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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*

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SUMMARY

***tinman* is a *Drosophila* Nk-homeobox gene required for heart and visceral mesoderm specification. Mutations in *tinman* result in lack of formation of the *Drosophila* heart, the dorsal vessel. We have isolated an Nk-homeobox gene from *Xenopus laevis*, *XNkx-2.3*, which appears by sequence homology and expression pattern to be a homologue of *tinman*. The expression pattern of *XNkx-2.3* both during development and in adult tissues partially overlaps with that of another *tinman* homologue, *Csx*/*Nkx-2.5*/*XNkx-2.5*. We have found that embryonic expression of both *XNkx-2.3* and *XNkx-2.5* is induced at a time when cardiac specification is occurring. *XNkx-2.3* is expressed in early cardiac primordia before the expression of a marker of cardiac differentiation, *XMLC2*, as well as in pharyngeal endoderm. In adult tissues, *XNkx-2.3* is expressed in the heart and several visceral organs. As the helix-loop-helix factor Twist is thought to regulate *tinman* expression in *Drosophila*, we have compared the expression of *XNkx-2.3* and *Xtwist* during embryonic development in *Xenopus*. There appears to be no overlap in expression patterns of the two RNAs from the neurulae stages onward, the first time at which the RNAs can be visualized by in situ hybridization. The overlapping expression patterns of *XNkx-2.3* and *mNkx-2.5*/*XNkx-2.5* in conjunction with evidence presented here that other Nk-homeodomains are expressed in adult mouse and *Xenopus* heart suggests that *tinman* may be represented by a family of genes in vertebrates.**

Key words: Nk-homeobox, *tinman*, cardiac specification/determination, *Drosophila*, *XNkx-2.3*, vertebrate, *Xenopus laevis*

INTRODUCTION

Although mechanisms of cardiac muscle specification and determination are as yet unknown, some progress has been made recently in defining genes that may be involved in these processes. Striated cardiac and skeletal muscle express many genes in common, and employ many of the same transcription factors. However, cardiac muscle does not express members of the MyoD family which appear to be determination factors in the skeletal muscle context (reviewed by Edmondson and Olson, 1993). Several transcription factors that are involved in the transcription of cardiac-specific genes have been described, including SRF (Sartorelli et al., 1992), MEF2 (Yu et al., 1992), Hf1b (Navankasattusas et al., 1992), TEF (Farrance et al., 1992) and GATA-4 (Grepin et al., 1994). The expression of GATA-4 (Arceci et al., 1993) and specific members of the MEF2 family (Chambers et al., 1994; Edmondson et al., 1994) in the developing heart prior to the expression of other markers of the differentiated cardiac phenotype suggests that these factors may play a role in determinative events of cardiac muscle. Members of the MEF2 gene family may also be playing an important role in skeletal muscle determination, as there appear to be complex regulatory circuits between the MyoD family members themselves and MEF2 (Cheng et al., 1993; Kaushal et al., 1994).

Recently, a member of the homeobox transcription factor family has been described that may play a role in cardiac mesoderm specification/determination. The *Drosophila* gene *Nk-4*/*msh-2*/*tinman* (Kim and Nirenberg, 1989; Bodmer et al., 1990; Bodmer, 1993; Azpiazu and Frasch, 1993) is first expressed throughout the mesoderm in the blastoderm, then becomes restricted to the dorsal half of the mesoderm which will give rise to visceral mesoderm and dorsal somatic muscles. The visceral mesoderm will further partition into visceral and cardiac mesoderm which may be specified independently. Following this partition, *tinman* is transiently expressed in visceral mesoderm, while its expression persists in the cardiac mesoderm. *tinman* is expressed in the two major cell types of the *Drosophila* heart, the cardial and pericardial cells. In *tinman* mutants, no visceral mesoderm of the midgut or cardiac mesoderm forms. Tinman has been shown to regulate several genes that are important for visceral mesoderm development, including a related Nk-homeodomain gene, *bagpipe*/*Nk-3* (Azpiazu and Frasch, 1993).

A vertebrate homologue of *tinman* has been cloned, the mouse gene *Csx/mNkx-2.5* (Komuro and Izumo, 1993; Lints et al., 1993) and its *Xenopus* counterpart, *XNkx-2.5* (Tonissen et al., 1994). Expression of *Csx/mNkx-2.5* mRNA as first detected by RNA in situ hybridization occurs in a pattern consistent with its being in early cardiac primordia, suggesting that it, like *tinman*, may play a role in cardiac mesoderm determination. *MNkx-2.5* is also expressed embryonically in the pharyngeal endoderm, thyroid anlage, tongue muscle, spleen, stomach anlage, and in adult heart, spleen and tongue (Lints et al., 1993). *XNkx-2.5* is expressed in a bilaterally symmetrical pattern just ventral to the anterior neural folds in the *Xenopus* neurula, consistent with its being expressed in cardiac primordia. In tailbud embryos, it is also expressed in the pharyngeal region and in a region of the developing gut (Tonissen et al., 1994). Unlike mutation of *tinman*, however, knockout of the *mNKx-2.5* gene in transgenic mice does not result in a complete absence of heart formation, but rather results in abnormal development of the heart (R. Harvey, personal communication). This result suggests that if *mNkx-2.5* is a homologue of *tinman*, perhaps there is some redundancy of function in vertebrates that allows the heart to form in the *mNkx-2.5* mutant.

We have been interested in obtaining *Xenopus* homologues of *tinman* to investigate their potential role in cardiac mesoderm determination, as *Xenopus laevis* is a well-defined model of mesoderm induction (reviewed by Sive, 1993), and *Xenopus* offers many experimental advantages for the study of early developmental events in vertebrates. In this paper, we describe the cloning of a *Xenopus* Nk-homeobox gene whose expression pattern indicates that it is a second vertebrate *tinman* homologue. This, in conjunction with the phenotype of the *mNkx-2.5* mutant, and other results (see below) indicate that *tinman* may be represented by a family of genes in vertebrates, in a manner analogous to that in which the *Drosophila* MyoD homologue *nautilus* is represented by the MyoD family in vertebrates.

MATERIALS AND METHODS

Cloning by the polymerase chain reaction (PCR)

The degenerate oligonucleotide primers that were used to amplify NK-homeodomain related sequences encoded the amino acid sequences underlined in Fig. 1 and were as follows:

upstream primer (corresponding to aa AQVFELER):

`5′GC(ACGT)CA(AG)GT(ACGT)TA(TC)GA(AG)(CT)T(ACGT)GA(AG)(AC)G 3′;`

downstream primer (corresponding to TQVKIWFQN):

`5′(AG)TT(CT)TG(AG)AACCA(AGT)AT(CT)TT(ACGT)AC(CT)TG(ACGT)GT 3′.`

Approximatlely 5 μg of total RNA from adult mouse and *Xenopus* heart was transcribed into cDNA according to manufacturer's instructions (Superscript Kit; BRL). 1 μl of this cDNA reaction was used in a PCR amplification reaction, with 1 μg of each degenerate primer and Taq DNA polymerase (BRL) according to the manufacturer's instructions. Samples were denatured for 4 minutes at 94°C and amplified thirty times, each amplification cycle consisting of 1 minute at 94°C, 1 minute at 55°C, and 1 minute at 72°C. Samples were finally extended for 10 minutes at 72°C. Amplified DNA sequences were resolved on a 3% lomelt agarose gel, excised and ligated directly to the TA cloning vector, pCR II (Invitrogen).

Isolation of *XNkx-2.3* cDNAs

The *XNkx-2.3* homeodomain clone obtained by PCR was excised from the pCR vector, radiolabelled with ^{32}P and used to screen a λgt.10 library constructed from the anterior half of stage 17 neurulae (Kintner and Melton, 1987). Filters were hybridized in 50% formamide, 6× SSPE, 1× Denhardt's, 0.1% SDS and 100 mg/ml salmon sperm DNA at 42°C overnight and then were washed in 0.1% SDS, 0.5× SSPE at 68°C for approximately 4 hours. Positive clones were plaque purified and subcloned as *Eco*RI fragments into the plasmid vector pBluescript KS^+ (pKS) (Stratagene).

