHDX2-3 or EnHDX2-5 decreased activation of NkxLuc by the corresponding wild-type *tinman* homologue in a dose-dependent manner. No significant decrease was observed with the HDX2-3 or HDX2-5 control constructs,

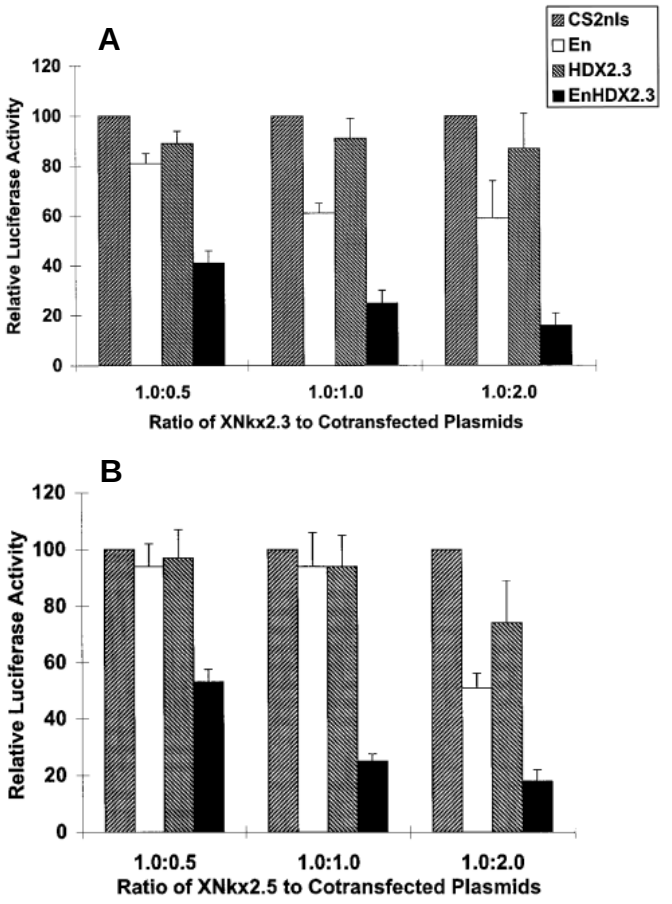


Fig. 2. Results of transient transfection assays in neonatal rat ventricular myocytes. These data demonstrate EnHDNkx-mediated repression of activation by *XNkx2-3* (A) or *XNkx2-5* (B) of an Nkx-luciferase reporter in a dose responsive manner. Details of the transfection protocol are given in Materials and Methods. Data shown are normalized for β-galactosidase activity of a cotransfected plasmid to control for transfection efficiency. Each experiment was performed a minimum of three times, and error bars indicate the standard error of the mean. CS2nls, parental CS2nls expression vector (Turner and Weintraub, 1994); En, CS2nls vector expressing the 296 amino acid engrailed repressor domain (Han and Manley, 1993) alone; HDX2.3/HDX2.5, CS2nls vector expressing the enlarged homeodomain fragment from *XNkx2-3* or *XNkx2-5* respectively (refer to Fig. 1D); EnHDX2.3/EnHDX2.5, CS2nls vector expressing the dominant repressor for either *XNkx2-3* or *XNkx2.5* respectively.

whereas some decrease was observed with the En control construct, although much less than observed with the EnHD constructs. Similar results were observed for transfections performed in C3H10T1/2 cells, an embryonic fibroblast cell line (data not shown). These results led us to ectopically express the EnHD constructs in developing *Xenopus* embryos, utilizing the En construct as a control for nonspecific effects.

Ectopic expression of EnHDNkx constructs in Xenopus embryos prevents the expression of markers specific for differentiated myocardium and abrogates heart formation

Our first attempts to ectopically express these EnHD constructs in embryos were by injecting mRNA encoding them into one cell of the two-cell stage embryo. These experiments resulted in high mortality of EnHD-injected embryos, which was little mitigated by attempts to target the injected mRNA to cardiogenic regions at the four-to-eight cell stage (Cleaver et al., 1996). The reasons for this mortality are not clear.

Table 1. Differentiated cardiac phenotype of embryos injected with dominant repressor constructs of *tinman* homologues

DNA	Amount of DNA injected (pg)	Embryos scored	Mutant (%)
En	200	403	6 (1.5)
EnHDX2.3	200	259	10 (3.9)
EnHDX2.5	200	271	9 (3.3)
EnHDX2.3 + EnHDX2.5	100 +100	303	58 (19.1)

Embryos were scored for expression of either XcTnI or XMLC2 mRNAs at stages 27-40, by whole mount in situ analyses. Only morphologically normal embryos were scored. Criteria for scoring an embryo as mutant were: asymmetric cardiac progenitors or heart, and/or a significant reduction in the region staining positively for differentiated cardiac markers (refer to Text and Figure 3).

Chi square analyses were performed for single and double EnHD injections. The hypothesis that control and experimental samples were equivalent was rejected at a significance level of $P<0.05$. For further experimental detail, refer to Materials and methods.

Consequently, we decided to inject the DNA constructs, as injected DNA begins to be expressed only at stage 8 during the mid-blastula transition (Vize et al., 1991). As an experimental approach, injection of DNAs into *Xenopus* embryos has been plagued by mosaicism of transgene expression (for discussion, see Kroll and Amaya, 1996). For our experiments, this mosaicism presented a particularly difficult problem as we needed to achieve expression of our dominantly acting mutants in cells which normally express *XNkx2-3* or *XNkx2-5*, which are a relatively small population in the developing embryo. The difficulty in targeting ectopic gene expression to cardiogenic tissues has been previously noted by other investigators (Cleaver et al., 1996; Gove et al., 1997).

To overcome these difficulties, we employed a new strategy for increased efficiency and reduced mosaicism of transgene expression following direct DNA injection into *Xenopus* embryos (Fu et al., 1998). This utilizes left and right inverted terminal repeat sequences (ITRs) from adeno-associated virus (AAV) on plasmid DNAs. Utilizing a cytomegalovirus promoter-enhancer (CMV) to drive β-galactosidase expression in developing embryos, we have previously been able to detect transgene activity from ITR plasmids in more than 50% of cells on the injected side of the embryo in 65% of injected embryos (Fu et al., 1998).

We utilized a similar strategy to create CMV-driven EnHDNkx and control CMV-En plasmids with one copy of the left, and one copy of the right AAV ITR flanking the expression cassette, as indicated in Fig. 1. These DNAs were then injected into one cell of two-cell stage embryos. In this manner, the uninjected side serves as an internal control for the experiment.

