Requirement of a novel gene, *Xin*, in cardiac morphogenesis

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Accepted 8 January; published on WWW 15 February 1999

SUMMARY

A novel gene, *Xin*, from chick (*cXin*) and mouse (*mXin*) embryonic hearts, may be required for cardiac morphogenesis and looping. Both cloned cDNAs have a single open reading frame, encoding proteins with 2,562 and 1,677 amino acids for *cXin* and *mXin*, respectively. The derived amino acid sequences share 46% similarity. The overall domain structures of the predicted *cXin* and *mXin* proteins, including proline-rich regions, 16 amino acid repeats, DNA-binding domains, SH3-binding motifs and nuclear localization signals, are highly conserved. Northern blot analyses detect a single message of 8.9 and 5.8 kilo base (kb) from both cardiac and skeletal muscle of chick and mouse, respectively. In situ hybridization reveals that the *cXin* gene is specifically expressed in cardiac progenitor cells of chick embryos as early as stage 8, prior to heart tube formation. *cXin* continues to be expressed in the myocardium of developing hearts. By stage 15, *cXin* expression is also detected in the myotomes of developing somites. Immunofluorescence microscopy reveals that the *mXin* protein is colocalized with N-cadherin and connexin-43 in the intercalated discs of adult mouse hearts.

Incubation of stage 6 chick embryos with *cXin* antisense oligonucleotides results in abnormal cardiac morphogenesis and an alteration of cardiac looping. The myocardium of the affected hearts becomes thickened and tends to form multiple invaginations into the heart cavity. This abnormal cellular process may account in part for the abnormal looping. *cXin* expression can be induced by bone morphogenetic protein (BMP) in explants of anterior medial mesoendoderm from stage 6 chick embryos, a tissue that is normally non-cardiogenic. This induction occurs following the BMP-mediated induction of two cardiac-restricted transcription factors, Nkx2.5 and MEF2C. Furthermore, either MEF2C or Nkx2.5 can transactivate a luciferase reporter driven by the *mXin* promoter in mouse fibroblasts. These results suggest that *Xin* may participate in a BMP-Nkx2.5-MEF2C pathway to control cardiac morphogenesis and looping.

Key words: Antisense oligonucleotide, Bone morphogenetic protein 2 (BMP2), Cardiac progenitor cell, Embryo culture, In situ hybridization, Intercalated disc, Cadherin signaling

INTRODUCTION

Cardiac progenitor cells originate from the rostral half of the primitive streak and form a pair of heart-forming fields within lateral plate mesoderm (DeHaan, 1965; Garcia-Martinez and Schoenwolf, 1993). The heart-forming fields migrate ventrally and anteriorly to fuse into a linear heart tube. In chick, the newly formed heart tube begins to contract at Hamburger-Hamilton (HH) stage 10 (Hamburger and Hamilton, 1951) and subsequently undergoes rightward looping at HH stage 11 (DeHaan, 1965). Molecular mechanisms controlling the process and direction of looping remain unclear, however, it may partly be linked to the expression of laterality genes (see review in Harvey, 1998). The looping will continue until the two limbs (conus and sinus) are brought together along their hingepoint. As a result, the conus and its outlet septum will align with atrioventricular septum, interventricular septum and interatrial septum. Also, it will create a common wall, mitroaortic continuity, between the anterior leaflet of the mitral valve and the outlet of the left ventricle. Therefore, looping can be considered as a central event of cardiac morphogenesis and it may affect the septation and valve formation. Recently, knockout or ablation of several transcription factor genes, including Nkx2.5, MEF2C, *eHand* and *dHand*, have been shown to abort cardiac development, disrupting cardiac morphogenesis (Srivastava et al., 1995, 1997; Lyons et al., 1995; Lin et al., 1997; Firulli et al., 1998). A link between extracellular signaling by bone morphogenetic proteins (BMPs) and intrinsic cardiogenesis (Nkx2.5 pathway, cardiac jogging and looping) has been recently established in both chick (Schultheiss et al., 1997) and zebrafish (Chen et al., 1997). However, the molecular components and mechanisms for the BMP pathway that regulates cardiac morphogenesis and looping are not completely known.

In this study, we have cloned a novel gene, *Xin* (which means heart in Chinese), from both chick and mouse. In chick, the expression of *Xin* can be detected as early as stage 8, prior to heart tube formation and looping. Treatment of cultured chick...
embryos with Xin antisense oligonucleotides resulted in abnormal cardiac morphogenesis and looping. These results suggest a crucial role for Xin in cardiac morphogenesis. As for expression of Nkx2.5 and MEF2C, Xin expression is induced in anterior medial mesendoderm by BMP-2. The time required for the induction of Xin is significantly longer than that for Nkx2.5 and MEF2C but is shorter than that for the ventricular myosin heavy chain (vMHc) gene. In transactivation experiments, MEF2C or Nkx2.5 is able to activate transcription of the vMHC gene. In vivo experiments, MEF2C or Nkx2.5 is able to stimulate the expression of a luciferase reporter driven by the mXin promoter in fibroblasts. These results suggest that Xin may be downstream of Nkx2.5 and MEF2C and participates in a BMP-Nkx2.5-MEF2C pathway to regulate cardiac morphogenesis and looping.

MATERIALS AND METHODS

Isolation and characterization of cDNA clones

The original 251 base pairs (bp) cDNA clone (21C), isolated from differential mRNA display (Wang et al., 1996), was used as a probe to screen a cDNA library prepared from stage 17-18 chick embryonic hearts (Stratagene, La Jolla, CA) following the protocol provided by the manufacturer. After plaque purification, 15 positive clones were obtained. Plasmids from positive plaques were isolated from their Uni-ZAP RX vector by in vivo excision with R408 helper phage as described by the manufacturer. After sequencing of overlapping clones, only 2.2 kb of sequences from the 3′-end of the message was obtained. Several other cDNA libraries, including a custom cDNA library prepared from stage 25 chick embryonic hearts (Stratagene, La Jolla, CA), a cDNA library prepared from stage 11 chick embryos (a generous gift from Dr David Bader, Vanderbilt University, TN), and a 5′-stretch cDNA library prepared from adult chick hearts (Clontech, Palo Alto, CA), were further screened and a total of 6.2 kb of sequences was obtained.