Sequencing

Subcloned PCR products and cDNA clones were sequenced with the dideoxy chain termination method using fluorescently labelled nucleotides and oligonucleotide primers (Taq Dideoxy Terminator; ABI). Sequencing reactions were run on an automated DNA sequencer (ABI). For the cDNA clones, each strand of DNA was sequenced a minimum of three times.

Northern blot analysis

RNA was extracted from adult *Xenopus* tissues using RNASTAT-60 (Tel-Test Inc.) according to the manufacturer's instructions. $Poly(A)^+$ RNA was isolated using biotinylated oligo(dT) (polyATtract; Promega). 2 μg of $poly(A)^+$ RNA were fractionated on formaldehyde agarose gels and capillary blotted overnight onto nylon filters. Filters were stained with methylene blue to monitor integrity and transfer efficiency of RNA (Sambrook et al., 1989). Hybridization was performed with standard Northern blot conditions at high stringency using random primed probes (Random Primers DNA Labelling; BRL). The probe for *XNkx-2.3* was the entire *Eco*RI cDNA fragment of *XNkx-2.3*a (approximately 2.5 kb). The probe for *XNkx-2.5* was a 622 bp *Eco*RI fragment excised from a pCR II subclone which we obtained by PCR using primers complementary to the published sequences (Tonissen et al., 1994) and cDNA from *Xenopus* heart as a template. This fragment covers nt 671-1293 (Tonissen et al., 1994), containing the 3′ coding region and untranslated sequences, omitting the homeodomain.

RNAse protection assays

RNA was extracted from staged *Xenopus* embryos and adult tissues using RNASTAT-60. RNAse protection analyses were performed as described previously (Zhu et al., 1991). Radiolabelled riboprobes were synthesized from subclones of *XNkx-2.3* alleles. An approximately 1800 bp *Pst*I-*Eco*RI fragment from *XNkx-2.3a* was subcloned into $pBluescriptKS^+$ (pKS2.3aPE1800), linearized with *Bgl*II and transcribed with T7 RNA polymerase to give an antisense probe. Full protection of this probe results in a fragment of 256 nt. An approximately 1900 bp *Xba*I-*Eco*RI fragment from *XNkx-2.3b2* was subcloned into pKS (pKS2.3b2XE1900), linearized with *Bgl*II, and transcribed with T7 to give an antisense probe, which when fully protected results in a fragment of 283 nt. Both of these probes are complementary to 3′ untranslated regions of alleles *XNkx-2.3a* and *XNkx-2.3b2*, which have many nucleotide differences and therefore should be specific for each allele. A riboprobe for *XNkx-2.5* was synthesized from a pCR II-XNkx-2.5 subclone (see above, Northern Blot Analysis). The plasmid was linearized with *Pvu*II and transcribed with T7 to give an antisense probe which when fully protected is 455 bp.

Riboprobes were hybridized to 20 μg of total RNA per reaction. Prior to each assay, RNA samples were quantitated by spectophotometry and fractionated on an analytical formaldehyde-agarose gel to monitor integrity and quantitation. Each RNAse protection assay was repeated a minimum of three times, with distinct RNA samples. Results were consistent with those shown.

Whole-mount in situ hybridization

Embryos were fixed and processed as described (Harland, 1991). Riboprobes were prepared by in vitro transcription of linearized DNA templates in the presence of digoxigenin-11-UTP (Boehringer Mannheim). Probes for *XNkx-2.3* were prepared by linearizing *XNkx-*

2.3 subclones pKS2.3aPE1800 or pKS2.3b2XE1900 (see above) with *Eco*RI and transcribing with T7 RNA polymerase for antisense probes, or with *Bam*HI or *Xba*I and transcribing with T3 RNA polymerase for sense probes. These probes share many common sequences and would cross react with both *XNkx-2.3a* and *XNkx-2.3b2*. The probe for *Xtwist* spans the coding region (Hopwood et al., 1989). The probe for *XMLC2* was obtained by subcloning into pCR II a fragment obtained from a PCR reaction containing degenerate oligonucleotide primers complementary to conserved regions of human and mouse MLC2 atrial light chain (Kubalak et al., 1994) and cDNA from adult *Xenopus* heart. The PCR fragment was sequenced, and its sequence found to agree with that reported for *XMLC2* (Chambers et al., 1994). Our *XMLC2* clone is 324 bp long, with its 3′ termini encoding the final amino acid of XMLC2. Antisense probes for *XMLC2* were generated by linearizing pCRMLC with *Bam*HI and transcribing with T7 RNA polymerase. For sense probes, pCRMLC was linearized with *Xba*I and transcribed with SP6 RNA polymerase. Embryos were processed for in situ analysis as described (Harland, 1991). The staining reaction was allowed to proceed from 4 hours to overnight. Stained embryos were fixed, dehydrated and sometimes cleared before photographing. Some specimens were sectioned after staining, and were fixed overnight, dehydrated in ethanol, permeabilized briefly in histoclear, followed by 1× 20 minute change in 1:1 xylene:paraffin wax at 60°C, 3× 20 minutes change in paraffin wax at 60°C and embedded in paraffin wax. Sections (10 μm) were cut, dried, dewaxed by passing through histoclear 2× 10 minutes, mounted in Permount and photographed, with or without Nomarski optics.

RESULTS

Cloning vertebrate homologues of *tinman*: several Nk-homeodomain genes are expressed in adult heart

In order to obtain *tinman* homologues, we designed degenerate oligonucleotide primers to the conserved Nk-homeodomain region of Tinman (See Materials and Methods, and Fig. 1). Our primers were designed to amplify, by means of the polymerase chain reaction (PCR), a 123 bp region of the homeodomain, and were used in conjunction with cDNA templates generated from adult mouse and *Xenopus* heart RNA. Amplified fragments were cloned, and several clones from mouse and *Xenopus* were sequenced. In this manner, at least four distinct Nk-homeodomain genes were found to be expressed in mouse and *Xenopus* adult heart. Three of these have been previously described: *Csx*/*mNkx-2.5*/*XNkx-2.5* (Komuro and Izumo, 1993; Lints et al., 1993; Tonissen et al., 1994), *TTf-1*/*mNkx-2.1* (Price et al., 1992), and *mNkx-2.3* (Price et al., 1992; Lints et al., 1993). The apparent *Xenopus* homologue of *mNkx-2.3*, *XNkx-2.3*, was also obtained (Fig. 1). The homeodomain and immediately surrounding DNA sequence of *mNkx-2.3* has been published (Price et al., 1992; Lints et al., 1993), but no further information regarding its DNA sequence or expression has been described. Of the four homeodomain clones obtained by PCR, only *Csx*/*mNkx-2.5*/*XNkx-2.5* has previously been described as being expressed in the heart. Two additional homeodomain clones, one from *Xenopus* heart (*XPCR-3*), and one from mouse heart (*mPCR-13*) were sequenced (Fig. 1) and are potential homologues of each other. Their sequence does not correspond to that of any previously described genes.

Preliminary RNAse protection experiments with the *Nkx-2.3* homeodomain clones from mouse and from *Xenopus* indicated that these genes were expressed during gastrulation, and in the adult heart, but not in adult skeletal muscle, liver or brain (data not shown). These preliminary results suggested that *mNkx-2.3*/*XNkx-2.3* was a potential candidate to be a *tinman* homologue.

Obtaining cDNA clones for a vertebrate *tinman* homologue, *XNkx-2.3*

As *XNkx-2.3* appeared to be a good candidate to be a *tinman* homologue, we screened a *Xenopus* neurula stage cDNA library (Kintner and Melton, 1987) with an *XNkx-2.3* PCR-generated clone. From screening approximately one million plaques, we obtained five cDNA clones, three of which appeared to be distinct by restriction enzyme and preliminary DNA sequence analysis. These three clones were selected for further analysis.