In preliminary studies, we injected varying doses of either EnHD construct alone. We found that injection of more than 200 pg of DNA resulted in a high mortality of the embryos, and therefore performed most of our experiments utilizing this dose as an upper limit. We performed injections utilizing 200

pg of each EnHD construct alone, 100 pg of each EnHD construct coinjected with 100 pg of its homologous counterpart, or with 200 pg of the En construct alone.

Injected embryos were allowed to develop to stage 27, when markers of the differentiated cardiac mesoderm are readily scored by in situ hybridization (Logan and Mohun, 1993; Drysdale et al., 1994; Evans et al., 1995). Various stages from 27 through to 40 were scored for the expression of one of two markers specific for differentiated cardiac mesoderm, the myosin light chain 2 (*MLC2*; Chambers et al., 1994; Evans et al., 1995), or cardiac troponin I (*cTnI*; Drysdale et al., 1994). Results obtained for these stages, and for these two markers were similar, and were consequently pooled, as presented in Table 1. Comparable numbers of embryos were injected for each sample. Only those embryos exhibiting overall normal morphology were scored, to eliminate phenotypes caused by, for example, abnormal gastrulation and to focus selectively on cardiac phenotypes.

Embryos exhibiting aberrant expression of differentiated cardiac mesoderm markers as compared to control uninjected embryos were scored as mutant. Mutant phenotypes included small hearts as evidenced by a decrease in the area staining for expression of differentiated cardiac mesoderm markers (Fig. 3A, control hearts versus mutant heart of lower embryo in B), unfused and sometimes asymmetrically staining cardiac primordia (Fig. 3B and D), or asymmetrically located smaller 'hearts' (Fig. 3E and F). The most extreme examples of the latter phenotype exhibited a complete absence of *MLC2* or *cTnI* expression on one side of the embryo (Fig. 3F). Less extreme examples exhibited greatly decreased staining on one side of the embryo (Fig. 3E). Each mutant phenotype was observed both with coinjection of the dominant repressor constructs, and with injection of either dominant repressor alone, albeit at a reduced frequency for the latter.

Serial sections through wild-type and mutant embryos confirmed the whole-mount observations (Fig. 4), and indicated an absence of morphologically recognizable differentiated heart tissue on the side of the embryo which did not stain with the differentiated myocardium markers (Fig. 4C).

Statistical analyses of the data in Table 1 indicated that injection of either EnHD construct resulted in a significant ($P<0.05$) increase in mutant phenotype relative to embryos injected with the En control construct. However, coinjection of both EnHD constructs, at individual doses equalling one half of that utilized for each alone, resulted in a significantly higher frequency of mutant phenotypes than that observed after injection of either EnHD construct alone (19% versus 3 to 4%). This synergistic effect at lower doses suggests that each EnHD construct is capable of acting in a specific fashion, and that *tinman* homologues may have functional redundancy at least with regards to cardiac differentiation.

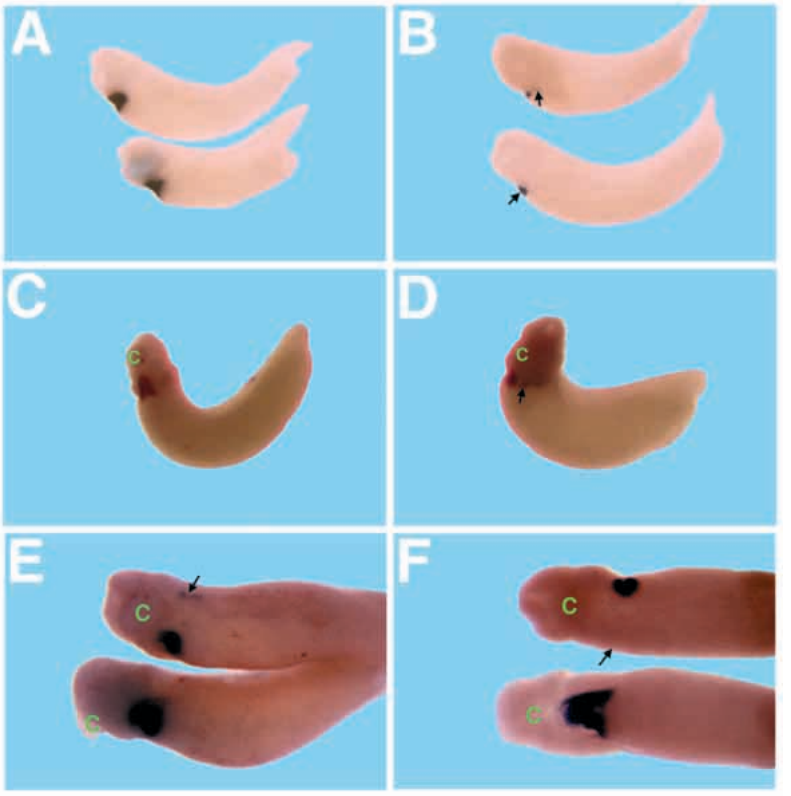


Fig. 3. Whole-mount in situ analyses of control and EnHD-injected embryos. Embryos were injected with a total of 200 pg of DNA into one cell at the two-cell stage, fixed, and processed for whole-mount in situ analyses utilizing probes for either *XMLC2* or *XcTnI* mRNA. All embryos shown are oriented with anterior to the left, and posterior to the right. The mutant phenotypes shown were observed in embryos injected with either EnHDX2.3 or EnHDX2.5, or with coinjection of both EnHD constructs. However, coinjection resulted in a significantly higher frequency of observed mutant phenotypes (refer to Table 1). The same mutant phenotypes were observed utilizing probes for either *XMLC2* or *XcTnI* mRNA. (A) Lateral view of control En-injected stage 34/37 embryos stained for expression of *XMLC2* mRNA. At these stages the normal *Xenopus* heart expresses high levels of *XMLC2* mRNA (Evans et al., 1995). (B) Lateral view of EnHD-injected stage 34/37 embryos stained for expression of *XMLC2* mRNA. In contrast to the control embryos (A), the upper embryo exhibits unfused heart primordia, with much lower expression of *XMLC2* mRNA on one side (arrow). The lower embryo has a much reduced area expressing *XMLC2* mRNA (arrow). The embryos shown were injected with DNA encoding the EnHDX2.5 repressor. (C) Ventrolateral view of control En-injected stage 32 embryo stained for expression of *XcTnI* mRNA, showing that fusion proceeds in an anterior to posterior direction (Balinsky, 1970). (D) Ventrolateral view of EnHDX2.3-injected stage 32 embryo stained for expression of *XcTnI* mRNA. This embryo exhibits unfused cardiac primordia, with one of the primordia showing greatly reduced staining (arrow). (E) Control and mutant stage 35/36 embryos stained for expression of *XcTnI* mRNA. The upper EnHD-injected embryo (ventral view) has severely reduced *XcTnI* staining on one side of the embryo (arrow), and a small asymmetrically located heart tube on the other side. This embryo was coinjected with both EnHDX2.3 and EnHDX2.5. The lower, control En-injected embryo, shown in a ventro-lateral view, exhibits a symmetrically located (centered beneath the cement gland) normal-sized heart. (F) Ventral views of control and mutant stage 32 embryos stained for expression of *XcTnI* mRNA. The upper EnHD-injected embryo exhibits a small heart tube on one side, and no apparent expression of *XcTnI* mRNA on the opposite side (arrow). This embryo resulted from a coinjection of both dominant repressor Nkx constructs. The lower En control-injected embryo demonstrates the normal-sized heart primordia. C, cement gland.