The mouse homologue (mXin) of chick Xin (cXin) gene was first identified when a public EST database was searched with the cXin cDNA sequence. One submission (GenBank accession number W70350) from a cDNA library prepared from mouse day 13.5-14.5 embryos was found to have homology (62% identity) to the cXin cDNA sequence. The bacterial stock containing this cDNA clone (L.M.A.G.E. Consortium Clone ID 390093) was obtained (Genome Systems, St Louis, MO). This clone was used as a probe to screen a cDNA library prepared from adult mouse skeletal muscle (Clontech, Palo Alto, CA). Twelve positive clones were obtained. From sequencing, a total of 4.4 kb of cDNA sequence from the 3′-end of the message was obtained. This cDNA clone was further proven to be the mouse homologue of chic Xin gene by various northern and Southern blot analyses, which will be described under Results.

DNA sequencing was performed using Sequenase Kit Version 2.0 (United States Biochemical, Cleveland, OH).

5′-RACE (Rapid Amplification of cDNA Ends) cloning

Northern analysis revealed that sizes of cXin and mXin mRNAs were about 8.9 kb and 5.8 kb, respectively, suggesting we had not obtained full-length cDNAs. Therefore, 5′-RACE cloning (Frohman et al., 1988) was further employed to obtain cDNAs extending further 5′. Total RNA was isolated from day 20 embryonic chick hearts using the Tri Reagent RNA isolation kit (Molecular Research Center, Inc., Cincinnati, OH) or from adult mouse hearts purchased from Clontech (Palo Alto, CA). 5 μg of RNA was used for first-strand cDNA synthesis with random hexamers. cDNA synthesis and ligation to adapter primers, as well as subsequent RACE-PCR were carried out using the Marathon cDNA amplification kit (Clontech, Palo Alto, CA). A ‘touchdown parameters’ PCR program was chosen from the provided protocol (Clontech, Palo Alto, CA) to increase the specificity of amplification. Briefly, after an initial denaturation step (94°C, 1 minute), reactions were cycled five times at 94°C, 30 seconds, 72°C, 5 minutes, five times at 94°C, 30 seconds, 70°C, 4 minutes and 25 times at 94°C, 20 seconds, 68°C, 4 minutes. The first round of PCR was performed with adapter primer (AP1, 5′-CCATCTATACGGACTCATAAGGGG 3′) and one of the Xin gene-specific primers (CRACEB, for cXin, 5′-TGGTTTGTGTTTGTATCTAAAGGC 3′ and M21C-RACE1, for mXin, 5′-CCACTTGGACCTGTCTCCTGAA 3′), using Advantage KlenTaq polymerase mix (Clontech, Palo Alto, CA).

An aliquot of the first PCR reaction mix was diluted 50-fold, and 5 μl was used as a template for subsequent PCR using the nested adapter primer (AP2, 5′-ACTCATAAGGGCTTGGAGGGC 3′) and Xin gene-specific nested primer (CRACEA, for cXin, 5′-TGGTTTGTGTTTGTATCTAAAGGC 3′ and M21C-RACE1, for mXin, 5′-CCACTTGGACCTGTCTCCTGAA 3′). PCR conditions were the same as the first round PCR. Amplified PCR fragments with a size of about 2.6 kb for cXin or 1.3 kb for mXin were gel-purified using a QIAEX II gel extraction kit (Qiagen, Chatsworth, CA) and subcloned into the TA vector (Invitrogen, San Diego, CA). Sequences of inserts from at least three independent clones were determined using the Sequenase Kit.

Primer extension

The cXin antisense primer 21CP3 (5′-AACCTCTGTGAAC-TCAACCAC 3′), corresponding to nucleotide (nt) 46-65, and mXin antisense primers M21C-RACE4 (5′-TCTGCGGGATTTCGTGGTG 3′) and M21C-P51 (5′-TTTTGGGTGTCTAGCCTTGGG 3′), corresponding to nt 60-79 and nt 89-109 respectively, were used for the primer extension experiments. Primers were end-labeled using T4 DNA polynucleotide kinase (Promega, Madison, WI) in the presence of γ-32P-ATP (NEN, Boston, MA). Labeled primers (2×105 cts/minute) were incubated with 15 μg of total RNA, prepared from day 20 embryonic chick hearts or from adult mouse hearts, at 50°C for 1 hour. The extension reaction was performed at 45°C for 30 minutes using the Superscript II RNAse H− reverse transcriptase (Life Technologies, Gaithersburg, MD). The reaction was stopped by phenol/chloroform extraction and the reaction products were then precipitated with ethanol. Pellets were resuspended into 12 μl of formamide sequencing loading buffer and 2 μl of the samples were loaded onto a 6% denaturing polyacrylamide gel. As a control, labeled primers without the addition of RNA were used for the same primer extension reaction. A sequence reaction of a known gene was loaded on the gel along with the primer extension products to serve as size markers.

Northern and Southern blot analysis

Total RNA was isolated from different tissues of a day 20 chick embryo as described above. 10 μg of total RNA was loaded in each lane. Northern blotting was performed as described (Wang et al., 1996). DNA probe containing 0.77 kb from the 3′-end of the cXin cDNA was labeled with α-32P-dATP (NEN, Boston, MA) using a random primed DNA labeling kit (Boehringer Mannheim, Indianapolis, IN). The hybridization was performed at 68°C for 1 hour in an ExpressHyb hybridization solution (Clontech, Palo Alto, CA). The filter was then washed twice in 2× SSC, 0.05% SDS at room temperature for 30 minutes each, followed by 30 minutes at 50°C in 0.1× SSC, 0.1% SDS. The filter was then exposed to X-ray film at −70°C with the presence of intensifying screens. The same membrane was boiled twice in 0.1× SSC, 0.1% SDS, for 30 minutes each and reprobed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to demonstrate equal loading.