DNA sequence analysis indicated that the three clones are likely to be alleles of the same gene, and they have been designated *XNkx-2.3*a, *XNkx-2.3b1* and *XNkx-2.3b2* (Fig. 2). The very high identities between *XNkx-2.3b1* and *XNkx-2.3b2*, and the nature of the sequence differences (several single nucleotide substitutions, and small deletions that do not alter the reading frame) indicate that these two cDNAs represent alleles. *XNkx-2.3a* demonstrates slightly more sequence divergence from *XNkx-2.3b1* and *XNkx-2.3b2*, yet is still very highly related, as indicated by the table in Fig. 2B. *Xenopus laevis* appears to be an ancient tetraploid species that is now diploidized (Graf, 1991). The number of conserved duplications is at least 54% of all loci, with the degree of sequence divergence beween examined duplicate gene copies varying from 4.5% to 24.9% in the translated regions. The divergence between *XNkx-2.3a* and *XNkx-2.3b1*/*b2* is well within that range, being about 4.7% in the coding regions. There is also a high degree of identity in the untranslated regions, supporting the idea that these cDNAs represent the tetraploid past. The nature of the sequence differences make it unlikely that *XNkx-2.3a* is a splice variant (refer to Fig. 2A). The lengths of the *XNkx-2.3a*, *XNkx-2.3b1* and *XNkx-2.3b2* cDNAs are 2482, 1910, and 2350 nucleotides, with 1304, 803, and 1184