A low frequency of mutant phenotypes (1%) was observed in En-control injected embryos. The predominant phenotype observed was the delayed fusion of heart primordia, infrequently accompanied by decreased cardiac marker staining on one side, the 'unfused' phenotype. This phenotype may result from nonspecific gastrulation defects, resulting in abnormal migrations of tissues which prevent fusion of the cardiac primordia. Although the heart tube in amphibia normally forms following the convergence of the two cardiac primordia at the ventral midline (Balinsky, 1970), sections through embryos where fusion of the heart primordia had not occurred by stage 32 revealed the formation of two separate heart tubes on either side of the embryo (Fig. 4B), consistent with earlier embryological experiments in amphibia where cardiac primordia were prevented from fusing (Fales, 1946, and references therein).

Reduction or absence of differentiated myocardium occurs on the EnHD-injected side of the embryo

To confirm that the asymmetric heart phenotype that we were observing was a result of reduction or absence of differentiated myocardium on the EnHD-injected side of the embryo, we performed lineage tracer studies, coinjecting mRNA encoding β-galactosidase with our dominant repressor constructs. This approach is used frequently in ectopic expression studies in *Xenopus* (Vize et al., 1991; Gove et al., 1997). Results of these studies are included in the data in Table 1, and shown in Fig. 5. Of 57 embryos scored following coinjection with both dominant repressor EnHD constructs and lacZ mRNA, 12 (21%) exhibited a mutant phenotype. Of these 12 mutant embryos, 9 exhibited extensive one-sided staining for β-galactosidase activity. In each of these mutants, the mutant phenotype was associated with the injected side of the embryo. An example is shown in Fig. 5, where an embryo exhibiting an asymmetric small heart stained positively for *XcTnI* mRNA (purple) exhibited positive staining for β-galactosidase activity (blue) on the side opposite to that where the heart is. These results indicate that the EnHD-injected side of the embryo is the one exhibiting reduced or absent staining for markers of differentiated myocardium.

Do dominant repressor constructs selectively prevent differentiation of myocardium and heart formation, or also affect gene expression in cardiogenic mesoderm?

The foregoing results indicated that dominant repressor derivatives of *tinman* homologues can prevent expression of markers of differentiated myocardium, but left open the question as to whether gene expression in cardiogenic mesoderm was likewise affected. Results of other investigators have demonstrated that ectopic overexpression of *GATA-6* can prevent cardiac differentiation, but does not affect formation of cardiogenic mesoderm, as evidenced by expression of *XNkx2-5* (Gove et al., 1997). To examine this issue, we repeated the injection studies, this time scoring for a marker of cardiogenic mesoderm, *XNkx2-5*. The probe utilized for these studies was specific for endogenous *XNkx2-5*, to avoid cross-reactivity with the ectopically expressed EnHDX2.5 mRNA (see Materials and methods). Injected embryos were harvested, at stages 17 to 23, and stained for expression of *XNkx2-5* mRNA by whole-mount in situ hybridization. As noted previously, markers of the differentiated cardiac mesoderm are not expressed until stage 27. In addition, injected embryos were harvested following cardiac differentiation post stage 27, and costained for the expression of *XNkx2-5* and *XcTnI* by double-label whole-mount in situ analysis to investigate whether expression of both markers was equally affected in EnHD injected embryos.

Results of these injection studies are shown in Table 2, and examples of observed staining patterns in mutants are shown in Fig. 6. As seen previously, the incidence of mutant phenotypes as evidenced by asymmetric staining for mRNA expression of either *XNkx2-5* or *XcTnI* was greatly increased in embryos coinjected with both dominantly acting Nkx constructs. In stage 17-23 embryos, *XNkx2-5* expression, in a manner analogous to that of *XcTnI* expression, was asymmetric, being partially or entirely absent from one side of the embryo. Comparable asymmetric staining was not observed in En control-injected embryos. Mutants of later stage embryos, costained for *XcTnI* and *XNkx2-5* mRNA expression, exhibited reduced staining for both *XcTnI* and *XNkx2-5* (Table 2). Sections through these embryos confirmed the whole-mount in situ observations (Fig. 7), and indicated that the dominantly acting EnHD constructs repress expression of *Nkx2-5* mRNA in mesodermal primordia at stages both prior to and following the usual onset of cardiac differentiation.

Rescue of dominantly acting repressor mutant phenotypes by coinjection of wild-type tinman homologues

One of the problems associated with ectopically expressing dominant negative proteins in developing embryos is the

Table 2. Asymmetric XNkx2.5 and XcTnI expression in *Xenopus* embryos injected with dominant repressor constructs of *tinman* homologues

		XNkx2.5 expression (Stages 17-27)		XNkx2.5+XcTnI expression (Stages 27-36)	
DNA	Amount of DNA injected (pg)	Embryos scored	Mutant (%)	Embryos scored	Mutant (%)
En	200	30	0	83	1 (1.2)
EnHDX2.3	200	24	1 (4.2)	37	3 (8.1)
EnHDX2.5	200	22	1 (4.5)	46	3 (6.5)
EnHDX2.3 + EnHDX2.5	100+100	37	6 (16.2)	55	9 (16.4)

Only morphologically normal embryos were scored.

Chi square analyses were performed for single and double EnHD injections. The hypothesis that control and experimental samples were equivalent was rejected at a significance level of $P<0.05$. For further experimental detail, refer to Materials and methods.