Similarly, adult mouse multiple tissue northern blot purchased from Clontech (Palo Alto, CA) was used for northern blot analysis with probe derived from the Norl-EcoRI insert (900 bp) of the Clone.
MgCl$_2$, 0.75 mM CaCl$_2$, 1.5 g/l glucose, pH 7.2) and placed on an
incubator to reach embryonic stage 6 (~24 hours
after exposure to X-ray film, the same membrane was
centrifuged at 10,000
leftward or abnormal looping. Abnormal looping hearts had similar
heart looping, using the criteria described by Easton et al. (1992).

Whole-mount in situ hybridization
Whole-mount in situ hybridization was performed as described previously (Wang et al., 1996). Plasmid containing nucleotides 8,044-
8,814 or 7,384-8,784 of the cXin cDNA was used as template for making digoxigenin (DIG)-labeled riboprobes. For in situ
hybridization of mouse embryos, nucleotides 5,110-5,800 of the mXin
cDNA was used as a template for making DIG-labeled riboprobe. After whole-mount in situ hybridization, selected embryos were
embedded in Paraffin Plus (Oxford, St Louis, MO) and sectioned at
10 µm as described previously (Wang et al., 1996).

Embryo culture and antisense oligonucleotide treatment
Fertilized White Leghorn chicken eggs were incubated at 38°C in a
humidified incubator with the method of Strauss (1998). Restriction-enzyme-digested genomic DNA fragments were separated by 0.8% agarose gel electrophoresis and transferred to
nylon membranes (Schleicher & Schull, Keene, NH). Mouse cDNA
probe 5a (nt 1,441-1,806) and chick cDNA probe 3 (nt 1,977-2,649)
were used for Southern hybridization under both low- and high-
stringency conditions as described above for northern blot.

Western blot analysis was performed as described previously (Warren et al., 1995), except that horseradish peroxidase-conjugated
secondary antibodies and ECL western blotting detection reagents
were used for immunoblot analysis.

Polycystic kidney disease
Nineteen aniridia patients (aged 6 to 56 years) with a diagnosis of
polycystic kidney disease were included in the study. The
mechanisms of the disease in these patients were examined using
cDNA microarray analysis and RT-PCR.

Immunochemistry
Immunofluorescence microscopy and Western blot
analysis
Double-label immunofluorescence microscopy on frozen sections of
adult mouse heart was carried out as previously described (Hegmann et al., 1989). The primary antibodies used included monoclonal
antibodies, CT3 anti-cardiac Troponin T (Warren and Lin, 1993), CH-
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described below.
RESULTS

Cloning and characterization of Xin cDNAs

A novel cardiac cDNA, referred to as 21C, was initially cloned using differential mRNA display to identify genes expressed differentially in stage 15 or stage 21 atrioventricular canal regions of embryonic chick hearts. In situ hybridization revealed that the 21C transcript was present specifically in early developing hearts (Wang et al., 1996). Using 21C as a probe to screen embryonic chick heart cDNA libraries, several overlapping clones were obtained. However, 5'-RACE cloning (Frohman et al., 1988) was required to obtain the most 5'-end of the message. A total sequence of 8,814 bp was obtained from sequencing multiple overlapping cDNA clones. The corresponding gene, named cXin (GenBank accession number AF051944), has a single open reading frame (ORF) starting with a conserved Kozak (1987) translation initiation codon and encoding a putative protein of 2,562 amino acids. The lengths of the 5' and 3' untranslated regions (UTR) that we obtained are 337 bp and 789 bp, respectively.

In an effort to identify the mammalian homologue of the chick Xin gene, we searched EST databases. These searches revealed a mouse clone with homology to the carboxy-terminal region of the predicted cXin peptide sequences. Using this cDNA fragment as a probe to screen an adult mouse skeletal muscle cDNA library (mXin is also expressed in adult skeletal muscle as revealed by northern blot hybridization, see Fig. 8B), a total of ~4.5 kb cDNA sequences were obtained. A 5'-RACE cloning approach was then used to obtain the remaining ~1.3 kb for the most 5'-end of the mXin message. Mouse mXin cDNA has 5,800 bp (GenBank accession number AF051945) with a single ORF encoding a novel putative protein of 1,677 amino acids. A UGA stop codon was found 6 bp upstream of the AUG translation initiation site in-frame with the predicted ORF. The 5' and 3' UTRs of the mXin cDNA that we obtained are 140 bp and 625 bp, respectively.

Comparison of the predicted amino acid sequences from mXin and cXin cDNA sequences reveals very similar organization of the putative domains (Fig. 1A). Putative nuclear localization signals (NLS) are found in both cXin and mXin proteins (LRKKP, aa 1,800-1,804 in the cXin protein; RRKKP, aa 1,019-1,022 and PRKKP, aa 1,185-1,189 in the mXin protein) (Fig. 1A). A potential DNA-binding domain (DBD) which is very similar to that found in oncogenes Myb-A and Myb-B. 35.7% identity and 85.7% similarity are found between the DNA-binding domains of these two gene products. + represents residue with similarity. (C) Comparisons of putative SH3-binding domains of Xin proteins with that found in human phosphatidylinositol 3-kinase (PI3K). Amino acid residues with identity are linked with a vertical line while amino acid residues with similarity are indicated by a colon (:).

(A) Schematic diagrams of cXin and mXin proteins with putative domains indicated. (B) Comparisons of the putative DNA-binding domains in the Xin proteins with that found in oncogenes Myb-A and Myb-B. 35.7% identity and 85.7% similarity are found between the DNA-binding domains of these two gene products.

Fig. 1. Comparisons of conserved domains of predicted cXin and mXin proteins. (A) Schematic diagrams of cXin and mXin proteins with putative domains indicated. (B) Comparisons of the putative DNA-binding domains in the Xin proteins with that found in oncogenes Myb-A and Myb-B. 35.7% identity and 85.7% similarity are found between the DNA-binding domains of these two gene products.