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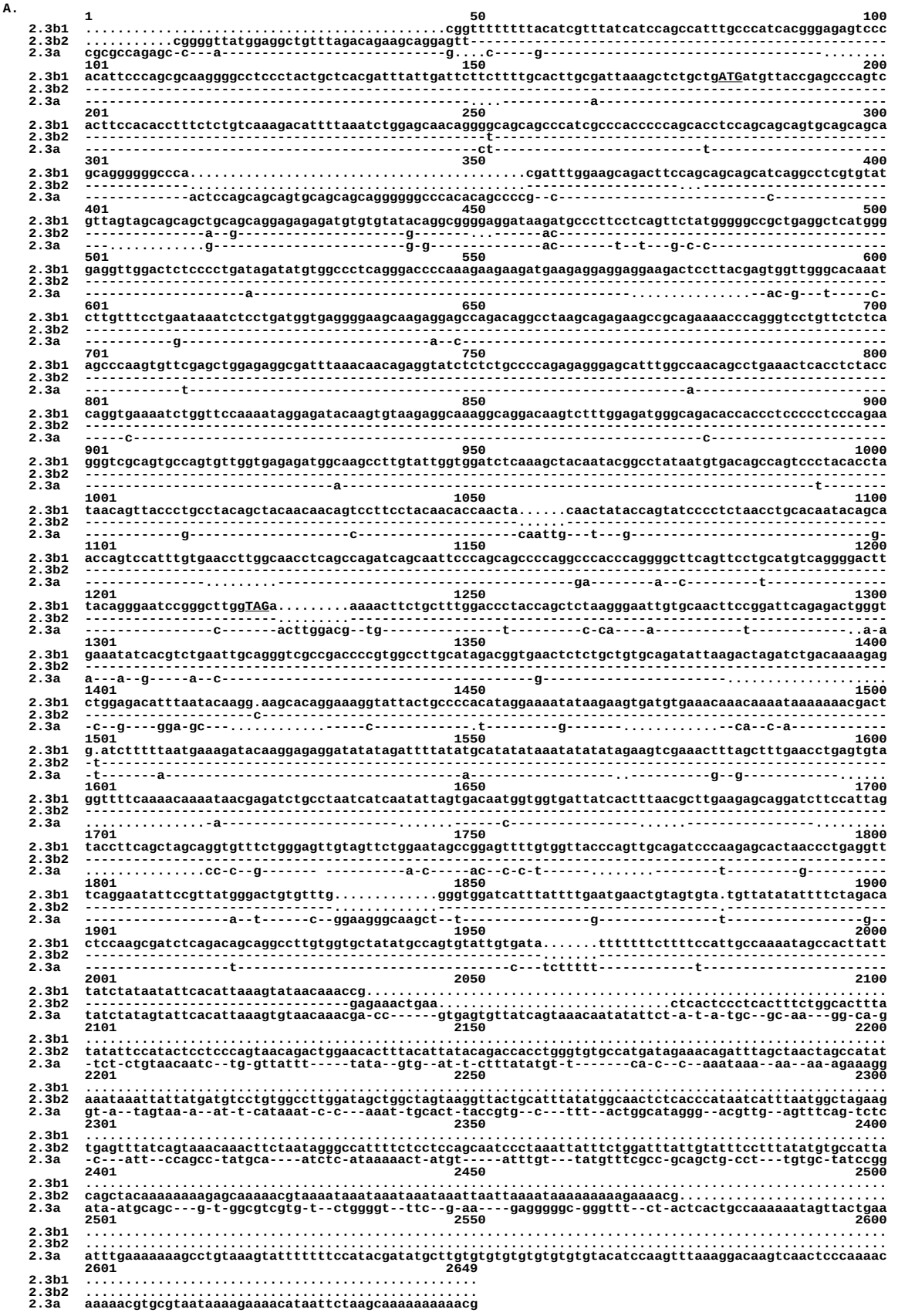
                 R  Y   Q   L   F    Y     R   A  I L   Q KIWFQNRR K K
XNkx-2.3 RRKPRVLFSQAQVFELERRFKQQRYLSAPEREHLANSLKLTSTQVKIWFQNRRYKCKRQR
mNkx-2.3 ----------------------------------S------------------------
XNkx-2.5 ------------Y---------K-------D----V------------------------
mNkx-2.5 ------------Y-----------------DQ--SV------------------------
mNkx-2.1 ---R---------Y---------K-----------SMIH--P---------H---M---A
Bagpipe  KKRS-AA--H----------A------G---SEM-K--R--E-------------T--KQ
Tinman   K-----------L---C--RLKK--TGA---II-QK-N-SA-------------S--GD
XPCR-3             -------------------------KN--------------
mPCR-13            ----------SH---------A---KN----E---------
```

Fig. 1. A Comparison of NK-homeodomains to the homeodomain of XNkx-2.3. The top line shows the consensus sequences for homeodomains (Guazzi et al., 1990). To obtain PCR clones from *Xenopus* and mouse adult heart, degenerate oligonucleotide primers were designed to the underlined amino acid regions (for details, see Materials and Methods). The PCR clones obtained in this manner corresponded to each homeodomain shown here, with the exception of Bagpipe and Tinman, which are shown for purposes of comparison only. XPCR-3 and mPCR-13 are two clones that were obtained and which appear to have novel NK-homeodomains. References for all the listed homeodomains are in the text. The amino acid sequence is shown as single-letter code, and dashes indicate identity with XNkx-2.3.

A.



B.

	5′UT	CODING	3′UT
2.3b1 x 2.3b2	97.8	99.4	99.6
2.3b1 x 2.3a	94.3	95.4	90.4
2.3b2 x 2.3a	96.0	95.3	71.5

Fig. 2. DNA sequence comparison of the three *XNkx-2.3* cDNA clones. (A) DNA sequences of *XNkx-2.3b1*, *XNkx-2.3b2*, and *XNkx-2.3a* were aligned with the Gap Program, University of Wisconson. The ATG start and TAG stop codons are indicated in capital letters and underlined to demarcate the coding sequences. Dashed lines indicate nucleotide identity with *XNkx-2.3b1*, or, in the 3′ untranslated regions where *XNkx-2.3b1* is truncated, identity to *XNkx-2.3b2*. Dots represent gaps. GenBank accession numbers for *XNkx-2.3a*, *XNkx-2.3b1* and *XNkx-2.3b2* are L38674, L38675 and L38676, respectively. (B) Percent identities for pairwise comparisons between the three cDNAs are shown for the 5′ untranslated (5′ UT), coding, and 3′ untranslated (3′UT) regions.

nucleotides of 3′ untranslated sequence respectively. *XNkx-2.3a* and *XNkx-2.3b2* each have a single out-of-frame methionine codon in their 5′ untranslated regions.

Protein sequence of the products of the *XNkx-2.3* alleles

The predicted amino acid sequences are indicated in Fig. 3, and aligned to that of XNkx-2.5, with which they share approximately 50% identity overall. An alignment of XNkx-2.3 to Tinman (Bodmer et al., 1990) reveals an overall amino acid identity of 41%, with 59% similarity. This is greater than the overall amino acid identity of XNkx-2.3 to Bagpipe (Azpiazu and Frasch, 1993), which is 32% identical, with 52% similarity. Translation of the DNA sequence predicts 335, 331, and 329 amino acids for XNkx-2.3a, XNkx-2.3b1 and XNkx-2.3b2, respectively. XNkx-2.3b1 and XNkx-2.3b2 have 99% amino acid sequence identity, and are 93 and 94% identical respectively to XNkx-2.3a. We think it likely that the double methionine codons which are the first in-frame start codons are the true start, as they are immediately upstream of a conserved decapeptide region (see below).

Comparison of XNkx-2.3 homeodomain and other conserved regions to those of other Nk-homeodomain family members

A comparison of the XNkx-2.3 homeodomain to that of mNkx-2.3 (Price et al., 1992) and other Nk family members is indicated in Fig. 1. The designation of our clones as XNkx-2.3 is solely based upon the high relatedness of the homeodomain to that previously published for mNkx-2.3 (one amino acid difference). As indicated in Fig. 3, XNkx-2.3 also contains two other conserved sequences found in other Nk family members. The first is a decapeptide near the predicted amino-termini, which is found in mNkx-2.5/XNkx-2.5, mNkx-2.1 and Tinman (Lints et al., 1993; Tonissen et al., 1994). The second is carboxy-terminal to the homeodomain and is a 17 amino acid motif found in NK2 relatives (Price et al. 1992; Lints et al., 1993). Mutagenesis of a central hydrophobic cluster within this domain (VPVLV) does not affect the ability of mNkx-2.1 to bind to DNA in vitro and may be involved in protein-protein interactions (Guazzi et al., 1990). An acidic region is located just upstream of the homeodomain in XNkx-2.3, as with other members of the Nk-2 family (Kim and Nirenberg, 1989; Lints et al., 1994).

XNkx-2.3 mRNA is expressed in adult heart and other tissues

Northern blot analysis of poly(A)$^+$ RNA from several adult tissues using a probe that would hybridize to all three *XNkx-2.3* alleles indicated that *XNkx-2.3* mRNA is expressed in adult heart, intestine, spleen and tongue, with lower amounts in stomach (Fig. 4). The principal mRNA species in all these tissues appears to be approximately 2.4 kb in length, which corresponds closely to the sizes of *XNkx-2.3a* and *XNkx-2.3b2* cDNAs (2495 and 2364 bp, respectively), suggesting that these clones are full length. Minor hybridizing species are a band of approximately 3 kb in all positive tissues, and a smaller band of about 1.9 kb in adult intestine (Fig. 4). Hybridization of the same blot to a radiolabelled probe specific for *XNkx-2.5* (see Materials and Methods) indicated that *XNkx-2.5* is expressed to detectable levels in adult heart and spleen (data not shown).

All three *XNkx2.3* alleles are expressed in the same adult tissues

To investigate whether there were any differences in expression of the *XNkx-2.3* alleles, we used probes specific to the *XNkx-2.3a* and *XNkx-2.3b* alleles in RNAse protection assays, a technique that is also more sensitive than northern blot analysis. Results of the RNAse protection assays are shown in Fig. 5. Both alleles appear to be expressed in adult heart, intestine, spleen, stomach and tongue, as seen previously with the northern blot analysis. In addition, they are expressed in pancreas. Adult tissues that do not express *XNkx-2.3* mRNA to any appreciable level include kidney, liver, lung and skeletal muscle.

Expression of both *XNkx-2.3* and *XNkx-2.5* RNAs is induced between late gastrula and early neurula stages

