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 skeletal 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 cardiac 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 Cx/mNkx-2.5 (Komuro and Izumo, 1993; Lints et al., 1993) and its Xenopus counterpart, XNkx-2.5 (Tonissen et al., 1994). Expression of Cx/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. XNkx-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 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 AQPVELER):**

\[5'GC(AGCT)CA(AG)GT(AGCT)TA(TC)GA(AG)(CT)AT(AGCT)GA(AG)(AC)G3';

**downstream primer (corresponding to TQVKIWFQN):**

\[5'(AG)TT(CT)TG(AG)AACCAGT(AG)AT(CT)T(AGCT)AC(CT)TG(AGCT)GT3'.

Approximately 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% low melt 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 32P 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, 6x SSPE, 1x Denhardt’s, 0.1% SDS and 100 mg/ml salmon sperm DNA at 42°C overnight and then were washed in 0.1% SDS, 0.5x SSPE at 68°C for approximately 4 hours. Positive clones were plaque purified and subcloned as EcoRI 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 formamide 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 EcoRI cDNA fragment of XNkx-2.3a (approximately 2.5 kb). The probe for XNkx-2.5 was a 622 bp EcoRI 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 previously described (Zhu et al., 1991). Radiolabelled riboprobes were synthesized from subclones of XNkx-2.3 alleles. An approximately 1800 bp Psil-EcoRI fragment from XNkx-2.3a was subcloned into pBluescript KS+ (pKS2.3aPE1800), linearized with BglII 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 XbaI-EcoRI fragment from XNkx-2.3b2 was subcloned into pKS (pKS2.3b2XE1900), linearized with BglII, 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 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 PvuII 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.
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; Tomissen 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-2.3a, 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 between 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 tetraptidial 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, 2482, and 2350 nucleotides, with 1304, 803, and 1184

![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.](image-url)
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 Wisconsin. 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 52% 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 decapetide 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 decapetide near the predicted amino-termini, which is found in mNkx-2.5/XNkx-2.3, 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 (Bodmer et al., 1990) reveals an overall amino acid identity of XNkx-2.3 to Bagpipe (Azpiazu and Frasch, 1993), which is 32% identical, with 52% similarity. Expression of both XNkx-2.3 and XNkx-2.5 RNAs is induced between late gastrula and early neurula stages

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. 3.

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

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formaldehyde agarose gels and probed 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. 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 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.

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-2.3, 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 XNkx-2.3 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). In contrast, expression of tinman extends continuously 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 XNkx-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
**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 16, 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 cephatic 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. 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.
XNkx-2.3, a second vertebrate homologue of tinman

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

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 orientation. 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.
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 reminiscent 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 cardiac muscle 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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