Table 3. Rescue of mutant phenotypes by coinjection with wild-type *tinman* homologues

DNA	Amount of DNA injected (pg)	Embryos scored	Mutant (%)	Large heart (%)
En	100	27	0	
EnHDX2.3 + EnHDX2.5	50+50	12	2 (16)	
EnHDX2.3 + EnHDX2.5 + Wt X2.3	50+50+50	35	2 (6)	1 (3)
EnHDX2.3 + EnHDX2.5 + Wt X2.5	50+50+50	46	1 (2)	1 (2)
Wt X2.3	100	58		2 (4)
Wt X2.5	100	35		4 (11)

Embryos were injected into one cell of two-cell stage embryos, harvested following cardiac differentiation at stages 27-40, embryos were scored for expression of *XcTnI* at stages by whole mount in situ analyses. Only morphologically normal embryos were scored.

Chi square analyses were performed for each experimental sample relative to the pertinent control sample. The hypothesis that control and experimental samples were equivalent was rejected at a significance level of $P<0.05$. For further experimental detail, refer to Materials and methods.

possibility that these proteins are not specifically affecting the intended target, in this case, the action of wild-type *XNkx2-3* or *XNkx2-5*. Our experiments suggest that *XNkx2-3* and *XNkx2-5* are functionally redundant with regards to affecting gene expression in both cardiogenic mesoderm and differentiated heart, and early heart formation. Therefore, if the EnHDNkx proteins are specifically targeting wild-type *XNkx2-3* or *XNkx2-5*, mutant phenotypes should be rescued by co-expressing either wild-type *XNkx2-3* or *XNkx2-5*.

Results of rescue experiments are shown in Table 3 and Fig. 8. Coinjection of either wild-type homologue significantly reduced the frequency of mutant phenotypes observed, suggesting that the EnHD proteins were specifically targeting *tinman* homologue action. In addition, several embryos injected with wild-type *tinman* homologues exhibited enlarged hearts (Fig. 8), at a greater frequency when injected with wild-type homologues alone than when coinjected with the dominantly acting repressor constructs. This result is consistent with the phenotype previously observed by injection of mRNA encoding either *XNkx2-3* or *XNkx2-5*, and indicates that the ability of these *tinman* homologues to affect cardiac cell fate does not require expression prior to the mid-blastula transition, when the injected plasmid DNA begins to be expressed (Vize et al., 1991).

DISCUSSION

Synergistic activity of the EnHDNkx proteins on cardiogenesis

The principal finding of our studies is that despite their sequence similarity, the EnHDNkx proteins act synergistically to inhibit normal heart formation in *Xenopus* embryos. Coinjection of plasmids encoding *XNkx2-3* and *XNkx2-5* homeodomains fused to the *engrailed* repressor domain proved between five and eight times more effective in producing a mutant phenotype compared with injection of either plasmid alone at a higher individual concentration. This result suggests two important conclusions about the role of the *tinman* homologues in cardiogenesis. Firstly, *XNkx2-3* and *XNkx2-5* are functionally redundant during amphibian heart formation, at least as judged by the appearance of heart-specific markers used in our experiments. Hence the expression of each dominant negative alone in the embryo produces only a low proportion of mutant phenotypes (see below). This conclusion is supported by previous studies in which ectopic expression of either *XNkx2-3* or *XNkx2-5* alone in *Xenopus* embryos produces a similar enlarged heart phenotype (Cleaver et al., 1996). It is also supported by our observation that mutant phenotypes resulting from coinjection of both EnHD constructs can be rescued by injection of either wild-type homologue alone (Table 3).

Intuitively, one might expect that functional redundancy of highly related transcription factors would be due to their common recognition of DNA target sites. Indeed, such a mechanism could well be operating with *tinman* homologues. However, if this were the sole case for the *tinman* homologues, we would expect similar proportions of mutant phenotypes to be produced by expression of the dominant negative constructs either individually or in combination. Our finding of profound synergy between the two dominant negative constructs suggests an alternative model for functional redundancy between *XNkx2-3* and *XNkx2-5*. We presume that each EnHDNkx protein must be targeting distinct DNA binding sites, on the same or different gene(s). Each set of targets must also lie within alternative and redundant pathways for cardiogenesis. Repression of either individual pathway by the appropriate EnHDNkx protein would then have little effect due to compensation by the unaffected alternative cardiogenic pathway. However, coexpression of the two EnHDNkx proteins would block both cardiogenic pathways and result in a dramatic increase in the incidence of mutant phenotypes. This model is consistent with our experimental findings.

Target specificity amongst the vertebrate tinman homologues

Our dominant repressor constructs were made by fusing the active repressor domain of the *Drosophila* engrailed homeoprotein (Han and Manley, 1993) to a DNA fragment containing the homeodomain of either *XNkx2-3* or *XNkx2-5*. Such chimaeric transcription factors have been used previously by others to create dominantly acting repressors of homeodomain proteins and other transcription factors in *Xenopus* (Conlon et al., 1996; Ryan et al., 1996; Bellefroid et al., 1996; Fan and Sokol, 1997), and we have utilized a similar strategy to create a dominant repressor of rat *Nkx2-5* (Zou et al., 1997).

The homeodomains of *XNkx2-3* and *XNkx2-5* are highly related, and in contrast to the model suggested by our present studies, the purified proteins recognize a consensus site common to several NK2-type protein families in vitro (T. J. M. and S. E. M., unpublished data). However, the two homeodomains vary at several amino acid residues, and there are precedents which indicate that transcriptional specificity

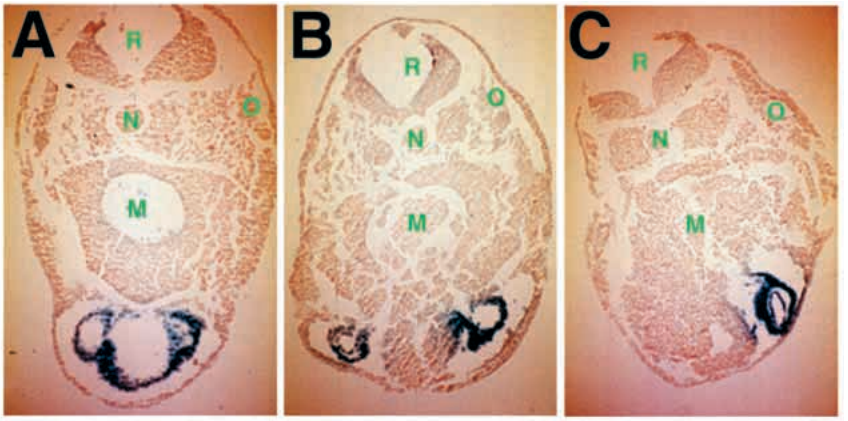


Fig. 4. Transverse paraffin sections of stage 36 control and mutant embryos following whole-mount in situ analyses for expression of either *XMLC2* or *XcTnI* mRNA. Sections were stained for expression of *XcTnI*, but similar results were observed in sections stained for *XMLC2*. Dorsal is at the top. (A) En-control injected embryo. At this stage, the heart is looped and has partitioned atria and ventricular chambers (Nieuwkoop and Faber, 1956). Note the relatively large size of myocardial tissue in comparison to *XcTnI* mRNA-positive tissue in the mutant sections shown in B and C. (B) Embryo injected with both EnHDX2.3 and EnHDX2.5 showing the unfused phenotype. Two distinct heart tubes can be seen forming, despite the fact that the primordia have not contacted each other. (C) Embryo injected with both EnHDX2.3 and EnHDX2.5. Whole mount in situ analysis of this embryo revealed a small asymmetrically located heart on one side, with no visible staining for *XcTnI* on the other. This external phenotype was confirmed by sectioning. Note that there is no morphological evidence of a heart tube on the side which lacks expression of *XcTnI*. Note general lack of development on this side of the embryo. M, midgut; N, notochord; O, otic vesicle and R, rhombencephalon.