To determine at what stage *XNkx-2.3* begins to be expressed in the embryo, we performed RNAse protection assays with probes specific for the *XNkx-2.3a* and *XNkx-2.3b* alleles, hybridizing to RNAs extracted from staged embryos (Nieuwkoop and Faber, 1967). Our results are shown in Fig.

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XNkx-2.3b1  MMLPSPVTSTPFSVKDILNLEQQGQQPIAHPQHLQQQCSSSRGAH.............D   46
XNkx-2.3b2  ----------------------------------------------------------   46
XNkx-2.3a   -------------------------------------------QLQQQCSSRGAHTAP-   60
XNkx-2.5     -FA-----------------.............-H-SGL-P...............M-   30

XNkx-2.3b1  LEADFQQQHQASCMLVAAAAAGERCVYTGGEDKMPFLSSMGAAEAHGEVGLSPDRYVALR  106
XNkx-2.3b2  -----.-------------------------.--L--------------------------  104
XNkx-2.3a   ---------------....---------------L----AT-------------E------  116
XNkx-2.5    ITSRLE...NS----STFKQES....-P-T....-C--ELTEEMSQRDTAKG-SSFPGSF   79

XNkx-2.3b1  DPKEEDEEEEEDSLRVVGHKSCFLNKSPDGEGKQEEPDRPKQRSRRKPRVLFSQAQVFEL  166
XNkx-2.3b2  ----------------------------------------------------------  164
XNkx-2.3a   ----------DG.....--N----D----------D----------------------  171
XNkx-2.5    FV-NYL-MDSK-PKDHKKDICPLQKTLEHDKREAED-E-----K-------------Y--  139

XNkx-2.3b1  ERRFKQQRYLSAPEREHLANSLKLTSTQVKIWFQNRRYKCKRQRQDKSLEMGRHHPPPPR  226
XNkx-2.3b2  ----------------------------------------------------------  224
XNkx-2.3a   ----------------------------------------------------------  231
XNkx-2.5    -------K--------D---V------------------------QT---VGL..----  197

XNkx-2.3b1  RVAVPVLVRDGKPCIGGSQSYNTAYNVTASPYTYNSYPAYSYNNSPSYNTNY..NYTSIP  284
XNkx-2.3b2  ----------------------------------------------------------  282
XNkx-2.3a   ----------------------------------------------------NC--A---  291
XNkx-2.5    -I------------L-E-SP--SP---SIN--S--T-----NYSN-ACSGS-NCS-S-MP  257

XNkx-2.3b1  SNLHNTATSPFVNLGNLSQISNSQQPQAHPGASVPACQGTLQGIRAW               331
XNkx-2.3b2  ------------------------------------------------              329
XNkx-2.3a   ------G-----...------------G---TP--S-----------              335
XNkx-2.5    -MQPTS-GNNFMNFS.VGDL.-TVQTPIQQASSVSALH...H-----              299
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Fig. 3. Predicted amino acid sequences of XNkx-2.3a, XNkx-2.3b1 and XNkx-2.3b2, aligned to that of XNkx-2.5. The homeobox domain is boxed. A decapeptide conserved in mNkx-2.5/XNkx-2.5, mNkx-2.1 and Tinman (Lints et al., 1993; Tonissen et al., 1994) is found just downstream of the predicted amino terminus and is underlined. The NK2 domain downstream of the homeodomain (Price et al., 1992) is doubly underlined. Amino acid numbering is indicated on the right.

6A,B, and indicate that RNAs for *XNkx-2.3a* and *XNkx-2.3b* are present at earlier stages, but appear to be significantly induced between stages 12.5 and 14 (late gastrula, early neurula). Similar assays were performed using a probe for *XNkx-2.5*, and indicate a very similar profile of expression for the two genes (Fig. 6C).

XNkx-2.3 RNA is expressed in early cardiac progenitors prior to expression of a marker for cardiac differentiation

In order to determine where in the embryo *XNkx2.3* is expressed, we performed whole-mount in situ hybridization analyses (Harland, 1991) on embryos from stages 10 through 36. Biotinylated probes for *XNkx-2.3* and two other genes, *Xtwist*, and *Xmyosin-light-chain 2* (*XMLC2*) were synthesized and hybridized to embryos prepared for whole-mount in situ analysis. We wanted to compare expression of *XNkx-2.3* to that of *Xtwist*, as expression of the *Drosophila twist* gene precedes the earliest expression of *tinman*, and is thought to regulate *tinman* expression (Bodmer et al., 1990). XMLC2 has been shown to be a very specific marker for cardiac mesoderm, and is one of the earliest markers of cardiac differentiation (Chambers et al., 1994). As we are interested in the potential role of *XNkx-2.3* in cardiac determination, we wanted to compare the expression of *XNkx-2.3* to a known marker of the cardiac phenotype.

Results of the in situ analyses are shown in Fig. 7. We were first able to detect expression of *XNkx-2.3* at stage 13.5 (Fig. 7A), in an anteroventral position. Slightly later, at stage 16, *XNkx-2.3* staining is observed just ventral to the anterior neural folds and immediately posterior to the cement gland. (Fig. 7B). Sectioning of stage 16 embryos revealed that this staining is mostly mesodermal, although there is weaker staining in endoderm at the floor of the prospective pharynx (Fig. 8). At early and mid-neurula stages the prospective heart mesoderm lies at the anterior edge of the neural plate (Jacobson and Sater, 1988). The anterior location of the *XNkx-2.3* expression domain is consistent with the location of the prospective heart mesoderm. However, at this stage, the prospective heart primordia are bilaterally located at the lateral edges of the anterior neural plate and are not thought to have fused yet (Jacobson and Sater, 1988). In contrast, *XNkx-2.3* expression extends continously form one lateral side of the embryo to the other. Therefore it is unclear whether this early *XNkx-2.3* expression corresponds precisely to the location of the heart primordia. One possibility is that the domain of *XNk-2.3* expression includes heart primordia but also noncardiogenic mesoderm, such as visceral mesoderm. This interpretation would be consistent with the early expression of *tinman* in visceral mesoderm. *Xtwist* expression at this time was observed dorsally, in the newly formed cephalic neural crest, as previously described (Hopwood et al., 1989; Fig. 7C). As development proceeds, *XNkx-2.3* expression moves more ventrally

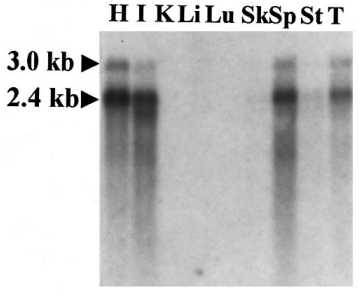

Fig. 4. Northern blot analysis of *XNkx-2.3* expression in adult *Xenopus* tissues. 2 μg of poly(A)$^+$ RNA were fractionated on formaldehyde agarose gels and probed for *XNkx-2.3* mRNA (see Materials and Methods). In tissues positive for *XNkx-2.3* mRNA expression, a major RNA species of 2.4 kb was observed. Secondary bands of approximately 3.0 kb were also observed in all positive tissues. A smaller RNA band of approximately 2.0 kb was also observed in the intestine. The autoradiogram shown was exposed for 4 days at –70°C. The origin of the A$^+$ RNAs: H, heart; I, intestine; K, kidney; Li, liver; Lu, lung; Sk, skeletal muscle; Sp, spleen; St, stomach; T, tongue.

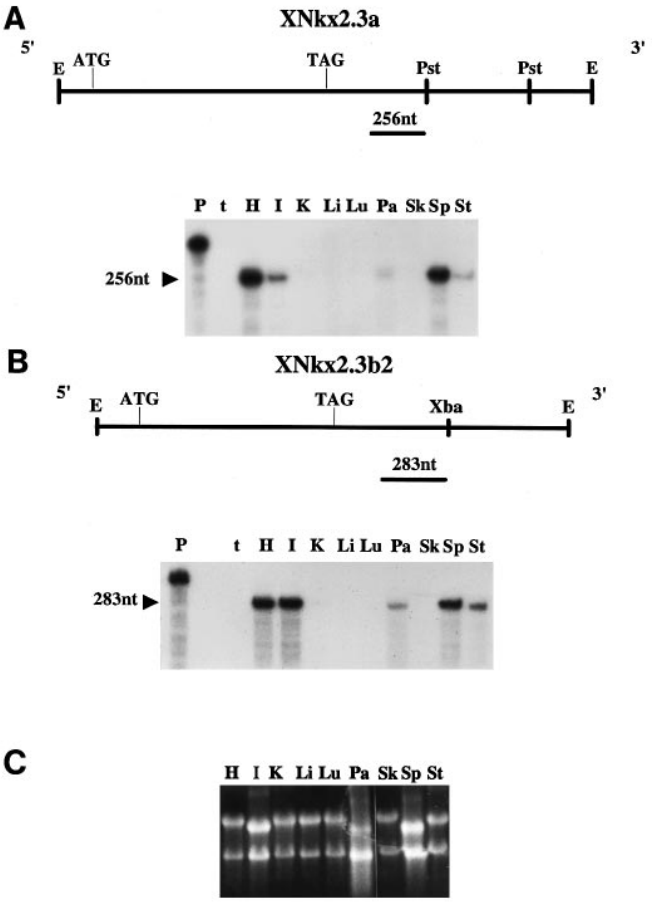

