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Tbx5 is essential for heart development

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SUMMARY

Mutations in the *Tbx5* transcription factor cause heart septal defects found in human Holt-Oram Syndrome. The complete extent to which *Tbx5* functions in heart development, however, has not been established. Here we show that, in *Xenopus* embryos, *Tbx5* is expressed in the early heart field, posterior to the cardiac homeobox transcription factor, *Nkx2.5*. During morphogenesis, *Tbx5* is expressed throughout the heart tube except the anterior portion, the bulbus cordis. When *Tbx5* activity is antagonized with a hormone-inducible, dominant negative version of the protein, the heart fails to develop. These results suggest that, in addition to its function in heart septation, *Tbx5* has a more global role in cardiac specification and heart development in vertebrate embryos.

Key words: Heart, T-box, Tbx-5, Cardiac development, Organogenesis, Mesoderm, Xenopus, Holt-Oram syndrome

INTRODUCTION

The heart is one of the first organs to develop and function in vertebrate embryos. In general, cardiac tissue is specified by inductive signals in the early gastrula that act on precardiac mesoderm as it migrates into the heart field, located at anterior-lateral positions on both sides of the embryo. These nascent cardiac tissues then migrate toward the ventral midline of the body where they fuse to form the heart tube. Rythmic contractions of the heart tube begin soon after its formation, followed by looping and septation of the tube as it completes morphogenesis (DeHaan, 1965; Fishman and Chien, 1997; Jacobson, 1961; Wilens, 1955).

In *Xenopus* embryos, the heart arises from the dorsolateral mesoderm adjacent to the Spemann Organizer (dorsal blastopore lip; Nieuwkoop and Faber stage 10.5; Keller, 1976). Cardiac tissue is specified during gastrulation within the dorsolateral mesoderm by inductive signals from the organizer and the deep dorsoanterior endoderm. Convergent-extension movements bring the paired, precardiac mesoderm into the anterior lateral plate. The bilateral cardiac primordia then migrate ventrally during neurula and early tadpole phases of development (stages 17-28) (Wilens, 1955). Heart tissue is fully specified within the anterior/lateral cardiac field by late neurulation as explants of these regions will form beating tissue when cultured in vitro (Sater and Jacobson, 1990). The migrating cardiac mesoderm fuses at the ventral midline in the stage 28 tailbud tadpole and, by stage 30, a linear heart tube forms consisting of the bulbus cordis, ventricle, atrium and sinus venosus, oriented from anterior to posterior. The heart tube begins beating by stage 35, after which heart septation and looping occur, separating the atrium into right and left halves and reorienting the atria to position them dorsal to the ventricle. In contrast to humans and other mammals, the *Xenopus* heart has only three chambers: two atria and one ventricle (de Graaf, 1957).

The molecular identity of inductive signals that specify the heart are not well understood, but various transcription factors that may regulate cardiac commitment and differentiation have been isolated, and some of these have been directly implicated in the control of cardiac differentiation. Several of these factors are expressed in the anterior lateral mesoderm at the proper time to play a role in heart development, including the Nkx and GATA gene families (Harvey, 1996; Heikinheimo et al., 1994; Jiang and Evans, 1996; Kelley et al., 1993; Laverriere et al., 1994; Morrisey et al., 1996). None of these factors individually have been shown to be sufficient to specify cardiac differentiation or required in the embryo for heart tube formation. Data from overexpression studies and loss-of-function mutations in frogs, zebrafish, mice and humans have demonstrated that *Nkx-2.5* can induce myocardial gene expression (Cleaver et al., 1996) and that it is necessary for the specification of the right ventricle, heart looping and atrial septation (Lyons et al., 1995; Schott et al., 1998). The GATA factors can induce cardiac gene expression when overexpressed in frog embryos (Jiang and Evans, 1996), and the ventral migration of precardiac mesoderm is disrupted in *GATA-4* mutant mice. These mutants do, however, form a pair of relatively normal hearts on their left and right sides, indicating that *GATA-4* is not essential for heart tube formation (Kuo et al., 1997; Molkentin et al., 1997). Other gene families implicated in heart development (Fishman and Olson, 1997; Lyons, 1996; Mohun and Sparrow, 1997) include bHLH factors, MEF2

genes, retinoic acid receptors and particular T-box genes (Basson et al., 1997; Chapman et al., 1996; Fishman and Olson, 1997; Gibson-Brown et al., 1998b; Lyons, 1996; Mohun and Sparrow, 1997).

T-box genes are a growing family of transcription factors that are expressed in diverse patterns throughout vertebrate development (Herrmann, 1995; Papaioannou and Silver, 1998; Smith, 1997). Since the founding member, *Brachyury (T)*, was shown to be necessary for proper mesoderm formation and notochord development (Herrmann, 1995), several other T-box genes, that are essential for normal embryonic growth have been isolated from frogs, zebrafish and mice (Chapman and Papaioannou, 1998; Horb and Thomsen, 1997; Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996). One of the first attempts to clone new members of the T-box gene family established that the mouse *Tbx1-5* genes are localized to distinct regions of the embryo involved in inductive interactions (Chapman et al., 1996). More recently, three groups have cloned the chick *Tbx2-5* genes and have examined the role of these genes in vertebrate limb development (Gibson-Brown et al., 1998a; Isaac et al., 1998; Ohuchi et al., 1998). Whereas *Tbx2* and *Tbx3* are expressed in overlapping patterns within the anterior and posterior portions of the forelimb and the hindlimb, *Tbx4* and *Tbx5* are localized to larger non-overlapping domains within the hindlimb and the forelimb, respectively. *Tbx5* has also been isolated from newt embryos and its expression is upregulated in the limb mesoderm during regeneration (Simon et al., 1997). The expression of *Tbx5* mRNA has been detected at late embryonic stages in the heart, forelimb and eye of both mouse and chick (Chapman et al., 1996; Gibson-Brown et al., 1998a,b; Isaac et al., 1998; Ohuchi et al., 1998), but a detailed analysis of *Tbx5* expression in these developing embryos has not been carried out. The clinical importance of T-box genes in embryonic development is illustrated by the recent link between mutations in human *Tbx3* and *Tbx5* and Ulnar-Mammary syndrome and Holt-Oram syndrome, respectively (Bamshad et al., 1997; Basson et al., 1997; Li et al., 1997b).