can be conferred by individual amino acids within, or in sequences immediately adjacent to, homeodomains. For example, despite the fact that the highly related homeodomain proteins *Oct-1* and *Oct-2* can recognize the same DNA sequences, including an octamer motif, differences in four amino acids within the homeodomain have been shown to affect their ability to interact with a specific cofactor, the herpesvirus *VP16* trans-activator. The *Oct-1* homedomain, but not the *Oct-2* homeodomain, can effect this interaction, and in concert with VP16 can activate transcription from a DNA binding site not recognized by *Oct-2* in the same environment (Cleary et al., 1993). A single amino acid substitution from the *Phox1* homeodomain to the distantly related *deformed* homeodomain can confer the ability to activate a serum response element, a property otherwise specific to the *Phox1* homeodomain (Simon et al., 1997). In the light of these examples, it may well be possible for the limited differences in sequence between the *XNkx2-3* and *XNkx2-5* homeodomains (and immediately flanking residues) to confer distinct DNA binding properties in vivo (Fig. 1D).

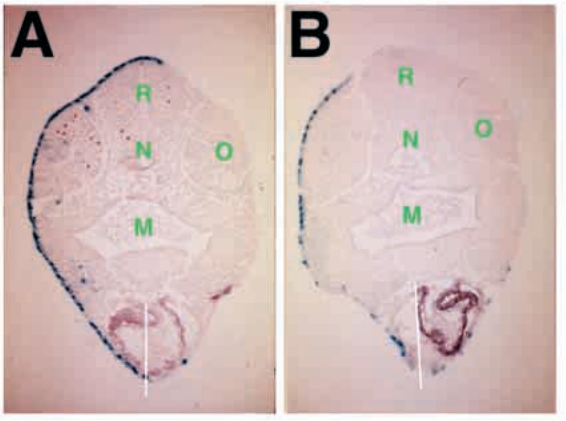


Fig. 5. Paraffin sections of embryos stained for expression of β-galactosidase, and processed by whole-mount in situ for expression of *XcTnI* mRNA. Sections shown are from stage 40 embryos, dorsal is at the top. 100 pg of mRNA encoding β-galactosidase was injected as a lineage tracer to indicate the injected side of the embryo. A white line is drawn through the midline of the sections, to emphasize the positioning of the heart. (A) Control embryo, coinjected with En control plasmid and mRNA encoding β-galactosidase. (B). Mutant embryo, coinjected with both dominant repressor Nkx constructs, and lineage tracer mRNA encoding β-galactosidase. Note the asymmetric placement of the *XcTnI*-positive myocardial the smaller size of the heart relative to that of the control (A). The small heart is formed on the side opposite to that of injection. M, midgut; N, notochord; O, otic vesicle and R, rhombencephalon.

We imagine that such specificity is mediated by cofactor interactions with each tinman homologue (cf. Cleary et al., 1993; Simon et al., 1997). Mouse *Nkx2-5* has previously been shown to interact with serum response factor (SRF) and *GATA-4* to activate transcription (Chen and Schwartz, 1996; Durocher et al., 1997). If either *XNkx2-3* or *XNkx2-5* were capable of selective interaction with such a cofactor, the result might be the selective ability to affect transcription from a DNA target, perhaps by affecting DNA binding. Future experiments will be aimed at exploring this issue.

Mutant phenotypes from ectopic expression of single dominant negative constructs

Our data indicate that each individual EnHDNkx construct is apparently capable of producing a low, but statistically significant incidence of mutant phenotypes. Furthermore, in cultured explant assays, we have found that putative dominant negative constructs comprising the engrailed repressor domain fused to the entire *XNkx2-3* or *XNkx2-5* coding region can each block the induction of cardiac muscle differentiation (T. J. M. and S. E. M., unpublished data). How can such observations be reconciled with a model of selective DNA binding which mediates functional redundancy between the *tinman* homologues?

One possibility is that the phenotypes resulting from expression of the dominantly acting constructs are critically dependent on the effective concentration of the ectopically expressed protein within a given target cell. In the case of DNA injections, it is entirely plausible that ectopic expression is variable both in the level of mosaicism within Nkx-expressing tissues and in the effective concentration of the