Fig. 5. RNAse protection analyses of *XNkx-2.3a* and *XNkx-2.3b* expression in adult *Xenopus* tissues: (A,B) Radiolabelled riboprobes specific for each *XNkx-2.3* allele were hybridized to 20 μg of total RNA from various adult *Xenopus* tissues. *XNkx-2.3* sequences contained in each probe are diagrammed above each autoradiogram (for details, see Materials and Methods). The probe for *XNkx-2.3b2* also recognizes *XNkx-2.3b1*. (C) Analytical formaldehyde agarose gel of RNA samples. RNA samples were monitored for integrity and quantity on analytical formaldehyde agarose gels before using. 10 μg of each RNA were loaded per lane. P, radiolabelled probe alone. These probes include the *XNkx-2.3* sequences as diagrammed, plus vector sequences, resulting in a radiolabelled probe of greater length than the fully protected *XNkx-2.3* sequences; t, tRNA control lane; H, heart RNA; I, intestine RNA; K, kidney RNA; Li, liver RNA; Lu, lung RNA; Pa, pancreas RNA; Sk, Skeletal muscle RNA; Sp, spleen RNA; St, stomach RNA.

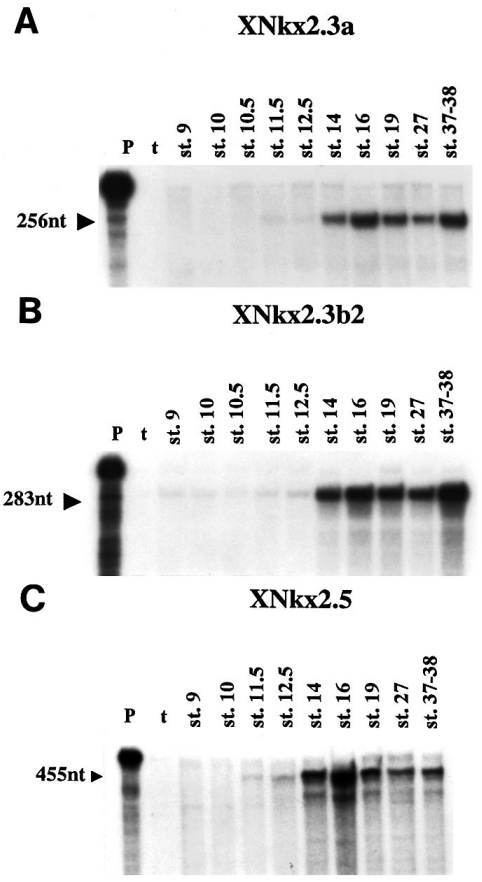

Fig. 6. RNAse protection analyses of *XNkx-2.3* and *XNkx-2.5* expression during *Xenopus* embryonic development. Radiolabelled probes for each *XNkx-2.3* allele (A,B) and for *XNkx-2.5* (C) were hybridized to 20 μg of total RNA extracted from staged *Xenopus* embryos (Nieuwkoop and Faber, 1967). RNA samples were monitored for integrity and quantity on analytical formaldehyde agarose gels before using. The protected RNA species as indicated by arrowheads in each figure correspond to full protection of probe sequences complementary to each cDNA. For probe details, see Materials and Methods. P, probe alone, including vector sequences and regions complementary to each cDNA; t, tRNA control; stage 9-38, RNA from stages 9 through 38 embryos.

(Fig. 7D). This apparent movement of *XNkx-2.3*-expressing cells is coincident with that previously described for migrating cardiac primordia (Jacobson and Sater, 1988). At stage 23, two patches of XNkx-2.3 staining were observed on the anterolateral ventral surface of the embryo (Fig. 7E,F). The more posterior of these patches is clearly bilaterally symmetrical and is consistent with the location of heart primordia at this stage, while the more anterior is likely to be staining of pharyngeal endoderm (refer to Fig. 8). At stage 27, *XMLC2* expression can be detected as the cardiac primordia differentiate. A comparison of *XNkx-2.3* and *XMLC2* expression at this stage indicates that *XNkx-2.3* expression overlaps that of *XMLC2*, but extends further anteriorly, in the pharyngeal region. Note that the two cardiac primordia are still somewhat distinct (Fig. 7G,H). Lateral views of stages 26 and 34 enable a comparison of the expression patterns of *XNkx-2.3*, *XMLC2* and *Xtwist* (Fig. 7I-K). Looping of the heart tube begins at stage 28, and, by stage 34, the heart has fully looped and begun beating. Expression of *XNkx-2.3* can be seen throughout the looped heart, and in the pharyngeal region. Again, its expression is compared to that of *XMLC2*, which is expressed only in the heart, and to *Xtwist*, which is also expressed in the pharyngeal region (Fig. 7L-N).

XNkx-2.3 RNA is expressed in pharyngeal endoderm and cardiac mesoderm and does not overlap with expression of twist in pharyngeal arch neural crest

To look more closely at which cells express *XNkx-2.3* during development, embryos that had been stained for whole-mount in situ analysis were sectioned, the sections mounted and examined under the microscope. Representative sections are shown in Fig. 8. Examination of anterior transverse sections from a stage 16 embryo stained for expression of *XNkx-2.3* RNA indicated that there was strong staining in mesoderm, and in adjacent endoderm (Fig. 8A,B). A comparison of transverse sections from stage 30 embryos, one hybridized to a probe for *XNkx-2.3*, and the other to a probe for *Xtwist*, indicates that the pharyngeal staining observed for *XNkx-2.3* and *Xtwist* is in distinct populations of cells: in the pharyngeal endoderm and myocardium for *XNkx-2.3* and in the neural crest cells for *Xtwist* (Hopwood et al., 1989), (Fig. 8C-E).

DISCUSSION

In this paper, we report the cloning and characterization of a second vertebrate homologue of *tinman*, *XNkx-2.3*. We have sequenced three distinct cDNA clones which appear to be alleles of *XNkx-2.3*. Two of these cDNAs, *XNkx-2.3b1* and *XNkx-2.3b2*, appear to be almost identical. The third, *XNkx-2.3a*, is more divergent (Fig. 2). Whether or not these sequence differences have functional significance is unknown. Using probes specific for each cDNA in RNAse protection assays, we could find no evidence for distinct expression patterns, although previous investigators have reported distinct expression patterns for duplicated alleles in *Xenopus* (Graf, 1991).

The potential role of *XNkx-2.3* in cardiac mesoderm specification/determination

Our results with *XNkx-2.3* are consistent with its playing a role in cardiac specification/determination. Expression of its RNA appears to be significantly induced between late gastrula (stage 12.5) and early neurula (stage 14), a window of development during which cardiac specification has been shown to occur (Sater and Jacobson,1989). By whole-mount in situ hybridization, *XNkx-2.3* RNA is first visualized at stage 13.5 At least by stage l6, this early expression occurs in mesoderm and associated endoderm (Fig. 8A,B). Its pattern of expression at this time and thereafter includes, but may not be limited to, the previously described locations of cardiac primordia during development (Jacobson and Sater, 1988). From these results, *XNkx-2.3* appears to be expressed in cardiac primordia well before the onset of expression of other differentiated markers of the cardiac phenotype, such as MLC2 (Chambers et al., 1994). Although *XNkx-2.3* appears to be an early marker for cardiac mesoderm progenitors, its embryonic expression is not confined to this population, but is also found in pharyngeal endoderm (Figs 7, 8).

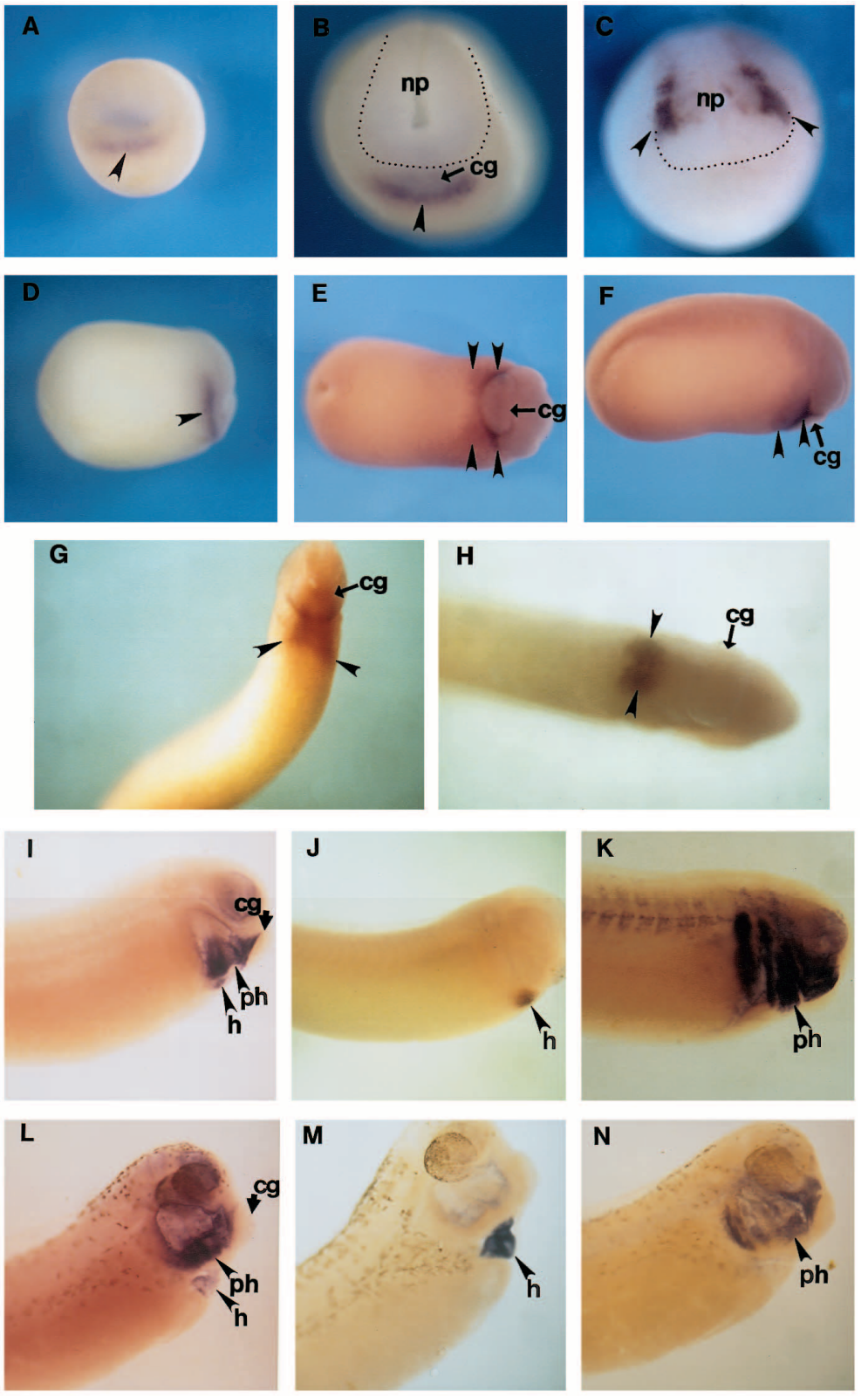