(B) DNA Binding Domains

<table>
<thead>
<tr>
<th>63 KRLYRHMHPELRKN 76</th>
<th>cXin</th>
</tr>
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<tbody>
<tr>
<td>54 RRLYKHIHPELRKN 67</td>
<td>mXin</td>
</tr>
<tr>
<td>ERWHNLNPEVKKS</td>
<td>Myb–A&amp;B</td>
</tr>
</tbody>
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(C) SH3-binding Motif

<table>
<thead>
<tr>
<th>1788 PKIPPKPEI 1796</th>
<th>cXin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1804 PVPPFPKEH 1812</td>
<td>cXin</td>
</tr>
<tr>
<td>1189 PPVFPPKAL 1197</td>
<td>mXin</td>
</tr>
<tr>
<td>303 PALPPPKPK 313</td>
<td>Human PI3K</td>
</tr>
</tbody>
</table>

signaling proteins contain SH3-binding motifs, which mediate protein-protein interactions (Yu et al., 1994). Thus, an additional possibility for Xin function is an involvement in signal transduction through protein-protein interaction. A 16-aa repeat unit is found in both predicted Xin proteins: 25 repeats in the cXin (Fig. 2A), and 13 repeats in the mXin (Fig. 2B). Fig 2C shows the consensus sequence for the 16-aa repeat unit. Although the role of this repeat unit remains unknown, the evolutionary conservation suggests it may provide an important role in Xin function.

Primer extension assays were used to determine whether Xin cDNA sequences extended to the 5'-end of the transcripts. When total RNA isolated from day 20 embryonic chick hearts was incubated with a cXin-specific antisense primer (21CP31) (nt 46-65 of the cloned cXin cDNA sequence), then extended with reverse transcriptase and analyzed on a denaturing polyacrylamide gel, a band of 155 bp was detected, representing the 5'-end of the cXin mRNA (nt 46-65) (Fig. 3A). This result suggests that the cXin cDNA sequence that we have obtained lacks the first 90 nucleotides at the 5'-end. On the contrary, two mXin-specific antisense primers at nt 60-79 and
nt 89-109 give rise to extension products of 79 and 109 bp, respectively (Fig. 3B), indicating that the 5′ end of mXin cDNA sequence corresponds to a transcription initiation site.

**mXin may be a mouse homologue of chick cXin**

To investigate whether mXin might be a mouse homologue of cXin, northern and Southern analyses were performed. The sequence regions with higher homology between cXin and mXin proteins were chosen for probes. When northern blots of adult mouse heart poly(A)+ RNAs were probed with either mouse probe 5a (nt 1,441-1,806) or chick probe 3 (nt 1,977-2,649) and washed under high stringency (lane A in Fig. 4, detected at 36-hour exposure) or low stringency (lane B in Fig. 4, detected at 3-hour exposure), respectively, a major transcript of about 5.8 kb was detected by both probes. There were also some minor bands detected by the chick probe. However, these bands disappeared when a moderate stringency for washing (at 45-48°C) was utilized. These results suggest that mXin is the closest homologue of cXin. However, we cannot completely rule out the possibility that the true homologue is the other minor band, since it can be expressed at a lower level. When Southern blots of mouse genomic DNAs digested with BamHI, EcoRI, HindIII or KpnI were hybridized with mouse probe 5a under high- (Fig. 5A) and low- (Fig. 5B) stringency conditions, a single genomic fragment from each restriction enzyme digest was detected. These results suggest that mXin may be a single gene in mouse. Similar Southern blot experiments with chick genomic DNA digested with BamHI, EcoRI or HindIII, hybridized with chick probe 3 (Fig. 5C,D) or mouse probe 5a (Fig. 5E) under high (Fig. 5C) or low stringency (Fig. 5D,E) also revealed a single DNA band from each restriction enzyme digest. These results indicate that mXin and cXin are closely related homologues. We could detect no evidence for paralogous genes even when hybridization was performed at low stringency. Therefore, it is likely that both mXin and cXin are orthologues.
Developmental expression pattern of Xin

Whole-mount in situ hybridization analyses of various staged embryos were carried out to determine Xin expression patterns. cXin transcripts were first detected at embryonic stage 8 (4 somites) in the paired lateral plate mesoderm that forms the primordia of the heart (Fig. 6A). There was no detectable expression of cXin in stage 7 embryos (data not shown). At stage 9 (7 somites), cXin expression was increased substantially in the heart-forming fields, which have migrated anteriorly and ventrally toward the midline of the embryo (Fig. 6B). As development proceeds, the bilateral heart-forming fields fuse at the midline to form a linear heart tube (Fig. 6C). At stage 10 (10 somites), although heart looping has not yet begun, the first evidence of regional variation in cXin expression was detected, with stronger staining in the caudal sinus venosus region, and on the sides, and relatively little staining in the medial part of the heart tube (Fig. 6C). When the heart tube begins to loop to the right at stage 11 (13 somites), an uneven expression of cXin was more pronounced, with stronger expression in the lateral parts and the developing outflow and sinus venosus of the heart tube than in the medial portions of the heart tube (Fig. 6D).

To further confirm the regional expression of cXin, embryos at various stages were paraffin-embedded and cross-sectioned following whole-mount in situ hybridization. At stage 8, cXin expression was found to be symmetric in the paired heart-forming fields (Wang et al., 1996; and Fig. 6E). At stages 10 and 11, the lateral regions of the developing heart tube had more cXin expression than the ventral parts (Fig. 6F,G, respectively). An asymmetric expression of cXin was observed at stage 13 with stronger expression in the right side (or inner curvature) of the looping heart (Fig. 6H,I). The regionally restricted expression of cXin prior to and during looping suggests that cXin may be involved in the heart looping process.

Expression of Xin in developing somites and skeletal muscle

Although cXin is not expressed in the somatic mesoderm of stage 8 chick embryos (Fig. 6E), somite expression begins to be detected at stage 15 (somite 24) (Fig. 7A). At this stage, cXin messages are only detected in the rostral-most somites (arrowhead in Fig. 7A) and are barely seen in the caudal somites. This expression sequence appears to correlate with somite maturation and myogenesis, suggesting that cXin may play a role in somite maturation. Transverse sections of stage 15 embryos after whole-mount in situ hybridization revealed that cXin messages were localized to the newly formed myotomes (arrowheads in Fig. 7D), but not the dermatome or sclerotome. By stage 21, expression of cXin in both developing heart and somites was greatly increased. Within the developing somites cXin messages were preferentially found in the dorsal edge of the myotome (Fig. 7B,C,E). cXin expression in developing somites is very similar to that of MEF2C (Edmondson et al., 1994). Somite expression of mXin can also be seen in day 13 postcoitus (p.c.) mouse embryos (data not shown).