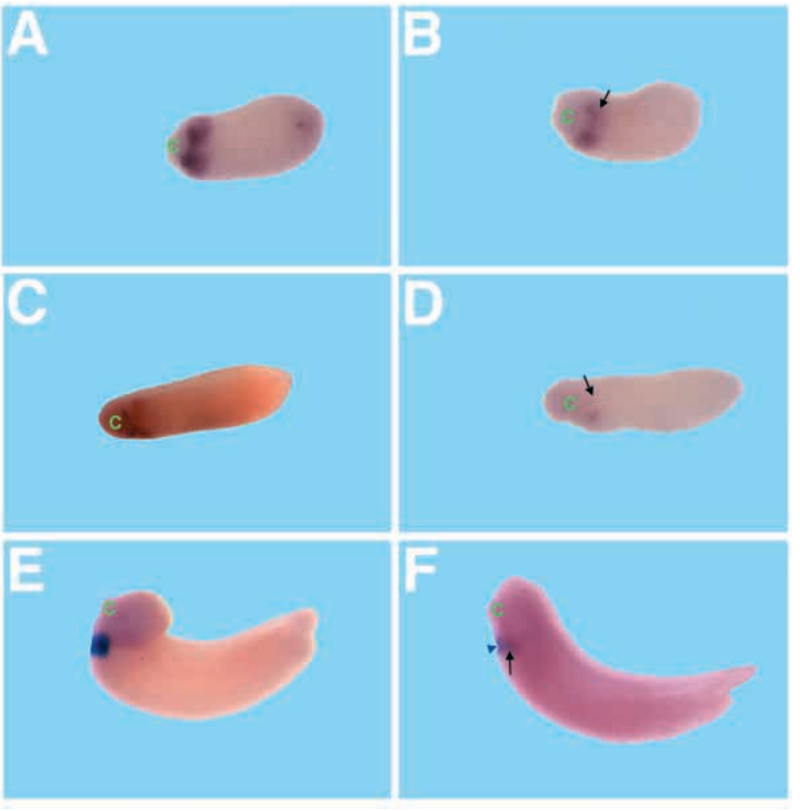


Fig. 6. Whole-mount in situ analyses of embryos stained for expression of *XNkx2-5*, or *XNkx2-5* and *XcTnI*. Embryos were injected with a total of 200 pg of DNA into one cell at the two-cell stage, fixed, and processed for whole-mount in situ analyses staining for *XNkx2-5* mRNA alone, or for both *XNkx2-5* and *XcTnI* mRNAs utilizing double-label in situ (see Materials and Methods for details). *XNkx2-5* mRNA signal was detected utilizing BCIP/NBT (brownish-purple color), and *XcTnI* mRNA signal was detected utilizing BCIP alone (light blue color). Overlap of the two signals gives a dark blue signal (E and F). Anterior is to the right. The mutant embryos shown were coinjected with both EnHDX2.3 and EnHDX2.5. (A) Control-injected stage 21 embryo, ventral view, showing symmetrical *XNkx2-5* mRNA staining just posterior to the cement gland. (B) EnHD-injected stage 21 embryo, ventral view, with arrow indicating reduction in *XNkx2-5* mRNA staining relative to the other side of the embryo. (C) Control injected stage 24 embryo, ventral view, showing *XNkx2-5* mRNA in fused cardiac primordia posterior to the cement gland. (D). EnHD-injected stage 24 embryo, ventral view, with arrow indicating absence of *XNkx2-5* mRNA staining on one side of the embryo. (E) Control injected stage 32 embryo, ventrolateral view, co-stained for *XNkx2-5* and *XcTnI* mRNAs (dark blue). (F) EnHD-injected stage 33 embryo, ventrolateral view, co-stained for *XNkx2-5* and *XcTnI* mRNAs. In this embryo, the positively staining regions have not yet fused. Although one side stains positively for both *XNkx2-5* and *XcTnI* mRNAs (dark blue staining as indicated by blue arrowhead), the other side shows very faint staining for both mRNAs (black arrow). C, cement gland.

repressor protein in expressing cells. This would account for the variation in severity of phenotype between individual embryos and also the absence of any mutant phenotype in the majority of normally developing injected embryos (Tables 1-3). Perhaps the low level of aberrant phenotypes obtained with injection of individual EnHDNkx constructs occurs in those embryos in which particularly high levels of EnHD protein are expressed. Under such conditions, subtle differences in target specificity may be lost, with the result that the ectopic protein represses both redundant cardiogenic pathways. Similarly, the use of RNA rather than DNA in explant assays might also produce high levels of ectopically expressed *engrailed* fusion protein, resulting in repression of cardiac muscle differentiation (T. J. M. and S. E. M., unpublished data).

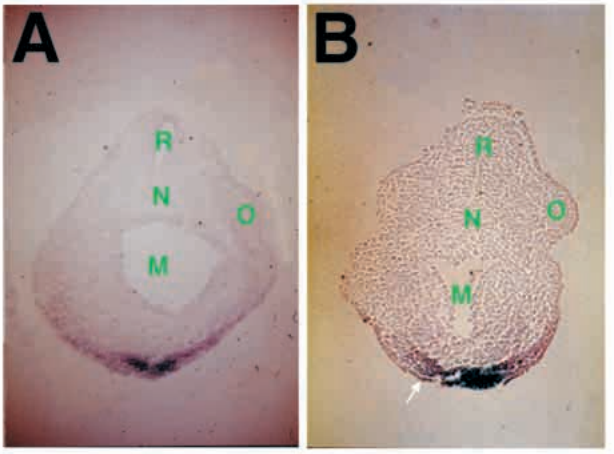


Fig. 7. Paraffin sections of control and mutant embryos following whole-mount in situ analyses for *XNkx2-5* and *XcTnI* mRNAs. Sections shown were from stage 28 embryos, co-stained for *XNkx2-5* mRNA (brownish-purple) and for *XcTnI* mRNA (blue); dorsal at the top. (A) Control embryo exhibits symmetrical staining of both *XcTnI* mRNA, in differentiating myocardial cells, and *XNkx2-5* mRNA in both differentiating myocardial cells and adjacent mesoderm. (B) Mutant embryo exhibits asymmetrical staining of both *XcTnI* and *XNkx2-5* mRNAs, with one side (arrow) exhibiting greatly reduced signal for both mRNAs. M, midgut; N, notochord; O, otic vesicle and R, rhombencephalon.

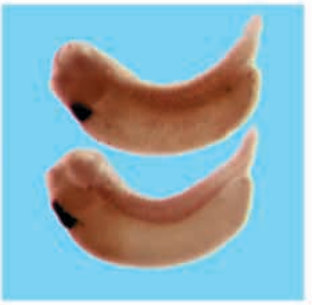


Fig. 8. Enlarged heart phenotype in embryos following injection with wild-type *XNkx2-5* DNA. The observation of this phenotype in DNA-injected embryos demonstrates that *tinman* homologues can affect cardiac cell fate when expressed following the mid-blastula transition. Lateral view of stage 35 embryos, anterior to the right, stained for expression of *XcTnI* mRNA. The lower embryo was injected with DNA encoding wild-type *XNkx2-5*. Note the greatly enlarged heart relative to the upper, control-injected embryo. Comparable enlarged hearts were also observed in embryos injected with DNA encoding wild-type *XNkx2-3* (data not shown).

Xenopus tinman homologues are required for maintenance of XNkx2-5 expression in cardiac mesoderm, for cardiac differentiation, and for heart formation

In *Drosophila*, mutation of *tinman* results in complete absence of dorsal vessel formation (Bodmer, 1993; Azpiazu and Frasch, 1993), raising the issue as to whether vertebrate *tinman* homologues are similarly required for earliest stages of heart formation. A mouse knockout of *Nkx2-5* aborts cardiac development before looping morphogenesis occurs, but does not prevent formation of the heart tube, nor expression of many differentiated cardiac mesoderm markers (Lyons et al., 1995).