Fig. 7. Whole-mount in situ analyses of *XNkx-2.3*, *Xtwist* and *XMLC2* RNA expression in *Xenopus* embryos. For details on whole-mount procedure and probes used, see Materials and Methods. Arrowheads indicate relevant stained areas. In B, C, the boundaries of the anterior neural plate are outlined with a dotted line. cg, cement gland, h, heart, np, neural plate, ph, pharyngeal region. (A) Anterior view of stage 13.5 embryo hybridized to probe for *XNkx-2.3* RNA. Dorsal is at the top. Bluish staining band above purple-brown hybridisation signal (arrowhead) is a result of artefactual trapping of probe in the archenteron (Harland, 1991). (B) Anterior view of stage 16 embryo stained for *XNkx-2.3* RNA expression. Dorsal is at the top. Staining is observed ventral to the neural folds, immediately posterior to the cement gland. (C) Anterior view of stage 16 embryo hybridized to probe for *Xtwist*. *twist* expression can be seen in the forming cephalic neural crest (Hopwood et al., 1989). (D) Ventral view of stage 19 embryo, hybridized to *XNkx-2.3* probe. Anterior is to the right. Staining is observed in an anteroventral position. (E) Ventral view of stage 23 embryo, showing two areas of staining for *XNkx-2.3* just posterior to the cement gland. The posterior staining is clearly bilateral and corresponds to the partially fused cardiac primordia. Anterior is to the right. (F) Lateral view of same embryo as in E. Anterior is to the right. Note two streaks of staining just caudal to the cement gland. (G) Ventral view of stage 27 embryo hybridized to probe for *XNkx-2.3*. Anterior is at the top right. Arrowheads mark the bilateral staining that corresponds to the prospective heart region (see H). The staining also extends further rostral, abutting the cement gland. (H) Ventral view of stage 27 embryo, hybridized to a probe for *XMLC2*, a marker for differentiated cardiac mesoderm. Anterior is to the right. Note that the bilateral staining here corresponding to the cardiac mesoderm does not extend rostrally to the cement gland, as did the staining shown in G for *XNkx-2.3*. (I) Lateral view of stage 26 embryo stained for *XNkx-2.3* RNA expression. Anterior is to the right, dorsal is at the top. Arrowheads indicate two regions of staining, in the heart tube (see J) and in the pharyngeal region more rostrally. (J) Lateral view of stage 26 embryo stained for *XMLC2* expression. Staining is confined in the heart tube. (K) Lateral view of stage 26 embryo stained for *Xtwist* expression. Note the heavy staining in the pharyngeal region, whereas there is no staining in the cardiac region. (L) Lateral view of stage 34 embryo, hybridized to probe for *XNkx-2.3*. Anterior is to the right, dorsal at the top. Staining is seen in the looped heart tube and more rostrally in the pharyngeal region. (M) Lateral view of stage 34 embryo, hybridized to probe for *XMLC2*. Staining is evident in the looped heart tube. (N) Lateral view of stage 34 embryo, hybridized to probe for *Xtwist*. Some staining remains in the pharyngeal region, and is absent from the heart. Embryos shown in I, K-N were cleared in benzyl:benzoate (Harland, 1991) before photographing.

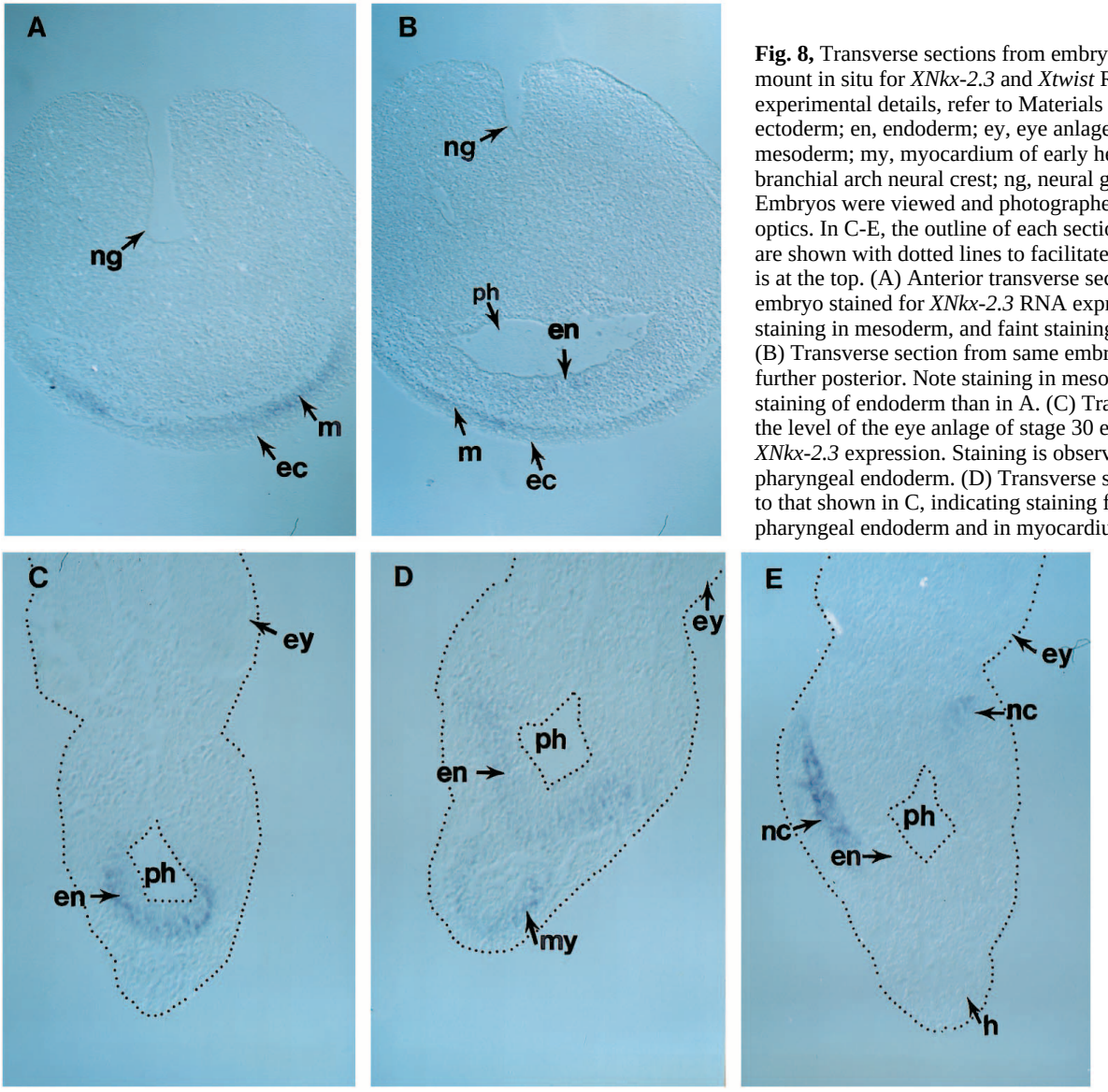

Fig. 8, Transverse sections from embryos stained by whole-mount in situ for *XNkx-2.3* and *Xtwist* RNA expression. For experimental details, refer to Materials and Methods. ec, ectoderm; en, endoderm; ey, eye anlage; h, heart region; m, mesoderm; my, myocardium of early heart tube; nc, branchial arch neural crest; ng, neural groove; ph, pharynx. Embryos were viewed and photographed using Nomarski optics. In C-E, the outline of each section and the pharynx are shown with dotted lines to facilitate orientiation. Dorsal is at the top. (A) Anterior transverse section from a stage 16 embryo stained for *XNkx-2.3* RNA expression. Note staining in mesoderm, and faint staining in the endoderm. (B) Transverse section from same embryo as in A, but further posterior. Note staining in mesoderm and stronger staining of endoderm than in A. (C) Transverse section at the level of the eye anlage of stage 30 embryo stained for *XNkx-2.3* expression. Staining is observed in the pharyngeal endoderm. (D) Transverse section just posterior to that shown in C, indicating staining for *XNkx-2.3* in pharyngeal endoderm and in myocardium. (E) Transverse section of stage 30 embryo stained for *Xtwist* RNA expression. The neural crest invading the pharyngeal mesoderm is stained for *twist*. This staining pattern was never observed for embryos stained for *XNkx-2.3* expression. The endoderm and the heart do not stain but their position is shown to facilitate comparison with D.

XNkx-2.3 RNA expression overlaps with that of *XNkx-2.5*

The early expression patterns of *XNkx-2.3* are remarkably coincident with those of *XNkx-2.5* (Tonissen et al., 1994), and *Nkx-2.5* (Lints et al., 1993). By RNase protection assays, we have found that *XNkx-2.5* expression increases significantly between stages 12.5 and 14, as does that of *XNkx-2.3* (Fig. 6). The pattern of hybridization observed for *XNkx-2.3* in embryos by whole-mount in situ analyses appears to be similar, if not identical to that observed for *XNkx-2.5* (Tonissen et al., 1994). In situ hybridization to sections of early mouse embryos indicated that *mNkx-2.5* RNA was expressed in pharyngeal endoderm, as well as cardiac mesoderm, as we have found for *XNkx-2.3*.

In adult tissues, there also appears to be overlap in the expression patterns of *XNkx-2.3* and *mNkx-2.5*. Both are expressed in adult heart, spleen, tongue and stomach (this paper, and Lints et al., 1994). In addition, *XNkx-2.3* appears to be expressed in the adult intestine, where *mNkx-2.5* is not expressed, and in the pancreas, which was not examined for *mNkx-2.5*. From our results with the embryonic heart, we might expect that the mesodermal component of the adult heart expresses *XNkx-2.3*. The spleen and pancreas are both mesodermally derived organs, originating from the mesogastrium. *MNkx-2.5* expression was seen in myoblasts and muscle of the tongue, and in a restricted region of the stomach, consistent with its being expressed in a mesodermal derivative, the pyloric sphincter (Lints et al., 1993). The intestine is composed of both endodermal and mesodermal components, and it is not known which one(s) express *XNkx-2.3* mRNA.

Expression of XNkx-2.3, XNkx-2.5 and tinman

In *Drosophila*, *tinman* is initially expressed throughout the newly formed mesoderm, and becomes progressively restricted to visceral mesoderm and finally cardiac mesoderm, persisting during dorsal vessel formation (Bodmer et al., 1990; Azpiazu and Frasch, 1993). There is also some *tinman* expression near