Northern blot analysis of total RNA isolated from various tissues of embryonic day 20 chick was performed to examine the tissue distribution of cXin mRNA. As shown in Fig. 8A, cXin transcripts are present in heart, skeletal muscle and tongue, but not in brain, intestine, liver, lung and stomach. Similarly, a single 5.8 kb message was detected from heart and skeletal muscle (Fig. 8B). mXin was not expressed in adult mouse brain, spleen, lung, liver, kidney or testis (Fig. 8B).

mXin protein is colocalized with N-cadherin and connexin-43 at intercalated discs of adult heart

Immunofluorescence microscopy performed on frozen sections...
**Fig. 6.** Expression of cXin in embryonic chick hearts. Expression analysis of cXin in chick embryos using whole-mount in situ hybridization with a digoxigenin-labeled riboprobe.  
(A-D) Ventral view of embryos. (E-H) Paraffin-embedded and transversely sectioned embryos after whole-mount in situ hybridization. The lines in A, C, D and I represent the transversely sectioned plane shown in E-H, respectively. (A) Stage 8 embryo. The cXin transcript is detected in a pair of heart-forming fields (arrowheads). (B) Stage 9 embryo. The cXin expression is markedly increased in the heart-forming fields (arrowheads). However, the expression appears to remain symmetrical between the two heart-forming fields. (C) Stage 10 embryo. Although looping has not begun, cXin expression begins to show regional variations in the fusing heart tube. The lateral (right and left) sides of the heart tube appear to have much higher amounts of cXin messages than the medial region. (D) Stage 11 embryo. The heart tube has begun to loop to the right. The expression of cXin is throughout the developing heart tube, but with stronger expression in the lateral sides as well as at the posterior and anterior ends of the heart tube. (E) Transverse section of a stage 8 embryo shows the cXin transcript symmetrically present in precardiogenic mesoderm (pm), but not present in somatic mesoderm (sm). (F) Before looping at stage 10, the expression of cXin is stronger in the myocardium (mc) of both lateral sides of the heart tube than in the ventromedial region of the heart tube. This difference in staining may be due to the difference in numbers of cell layers in these regions. The cXin transcript is not detected in the endocardium. (G) At stage 11, the asymmetric expression of cXin is more obvious, with stronger expression in both lateral sides than in the medial portion. Also, an asymmetry between the right (arrowhead) and left sides of the heart tube begins to be detected. This was further confirmed by examining the expression patterns in serial sections. (H) At stage 13, the cardiac looping is well-defined and cXin is expressed at a higher level in the right side (inner curvature) of the looping heart (arrowhead), as compared to that in the left side (outer curvature) of the heart tube. (I) Schematic sketch of the stage 13 embryo used for the section shown in H. Bars in A-D, 280 μm; in E-H, 90 μm.
of adult mouse heart with anti-mXin antibody revealed a banding pattern of mXin localization, possibly between two cardiomyocytes in structures known as intercalated discs. To further identify this Xin-containing structure, we performed double-label immunofluorescence microscopy with anti-Xin (U1013) antibody and with anti-connexin-43 (MAB3068), anti-N-cadherin (CH-19), or anti-cardiac troponin T (CT3) antibody. The majority of bands stained with anti-Xin antibody are colocalized with a gap junction protein, connexin-43 (Cn-43), detected by connexin-43 antibody (about 80% colocalization, Fig. 9A) and with a Ca\textsuperscript{2+}-dependent adhesion molecule, N-cadherin, detected by N-cadherin antibody (more than 95% colocalization, Fig. 9C). Both connexin-43 and N-cadherin are known to locate in the intercalated discs of adult cardiac muscle (Beyer et al., 1989; Ruangvoravat and Lo, 1992; Soler and Kundsen, 1994). In Fig. 9E, cardiac troponin T molecules detected by CT3 antibody are distributed in the sarcomere-containing myofibrils throughout the whole cell, except at sites where mXin is located (Fig. 9F). These results suggest that the majority of mXin in adult cardiac muscle is colocalized with both connexin-43 and N-cadherin at the intercalated discs.

**Inhibition of cXin function by antisense oligonucleotides disturbs cardiac morphogenesis**

To investigate the function of Xin in cardiac morphogenesis, we treated cultured stage 6 embryos with antisense oligonucleotide, as described (Issac et al., 1997; Srivastava et al., 1995). Antisense oligonucleotides were chosen from regions exhibiting high homology in DNA sequence between mXin and cXin. To ensure that the chosen oligonucleotides did not inhibit other unexpected genes with sequence similarity, their sequences were first compared against the GenBank database. No homologues were found. Oligonucleotides were synthesized as phosphorothioate derivatives to prevent degradation by endogenous nucleases and to increase uptake by cultured embryos (Akhtar and Juliano, 1992; Wagner 1994; Matteucci and Wagner, 1996). Random oligonucleotides with the same base composition as the antisense oligonucleotides were used as controls.

In the presence of antisense oligonucleotides, most embryos appeared to develop normally up to stage 9, when the hearts started to fuse in the midline. The heart fusion process seemed to be slightly retarded in antisense-treated embryos relative to random oligonucleotide-treated embryos. However, the majority of treated embryos eventually completed heart fusion.
and formed a linear heart tube. By 20 hours in culture (equivalent to stage 10), hearts of both antisense and random oligonucleotide-treated embryos started to beat. After another 4 hours in culture (equivalent to stage 10*-11), the tubular hearts began to show rightward looping. By 28 hours in culture (equivalent to stage 11-12), 80-82.1% of control (medium alone and random oligonucleotide treated) embryos had normal, rightward looping hearts, whereas only 34.8% and 51.9% of embryos treated with antisense 3 and antisense 2, respectively, underwent normal looping (Table 1). Conversely, there were significant increases in the percentages (47.8% and 29.6%) of antisense-treated embryos, which exhibited abnormal looping (Table 1). Abnormal looping hearts appeared as a medially situated block of tissue in the embryos, as described in Easton et al. (1992). Similar results were obtained with cultured embryos treated simultaneously with both antisense 2 and antisense 3 (Table 1). These data are statistically significant with P<0.01 and P<0.001, respectively. Of these affected embryos, about 30% developed pericardial edema and died at this stage of development. Of those surviving embryos, their hearts continued to grow as bulged tubes that remained in or were slightly to the left or right of the midline of the embryos (for examples, Figs 10B,F, 11B). In contrast, the hearts in control embryos developed into normal looped, S-shaped heart tubes (for examples, Figs 10A,E, 11C). In addition to the looping defect, the affected hearts also exhibited abnormal beating behavior such as non-rhythmic beating, and beating more slowly and less forcefully than normal hearts. The decrease in cXin expression in antisense-treated embryos is better demonstrated by dark-field microscopy (Fig. 10E,F) than by bright-field microscopy (Fig. 10A,B) of embryos after whole-mount in situ hybridization.