The results reported here indicate that ectopic expression of dominant repressors of *tinman* homologues resulted in a significant proportion of embryos completely lacking expression of *XNkx2-5*, *XcTnI*, or *XMLC2*, and without morphological evidence of heart formation on the EnHD-injected side of the embryo. These results indicate that, in *Xenopus*, *tinman* homologues *XNkx2-3* and *XNkx2-5* are required for the maintenance of *XNkx2-5* expression in cardiogenic mesoderm, for proper biochemical differentiation of cardiac primordia, and for the earliest stages of heart formation. To our knowledge, this is the most extreme mutant cardiac phenotype reported in a vertebrate system to date (Fishman and Chien, 1997), and underscores the importance of the *tinman* family for heart development.

Functional redundancy of *XNkx2-3* and *XNkx2-5* has previously been suggested by their largely overlapping expression patterns during cardiogenesis and in the differentiated heart, and by ectopic expression studies which demonstrated that expression of either homologue can result in an enlarged heart (Evans et al., 1995; Fu and Izumo, 1995; Cleaver et al., 1996). The synergism observed by coinjecting individual dominant repressor constructs for *XNkx2-3* or *XNkx2-5* at lower concentrations than those used for injections of either construct alone, provides the first evidence that *tinman* homologues fulfill their requirement for heart formation in a functionally redundant manner. This conclusion is strengthened by the observation that mutant phenotypes resulting from coinjection of both EnHD constructs can be rescued by injection of either wild-type homologue alone.

The absence of *XNkx2-5* expression in cardiogenic mesoderm of mutant embryos suggests that *XNkx2-5* expression is autoregulated by *tinman* homologues, in a direct or indirect manner. *Tinman* expression in developing dorsal vessel may also be autoregulatory (Bodmer, 1993). The absence of *XNkx2-5* expression in cardiogenic mesoderm suggests that perhaps other markers of cardiogenic mesoderm will also be affected, or that formation of cardiogenic mesoderm itself has been prevented by expression of *tinman* homologue repressors. Future experiments will be aimed at exploring this issue by using a range of markers for cardiogenic mesoderm.

Our conclusions have been arrived at by utilizing markers for cardiogenic mesoderm (*XNkx2-5*) and differentiated heart (*XNkx2-5*, *XcTnI*, *XMLC2*), and by histological examination of sections following whole-mount in situ staining for these markers. As we are not using a comprehensive panel of markers, we cannot rigorously exclude that the EnHD proteins are selectively affecting those markers we have examined. In the *Nkx2-5* knockout mice, expression of a select few markers of the differentiated heart was downregulated, whereas most were unaffected (Lyons et al., 1995). However, in the case of the *Nkx2-5* knockout, the heart tube appeared morphologically normal. In contrast, in *Xenopus* EnHDNkx-injected mutants, where staining for either *XcTnI* or *XMLC2* was absent, there was no morphological evidence of heart formation (Figs 4 and 5).

Do dominantly acting repressors selectively affect cardiogenesis?

In these experiments, we have focussed on the effects of EnHDNkx repressor protein expression on heart formation. However, as *tinman* in *Drosophila* also affects visceral mesoderm formation, and as both *XNkx2-3* and *XNkx2-5* are expressed early on in pharyngeal endoderm as well as cardiogenic mesoderm, and in visceral mesodermal progenitors (Tonissen et al., 1994; Evans et al., 1995; Cleaver et al., 1996), it is possible that expression of the EnHDNkx repressors affects lineages other than heart. In addition, as our experiments utilized the ubiquitously expressed CMV promoter, we cannot at present say whether our mutant phenotypes are a result of EnHD expression repressing *XNkx2-3* or *XNkx2-5* action in cardiogenic or other Nkx-expressing tissues, or both.

We thank Dr Kenneth Chien for his support and encouragement throughout this work. Thanks to Mahmoud Itani and Robyn Todd for expert technical support. We are greatly indebted to Dr Seigo Izumo for kindly providing *XCsx2* cDNA, Dr Qi-Cheng Yang for the P2L vector, and Dave Turner for CS2 plasmids. We are grateful to Duncan Sparrow for his helpful criticism of the manuscript. This work was supported by an NIHHLB First Award and American Heart Association Grant-in-Aid to S. M. E., and a British Heart Foundation Grant to T. J. M.

REFERENCES

Azpiazu, N. and Frasch, M. (1993). *tinman* and *bagpipe*: Two homeobox genes that determine cell fates in the dorsal mesoderm of *Drosophila*. *Genes Dev.* **7**, 1325-1340.

Badiani, P., Corbella, P., Kioussis, D., Marvel, J. and Weston, K. (1994). Dominant interfering alleles define a role for c-Myb in T-cell development. *Genes Dev.* **8**, 770-782.

Balinsky, B. I. (1970). Development of the Heart. In *An Introduction to Embryology* (Third Edition). pp. 454-465. Philadelphia, London, Toronto: W. B. Saunders Company.

Bellefroid, E. J., Bourguignon, C., Hollemann, T., Ma, T., Anderson, D. J., Kintner, C. and Pieler, T. (1996). X-MyT1, a *Xenopus* C2HC-type zinc finger protein with a regulatory function in neuronal differentiation. *Cell* **87**, 1191-1202.

Bodmer, R. (1993). The gene *tinman* is required for the specification of the heart and visceral muscles in *Drosophila*. *Development* **118**, 719-729.

Chambers, A. E., Logan, M., Kotecha, S., Towers, N., Sparrow, D. and Mohun, T. J. (1994). The RSRF/MEF2 protein SL1 regulates cardiac muscle-specific transcription of a myosin light-chain gene in *Xenopus* embryos. *Gene Dev.* **8**, 1324-1334.

Cherrington, J. M. and Mocarski, E. S. (1989). Human cytomegalovirus iel transactivates the α promoter-enhancer via an 18 basepair repeat element. *J. Virol.* **63**, 1435-1440.

Chen, C. and Okayama, H. (1987). High efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7**, 2745-2752.

Chen, J. N. and Fishman, M. C. (1996). Zebrafish *tinman* homology demarcates the heart field and initiates myocardial differentiation. *Development* **122**, 3809-3816.

Chen, C. Y. and Schwartz, R. J. (1996). Recruitment of the tinman homolog

Nkx2-5 by serum response factor activates cardiac α-actin gene transcription. *Mol. Cell. Biol.* **16**, 6372-6384.

Cleary, M. A., Stern, S., Tanaka, M. and Herr, W. (1993). Differential positive control by Oct-1 and Oct-2: activation of a transcriptionally silent motif through Oct-1 and VP16 corecruitment. *Genes Dev.* **7**, 72-83.