the stomodeum, the pharyngeal region, but this expression has not been fully characterized (Bodmer et al., 1990). From these observations, *tinman* appears to be playing a role in the progressive partitioning of the mesoderm. By the time that we are able to detect *XNkx-2.3* expression in *Xenopus* embryos by whole-mount in situ analysis at stage 13.5, the mesoderm has been partitioned (reviewed by Sive, 1993). There do appear to be low levels of *XNkx-2.3* transcripts present in the embryo prior to this time, however, as detected by RNAse protection assays (Fig. 6), which could correspond to the early *tinman* expression. Alternatively, there may be other vertebrate *tinman* homologues which fulfill this early function (see below).

The observed *XNkx-2.3* and *Nkx-2.5*/*XNkx-2.5* expression in cardiogenic regions (Lints et al., 1993; Tonissen et al., 1994) is consistent with the expression of *tinman* in cardiogenic mesoderm. The pharyngeal expression of *XNkx-2.3* and *Nkx-2.5*/*XNkx-2.5* is also remniscent of the stomodeal expression reported for *tinman*. Expression of *tinman* has not been examined in adult *Drosophila*. However, expression of *XNkx-2.3* and *Nkx-2.5*/*XNkx-2.5* in many derivatives of the visceral mesoderm is also consistent with *tinman*'s known role in visceral mesoderm development (Bodmer, 1993; Azpiazu and Frasch, 1993).

In *tinman* mutants, the visceral mesoderm of the midgut fails to form, perturbing development of the associated endoderm (Bodmer, 1993; Azpiazu and Frasch, 1993), and providing evidence that mesodermal/endodermal interactions are required for normal gut development. *XNkx-2.3* and *Nkx-2.5*/*XNkx-2.5* are also expressed in tissues where mesodermal/endodermal interactions are critical for development, such as the dependence of heart development on pharyngeal endoderm (Jacobson et al., 1988; Lints et al., 1993).

genes acting upstream and downstream of *tinman*: the role of twist

Genetic studies in *Drosophila* have suggested candidate genes that act upstream and downstream of *tinman* (Bodmer et al., 1989; Bodmer, 1993; Azpiazu and Frasch, 1993). Just as *tinman* itself may have functionally and structurally related counterparts in vertebrates, potential upstream activators and downstream targets for *tinman* action in *Drosophila* may have their counterparts in vertebrate systems, and are worth investigating in that regard.

One of the genes that appears to activate the early mesodermal expression of *tinman* directly or indirectly is *twist* (Bodmer et al., 1989). Twist is a helix-loop-helix protein which is thought to act positively to determine mesoderm in *Drosophila*. A *Xenopus* homologue of *twist*, *Xtwist*, has been described which is expressed very early in lateral mesoderm, notochord and sclerotome, suggesting that it, like its *Drosophila* counterpart, may play a role in mesoderm determination (Hopwood et al., 1989). A second induction of *Xtwist* expression occurs in the cephalic neural crest (Hopwood et al., 1989). We were interested in exploring the potential relationship between *Xtwist* and *XNkx-2.3* in *Xenopus* development by comparing their expression patterns. We observed positive signals for *Xtwist* and *XNkx-2.3* RNAs in the pharyngeal region of tailbud embryos, suggesting that there might be some overlap in their expression (Fig. 7). However, examination of sections prepared from stained embryos indicated that the population of cells expressing the two genes were in fact distinct, with probes for *Xtwist* RNA staining neural crest cells, and probes for *XNkx-2.3* RNA staining pharyngeal endoderm and cardiac mesoderm (Fig. 8). These results suggest that *Xtwist* is not responsible for the expression of *XNkx-2.3* which is observed from the neurula stage onward. They do not, however, rule out the possibility that the earlier expression of *XNkx-2.3* as observed by RNAse protection analyses is influenced by *Xtwist*.

The role of *tinman* and its homologues in cardiac determination

The precise role of *tinman* itself in cardiac determination remains to be uncovered. The persistence of *tinman* expression in major cardiac cell types, and the complete lack of dorsal vessel formation in *tinman* mutants, indicate that *tinman* is involved in cardiac mesoderm specification/determination. However, expression of *tinman* occurs in tissues other than cardiac cells (Bodmer, 1993; Azpiazu and Frasch, 1993). In a similar fashion, expression of *XNkx-2.3* and *Nkx-2.5*/*XNkx-2.5* is not confined to cardiac mesoderm. If these *tinman* homologues are playing a role in cardiac specification/determination, they are likely to be doing so in conjunction with other factors to confer tissue specificity. Candidates for these factors are others that are expressed very early in cardiogenic mesoderm, such as MEF2 family members (Edmondson et al., 1994; Chambers et al., 1994) and GATA-4 (Arceci et al., 1994).

XNkx-2.3 and *XNkx-2.5* may represent a family of *tinman* homologues in vertebrates

Do the parallels between *tinman*, *XNkx-2.3* and *mNkx-2.5*/*XNkx-2.5* expression indicate that the latter are functional homologues of *tinman*? One test of this would be to determine whether the vertebrate *tinman* homologues can functionally complement *tinman* mutants in *Drosophila*. The overall amino acid sequence identity of XNkx-2.3 to Tinman is 41%, with 59% similarity. That of XNkx-2.5 to Tinman is comparable, 35% identity and 56% similarity. The identity of the homeodomains to that of Tinman is even higher, (65%) but there are several members of the Nk-2 family which have highly related homeodomains (Fig. 1, and Lints et al., 1994). In particular, the homeodomain of mNkx-2.6 is 67% identical to that of Tinman (Lints et al., 1994). Some of these may also be expressed in adult heart as indicated by clones we have obtained from mouse and *Xenopus* heart by PCR cloning (Fig. 1). The expression patterns of these clones vis-a-vis cardiogenic mesoderm remain to be determined. Our results seem to indicate that there may be a family of *tinman* homologues in vertebrates that are involved in cardiac specification and determination, and the precise role each plays will await further cloning and characterization. The partially overlapping expression patterns of *XNkx-2.3* and *Nkx-2.5*/*XNkx-2.5* in conjunction with the fact that the heart can form in *mNkx-2.5* knockout mice (R. Harvey, personal communication), indicate that there may be some redundancy of *tinman* function in vertebrate heart formation, as with the MyoD family in skeletal muscle determination.

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