To further examine structural changes in affected hearts, embryos were embedded and sectioned following whole-mount in situ hybridization. The myocardium of the affected hearts became thickened and tended to form multiple invaginations into the heart cavity (Fig. 10D), as compared with the smooth and fully extended layer of myocardium in control hearts (Fig. 10C). Asymmetric expression of cXin in the control heart is also evident. This abnormal cellular process in the antisense oligonucleotide-treated embryo may account in part for the failure of the heart to loop. It should be noted,

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total</th>
<th>Rightward looping heart (%)</th>
<th>Leftward looping heart (%)</th>
<th>Abnormally looping heart (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium alone</td>
<td>28</td>
<td>23 (82.1)</td>
<td>4 (14.3)</td>
<td>1 (3.6)</td>
</tr>
<tr>
<td>Random oligo</td>
<td>80</td>
<td>64 (80.0)</td>
<td>10 (12.5)</td>
<td>6 (7.5)</td>
</tr>
<tr>
<td>Antisense 2</td>
<td>27</td>
<td>14 (51.9)**</td>
<td>5 (18.5)*</td>
<td>8 (29.6)**</td>
</tr>
<tr>
<td>Antisense 3</td>
<td>23</td>
<td>8 (34.8)*****</td>
<td>4 (17.4)*</td>
<td>11 (47.8)*****</td>
</tr>
<tr>
<td>Antisense 2 and 3</td>
<td>128</td>
<td>56 (43.8)*****</td>
<td>20 (15.6)*</td>
<td>52 (40.6)*****</td>
</tr>
</tbody>
</table>

*Statistically not significant (P>0.10) as compared to controls (medium alone or random oligo treated).

**Statistically significant (P<0.01) as compared to controls (medium alone or random oligo treated).

***Statistically significant (P<0.001) as compared to controls (medium alone or random oligo treated).

Fig. 9. Double-label immunofluorescence microscopy on frozen sections of adult mouse hearts. Sections were first incubated with an antibody mixture of rabbit polyclonal antibody against mXin and either one of mouse monoclonal antibodies against connexin-43 (Cn-43) (A,B), N-cadherin (C,D), or cardiac troponin T (cTnT) (E,F) and subsequently with a mixture of rhodamine-conjugated goat anti-rabbit IgG and fluorescein-conjugated goat anti-mouse IgG. (A,C,E) Sections viewed selectively for fluorescein fluorescence to allow the mouse antibody to be visualized. (B,D,F) Same fields seen in A, C and E, respectively, viewed selectively for rhodamine fluorescence to visualize the distribution of mXin. Arrowheads in A-D represent the regions where mXin is not colocalized with connexin-43 or N-cadherin. The arrows in E and F indicate the positions of mXin protein. Bar, 10 μm.
however, that the overall morphology of affected embryos appeared to be normal.

A slight increase in percentages of antisense-treated embryos having leftward looping over controls was observed (Table 1). However, it was not statistically significant ($P > 0.10$). RT-PCR of embryos with leftward looping hearts (Fig. 11D) from the control oligonucleotide-treated group revealed no significant decrease in expression of cXin (lane 4 in Fig. 11A). Similarly, there appeared to be no significant decrease in expression of cXin in those antisense oligonucleotide-treated embryos that have rightward looping hearts (lane 2 in Fig. 11A) or leftward looping hearts (data not shown), indicating that inhibition by antisense oligonucleotides was not completely penetrant. Expression of cXin was greatly reduced in antisense-treated embryos exhibiting abnormal looping (lane 1 in Fig. 11A), as compared to that in control embryos (lane 3 in Fig. 11A).

**cXin expression is induced by BMP**

Tongue expression of Xin in both chick and mouse, as revealed by in situ hybridization of developing embryos (data not shown) and northern blots of various tissues (Fig. 10A), was similar to that reported for Nkx2.5 (Lints et al., 1993). Furthermore, both targeted deletion of the Nkx2.5 gene in mice (Lyons et al., 1995) and antisense inhibition of the cXin gene in chick (this study) resulted in embryos with abortive cardiac morphogenesis and abnormal cardiac looping. Therefore, it was of interest to see whether Nkx2.5 and Xin are in the same regulatory pathway.

It has been previously reported that Nkx2.5 expression can be induced by BMP-2 in the anterior medial mesoendoderm of stage 6 chick embryos, a tissue that normally does not express cardiac muscle genes (Schultheiss et al., 1997). Thus, we used this explant system to examine whether cXin would also be induced. As shown in Fig. 12, as observed for Nkx2.5 and MEF2C, cXin expression was strongly induced by BMP-2 treatment of anterior medial mesoendoderm explants. The induction of cXin followed activation of Nkx2.5 and MEF2C, but preceded expression of the vMHC (Fig. 12). These results, together with similar tissue expression patterns in early embryos for Nkx2.5, MEF2C and cXin, as well as similar effects on cardiac morphogenesis observed in embryos after Nkx2.5 or MEF2C deletion or cXin antisense treatment, suggest that cXin may participate in a BMP-Nkx2.5-MEF2C pathway to affect cardiac morphogenesis.