Cleaver, O. B., Patterson, K. D. and Krieg, P. A. (1996). Overexpression of the *tinman*-related genes *XNkx-2.5* and *XNkx-2.3* in *Xenopus* embryos results in myocardial hyperplasia. *Development* **122**, 3549-3556.

Conlon, F. L., Sedgwick, S. G., Weston, K. M. and Smith, J. C. (1996). Inhibition of Xbra transcription activation causes defects in mesodermal patterning and reveals autoregulation of Xbra in dorsal mesoderm. *Development* **122**, 2427-2435.

Drysdale, T. A., Tonissen, K. F., Patterson, K. D., Crawford, M. J. and Krieg, P. A. (1994). Cardiac troponin I is a heart-specific marker in the *Xenopus* embryo: Expression during abnormal heart morphogenesis. *Dev. Biol.* **165**, 432-441.

Durocher, D., Charron, F., Warren, R., Schwartz, R. J. and Nemer, M. (1997). The cardiac transcription factors *Nkx2-5* and *GATA-4* are mutual cofactors. *EMBO J.* **16**. 5687-5696.

Evans, S. M., Yan, W., Murillo, M. P., Ponce, J. and Papalopulu, N. (1995). *Tinman*, a *Drosophila* homeobox gene required for heart and visceral mesoderm specification, may be represented by a family of genes in vertebrates: *XNkx-2.3*, a second vertebrate homologue of *tinman*. *Development* **121**, 3889-3899.

Fales, D. E. (1946). A study of double hearts produced experimentally in embryos of *Amblystoma Punctatum*. *J. Exp. Zool.* **101**, 281-298.

Fan, M. J. and Sokol, S. Y. (1997). A role for Siamois in Spemann organizer formation. *Development* **124**, 2581-2589.

Fishman, M. C. and Chien, K. R. (1997). Fashioning the vertebrate heart: earliest embryonic decisions. *Development* **124**, 2099-2117.

Fu, Y. and Izumo, S. (1995). Cardiac myogenesis: Overexpression of *XCsx2* or *XMEF2A* in whole *Xenopus* embryos induces the precocious expression of *XMHCα* gene. *Roux's Archives Dev. Biol.* **205**, 198-202.

Fu, Y., Wang, Y. and Evans, S. M. (1998). Viral sequences enable efficient and tissue specific expression of transgenes in *Xenopus*. *Nature Biotech.* **16**, 253-257.

Gove, C., Walmsley, M., Nijiar, S., Bertwistle, D., Guille, M., Partington, G., Bomford, A. and Patient, R. (1997). Over-expression of *GATA-6* in *Xenopus* embryos blocks differentiation of heart precursors. *EMBO J.* **2**, 355-368.

Han, K. and Manley, J. L. (1993). Functional domains of the *Drosophila* engrailed protein. *EMBO J.* **12**, 2723-2733.

Harland, R. M. (1991). In situ hybridization: An improved whole mount method for *Xenopus* embryos (ed. B. K. Kay and H. B. Peng), pp. 685-695. In *Xenopus laevis*: Practical Uses in Cell and Molecular Biology London, UK: Academic Press.

Harvey, R. P. (1996). *Nk-2* homeobox genes and heart development. *Dev. Biol.* **178**, 203-216.

Kelly, G. M., Eib, D. W. and Moon, R. T. (1991). Histological preparation of *Xenopus laevis* oocytes and embryos(ed. B. K. Kay and H. B. Peng). pp. 389-417. In *Xenopus laevis*: Practical uses in cell and molecular biology, London, UK: Academic Press.

Kroll, K. L. and Amaya, E. (1996). Transgenic *Xenopus* embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. *Development* **122**, 3173-3183.

Logan, M. and Mohun, T. (1993). Induction of cardiac muscle differentiation in isolated animal pole explants of *Xenopus laevis* embryos. *Development* **118**, 865-875.

Lyons, I., Parsons, L. M., Hartley, L., Li, R., Andrews, J. E., Robb, L. and Harvey, R. P. (1995). Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeobox gene *Nkx-2.5*. *Genes Dev.* **9**, 1654-1666.

Newman, C. S. and Krieg, P. A. (1998). *Tinman*-related genes expressed during heart development in *Xenopus*. *Dev. Genet.* in press.

Nieuwkoop, P. and Faber, J. (1956). Normal table of *Xenopus laevis* (Daudin). North-Holland, Amsterdam, The Netherlands.

Nordeen, S. K. (1988). Luciferase reporter gene vectors for analysis of promoters and enhancers. *Biotechniques* **6**, 454-458.

Peng, H. B. (1991). Solutions and protocols. (ed. B. K. Kay and H. B. Peng). pp. 657-662. In *Xenopus laevis*: Practical Uses in Cell and Molecular Biology. London, UK: Academic Press.

Ryan, K., Garrett, N., Mitchell, A. and Gurdon. J. B. (1996). *Eomesodermin*, a key early gene in *Xenopus* mesoderm differentiation. *Cell* **87**, 989-1000.

Simon, K. J., Grueneberg, D. A. and Gilman, M. (1997). Protein and DNA contact surfaces that mediate the selective action of the Phox1 homeodomain at the *c-fos* serum response element. *Mol. Cell. Biol.* **17**, 6653-6662.

Tonissen, K. F., Drysdale, T. F., Lints, T. J., Harvey, R. P. and Krieg, P. A. (1994). *XNkx-2.5*, a *Xenopus* gene related to *Nkx-2.5* and *tinman*: evidence for a conserved role in cardiac development. *Dev. Biol.* **162**, 325-328.

Turner, D. L. and Weintraub, H. (1994). Expression of *achaete-scute* homolog 3 in *Xenopus* embryos converts ectodermal cells to a neural fate. *Genes and Development* **8**, 1434-1447.

Vize, P. D., Hemmati-Brivanlou, A., Harland, R. M. and Melton, D. A. (1991). Assays for gene function in developing *Xenopus* embryos (ed. B. K. Kay and H. B. Peng). pp. 367-387. In *Xenopus laevis*: Practical uses in cell and molecular biology, London, UK: Academic Press.

Zhu, H., Garcia, A. V., Ross, R. S., Evans, S. M. and Chien, K. R. (1991). A conserved 28-base-pair element (HF-1) in the rat cardiac myosin light chain-2 gene confers cardiac-specific and a-adrenergic-inducible expression in cultured neonatal rat myocardial cells. *Mol. Cell. Biol.* **11**, 2273-2281.

Zou, Y., Evans, S., Chen, J., Kou, H.-C., Harvey, R. P. and Chien, K. R. (1997). CARP, a cardiac ankyrin repeat protein, is downstream in the *Nkx2-5* homeobox gene pathway. *Development* **124**, 793-804.