**MEF2C or Nkx2.5 alone is capable of transactivating the mXin promoter**

From sequence analysis of mXin genomic clones, we identified potential consensus binding sites (−286 TCAAGTG −280 and −106 CTAAAATAAC −96) for the-bindings of Nkx2.5 (Chen and Schwartz, 1995) and MEF2C (Gossett et al., 1989), respectively, within the promoter region of the mXin gene. To determine if the Xin gene was a potential target of Nkx2.5 and/or MEF2C, transactivation experiments were carried out by cotransfection of mouse fibroblast C3H10T1/2 cells with a mXin-luc reporter plasmid and various amounts of either MEF2C or Nkx2.5 expression plasmids. As a control, cells were cotransfected with plasmid containing the mXin-luc reporter and various amounts of parental expression vector pCDNA1.1. To normalize variations in transfection efficiencies, all samples were cotransfected with a plasmid containing Renilla luciferase driven by the thymidine kinase promoter. The resulting activities of firefly and Renilla

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**Fig. 10.** Inhibition of heart looping in chick embryos treated with cXin antisense oligonucleotides. Transcripts of cXin were detected by in situ hybridization in embryos exposed to random oligonucleotides (A,C,E) and cXin antisense oligonucleotides (B,D,F). (A,E) Control chick embryos treated with random oligonucleotides develop normally in culture and have a normal rightward-looping, S-shaped heart and a normal cXin expression. (B) Chick embryos treated with cXin antisense oligonucleotides develop normally in culture and have a normal rightward-looping, S-shaped heart and a normal cXin expression pattern similar to that of stage 12 embryo in ovo. (B,F) Chick embryos treated with cXin antisense oligonucleotides exhibit abnormal heart looping and a significant reduction in the cXin expression. (C) A transverse section of the embryo from A shows a heart tube with a very smooth myocardial layer and normal, asymmetric expression of cXin. (D) A transverse section of the embryo from B reveals that the myocardial layer has formed many invaginations into the cardiac cavity. Also, there is a great decrease in the expression of cXin in this antisense-treated embryo. (A-D) Bright-field micrographs; (E,F) dark-field micrographs. Bar, 280 μm for A,B,E, F or 90 μm for C,D.
Xin gene in cardiac looping

Luciferase in each transfection were measured and the ratio of their activities (relative luciferase activity) was used as an indication of the expression level. As shown in Fig. 13, expressed MEF2C or Nkx2.5 protein activated the Xin promoter about 10- or 3-fold, respectively, more than activation observed with the parental expression vector alone. These results suggest that Xin lies downstream of MEF2C and Nkx2.5 in a cardiogenic pathway.

DISCUSSION

Xin encodes a novel proline-rich protein

In the present study, we have cloned cDNAs for novel Xin genes from chick and mouse. Predicted amino acid sequences derived from both cXin and mXin cDNAs reveal that Xin proteins contain a nuclear localization signal, a DNA-binding domain, a SH3-binding motif and a 16 amino acid repeat unit. Although there are some differences between cXin and mXin, including predicted protein size, the number of nuclear localization signals, SH3-binding motifs, 16 amino acid repeats and proline-rich regions, these motifs and their overall location in both cXin and mXin proteins are very conserved. Results from northern and Southern blot analyses under both low- and high-stringency conditions for hybridization and washing suggest that mXin is likely a true mouse homologue of chick cXin. This is further supported by the fact that polyclonal antibody raised against mouse mXin fragments also recognized cXin protein from adult chick heart (data not shown). The overall amino acid sequence similarity between cXin and mXin proteins is 46%, and in certain regions (aa 150-738 of mXin) it can be as high as 70%. Furthermore, results of both northern and Southern blot analyses suggest that Xin is likely represented by a single gene in both chick and mouse.

Xin function and heart looping

The presence of both a nuclear localization signal and a DNA-binding motif suggests that the Xin protein may function as a transcriptional regulator in controlling cardiac and skeletal muscle differentiation. The proline-rich domains found in Xin may further support this function, since similar proline-rich domains acting as transactivation domains have been identified in a class of known transcription factors, such as CTF/NF-1 (Mermod et al., 1989), AP-2 (Williams and Tjian, 1991), MNF (myocyte nuclear factor) (Bassel-Duby et al., 1994) and skNAC (muscle-specific isoform of α-nascent polypeptide-associated complex, α-NAC) (Yotov and St-Arnaud, 1996). Using immunofluorescence microscopy on differentiated C2C12 myotubes with a polyclonal antibody against mXin, we have observed a speckled staining pattern at adhesion plaques, diffuse staining near membrane cortex and a few stainings associated with stress fibers. In addition, a trace amount of staining was found in nuclei of C2C12 myotubes (data not shown). Similarly, we have detected a major localization of

![Fig. 11. RT-PCR analysis of cXin gene expression (A) and morphological analysis (B-D) in both anti-sense and random oligonucleotide-treated embryos. Following treatments, embryos were scored for hearts with leftward, rightward or abnormal looping. The morphology of an embryo from each category is shown in B for abnormal looping, C for rightward looping and D for leftward looping. Some of these embryos were subjected to RNA isolation and RT-PCR to quantify expression of cXin and GAPDH. Lanes 1 and 2, antisense oligonucleotide-treated embryos with abnormal and rightward looping hearts, respectively. Lanes 3 and 4, random oligonucleotide-treated embryos with rightward and leftward looping hearts, respectively.](image)

![Fig. 12. Induction of cardiac gene expression by BMP2. Cultures of chick anterior medial mesoendoderm were cultured for the indicated length of time in the presence (+) or absence (−) of 200 ng/ml of human recombinant BMP-2 and RT-PCR analysis for expression of the indicated genes was performed as described (Schultheiss et al., 1997). Cultures were also observed for the presence of beating cardiac myocytes. Nkx2.5 is induced strongly within 6 hours of treatment. At 12 hours, MEF2C is also strongly expressed, while cXin is weakly expressed, suggesting that MEF2C expression begins somewhat earlier than that of cXin. By 24 hours of treatment, the cXin expression levels have increased dramatically, the myofibrillar gene Ventricular Myosin Heavy Chain (vMHC) has begun to be expressed and the cultures contain beating areas.](image)
mXin at cell-cell contacts and a minor localization of mXin in nuclei of cultured neonatal cardiomyocytes (data not shown). In adult mouse heart, we have shown that the majority of Xin appears to be colocalized with N-cadherin and connexin-43 at the intercalated discs (Fig. 9). During heart development, mXin is more closely associated with N-cadherin than with connexin-43 (data not shown), although there is a lot more mXin than N-cadherin in embryonic hearts. Given the fact that SH3-binding motifs are also found in Xin, membrane cortex, focal adhesion and intercalated disc localizations of mXin may further suggest a signal transduction role for Xin. For example, Xin might function as an intracellular signaling molecule in an N-cadherin signaling pathway by interacting with SH3-containing proteins. Therefore, based on DNA sequence information and our preliminary results from localization studies, it is possible that Xin may perform both transcriptional and signal transduction roles in myogenesis/cardiac morphogenesis. Under certain signaling conditions, Xin might migrate into the nucleus and act as a transcriptional regulator. There is precedent for this type of dual roles for an adherens junction-associated protein, α-catenin. β-catenin exhibits homology to plakoglobin, a component of desmosomal plaques, and to the product of the Drosophila segment polarity gene, Armadillo. It is known that β-catenin interacts with cadherin and α-catenin to form a cadherin-catenin complex, which is essential for cadherins to function as adhesion molecules in adherens junctions (Ben-Ze’ev and Geiger, 1998; Barth et al., 1997; Huber et al., 1996). In addition, α-catenin is homologous to an adhesion plaque molecule, vinculin, and is a candidate for linking the cadherin-catenin complex to the actin cytoskeleton. Like Armadillo, β-catenin can migrate into the nucleus in response to Wnt/wingless signals and this nuclear accumulation appears to be essential for β-catenin to function in determining cell fates (Larabell et al., 1997; Behrens et al., 1996; Molenaar et al., 1996; Orsulic and Peifer, 1996). Therefore, it is possible that muscle-specific Xin may play similar dual roles in cardiac morphogenesis.

Our results from antisense oligonucleotide experiments suggest that Xin may be required for cardiac morphogenesis. Significantly, the myocardia of affected hearts are often seen with multiple invaginations or folds into the cardiac cavity (Fig. 10D). This observation may provide a valuable clue to underlying mechanisms that explain why the developing heart failed to loop to the right. The Xin protein may play a role in regulating the dynamics and integrity of the cytoskeleton of the cardiomyocytes. Thus, the myocardium in the cXin-deficient embryos could not maintain normal cell shape and cell-cell interactions, leading to the abnormal looping. In support of this possibility, we have also found that hearts of cXin-deficient embryos beat slowly, less forcefully and arhythmically. Since embryo culture and antisense experiment can only be performed up to stages 12-13, it is possible that Xin may have other functions during the late stages of development and in the adult heart. In preliminary RNase protection assays, we found that there were two peaks of maximal level of cXin mRNA expression during chick heart development. One peak is at stage 21-24, which is believed to be a critical period for the formation of endocardial cushions. The other peak is at embryonic day 12-14.

**Signaling pathway for vertebrate cardiac morphogenesis**

Recent gene targeting and antisense oligonucleotide inhibition experiments have demonstrated that Nkx2.5, MEF2C, dHand and eHand are involved in cardiac morphogenesis and/or cardiac segmentation (Lyons et al., 1995; Lin et al., 1997;
Srivistava et al., 1995, 1997; Firulli et al., 1998). Our BMP-2 induction experiments clearly link the Xin gene to the cardiogenic pathway. Induction of cXin by BMP-2 was observed following induction of the homeodomain-containing transcription factor Nkx2.5 and the expression of the muscle-specific transcription factor MEF2C, but preceded the expression of the contractile protein gene vMHC. Moreover, cotransfection experiments shown in Fig. 13 demonstrated that Xin is a direct or indirect target for both MEF2C and Nkx2.5. Recently, it has been reported that dominant mutations in Nkx2.5 cause nonsyndromic human congenital heart defects (Schott et al., 1998). Since Xin appears to be lie downstream of Nkx2.5, it would be of interest to examine whether there is an association between Xin mutation and congenital heart disease.

At present, there is no direct evidence to show that BMP-2 is involved in cXin asymmetric localization during cardiac morphogenesis. However, the abundant expression of BMP-2 in the myocardium of early mouse embryos suggests that BMP-2 may play an important role in cardiac morphogenesis (Zhang and Bradley, 1996). Furthermore, mice deficient for BMP-2 die between day 7.0 and 10.5 of gestation. In addition to the malformation of the amnion/chorion, BMP-2-deficient embryos also show a defect in cardiac development. There is either no heart formation or hearts develop at abnormal sites and are arrested at the linear heart tube stage (Zhang and Bradley, 1996). Furthermore, the expression of Nkx2.5 is not detected in those mutants with no obvious heart formation, suggesting that Nkx2.5 may lie downstream of BMP-2. Similarly, it has been demonstrated that, in Drosophila, the Drosophila homologue of Nkx2.5, is regulated directly by dpp, one of TGFβ superfamily, signaling pathway (Xu et al., 1998). Recent mutational analysis and embryo injection with a dominant negative BMP-4 receptor in zebrafish have concluded that the asymmetric expression of cardiac BMP-4 may provide lateralizing signals for cardiac jogging and looping (Chen et al., 1997). Thus, BMP-4 or related molecules may be involved in one of several redundant pathways that control cardiac looping. It would be of interest to determine whether the expression of Xin is downregulated in the Nkx2.5 or MEF2C knockout mice and whether overexpression of Xin can rescue the morphogenetic defect in these knockout mice.

We thank Eric Olson for critical reading of the manuscript, David Bader for providing chick embryonic cDNA library, Dan Weeks for antisense oligonucleotide synthesis, Debashish Bhattacharya for analyzing the 16-amino acid repeat in the Xin protein and Janice Sistes for helping with the statistical analyses. BMP-2 was kindly supplied by Genetics Institute. T. M. S. is a Physician Postdoctoral Fellow of the Howard Hughes Medical Institute. This work was supported, in part, by a SCOR in Congenital Heart Disease grant HL42266 and a senior NRSA grant HL09464.

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