The identification of *Tbx5* mutations in patients afflicted with Holt-Oram Syndrome directly implicates this gene in heart development. Holt-Oram Syndrome (or HOS) is an autosomal dominant condition characterized by upper limb and cardiac defects (such as atrial or ventricular septal abnormalities) that vary in clinical magnitude from latent, and relatively benign, to life threatening (Basson et al., 1994; Holt and Oram, 1960; Hurst et al., 1991; Newbury-Ecob et al., 1996; Smith et al., 1979). Because of these defects, HOS is sometimes referred to as the Heart-Hand Syndrome, and it affects about 1 in 100,000 live births. All individuals studied thus far have been heterozygous for various mutant *Tbx5* genes, which suggests that the phenotypes described thus far result from a partial loss-of-function of *Tbx5*. The developmental consequences of a complete loss-of-function in Tbx5 however, have not been examined in any natural situation or experimental model organism.

We have turned to the amphibian *Xenopus laevis* to address details of *Tbx5* expression and function in vertebrate heart development. We have isolated a cDNA for *Xenopus Tbx5 (XTbx5)* and shown that the *XTbx5* gene is expressed exclusively in the developing heart and eye, which corresponds with the existing data on *Tbx5* expression in mouse, chick and human embryos. The cardiac expression of *XTbx5* begins within a subset of cardioblasts in the *Xenopus* heart field at neurula stages and continues within the heart during morphogenesis at later tadpole stages. Its expression pattern complements that of *XNkx2.5* at early stages, and partly overlaps with it as the heart tube forms. Within the heart tube, *Tbx5* is expressed in all but the most anterior domain, the bulbus cordis. At the functional level, we show that ectopic expression of a dominant negative, hormone-inducible XTbx5 protein in *Xenopus* embryos blocks heart tube formation nearly completely. The phenotypes of HOS patients originally suggested that Tbx-5 functions in septum formation, but our findings reveal that *Tbx5* participates in more global aspects of heart development.

MATERIALS AND METHODS

Isolation of *XTbx5*

Degenerate PCR primers used to amplify the T-box region of *Xenopus Tbx5* were as follows: GRRMFP, 5′-GG(ACGT) (CA)G(ACGT) (CA)G(ACGT) ATG TT(CT) CC-3′, and KADENN, 5′-(AG)TT (AG)TT (CT)TC (AG)TC (ACGT)GC (CT)TT-3′. First-strand cDNA, prepared by random priming of total embryonic stage 27 RNA, was used as the template in the PCR reaction to amplify the *XTbx5* T-box at an annealing temperature of 56°C. The 340 bp PCR product was subcloned into the *pCR-Script* vector (Stratagene) and sequenced to confirm that it encoded an insert that matched *Tbx5* sequences from other vertebrates. The cloned T-box region of *XTbx5* was then used to screen a stage 28 head cDNA library (R. Harland), from which two positive candidates were isolated. Sequences were obtained from the 5′ end of each clone, which revealed that both were most similar to human *Tbx5*, but that one of the clones *(XTbx5 #2)* contained a 15 bp insertion after the amino acid sequence FIAVTSYQ, which created a stop codon. The other clone, *XTbx5 #1*, was fully sequenced (Sequenase kit, US Biochemicals) and encodes a 520 a.a open reading frame that is similar to the length of one of the human Tbx5 proteins.

Nucleic acid constructs

To construct *XTbx5-EnR*, a *Hin*dIII-*Afl*III fragment containing the T-box of XTbx5 was cloned into the *Eco*RI (blunt) site of the *pCS2+* vector containing the *Drosophila* engrailed repressor domain (ENG-N; Gift of D. Kessler). To construct *XTbx5-En^R-GR*, the glucocorticoid receptor hormone-binding domain (GR) was amplified by PCR from *MyoD-GR* (gift of H. Sive) using the following primers: GR1 (*Xho*I) 5′-GGC GCC GCT CGA GCC CCT CTG AA-3′ and GR2 (*Xho*I) 5′-GGC GGG CAC TCG AGC ACT TTT GAT-3′. This PCR product was cut with *Xho*I and inserted into the *Xho*I site of *XTbx5-En^R-GR*. To construct *XTbx5-GR*, the *XTbx5* open reading frame (ORF) was amplified by PCR using the following primers: 5′ XTbx5, 5′-CAA TCC CTT GCC AGT GCC-3′ and XTbx5(XhoI), 5′-CTG ATT ACT CGA CGT AGG CAT-3′, and the product was subcloned into *pCS2+*. The GR hormone-binding domain was inserted into the *Xho*I site at the 3′ end of *XTbx5*. All constructs were sequenced at their cloning junctions to verify reading frame integrity and orientation. *XMLC2* was cloned into *pCR Script* after it was amplified from stage 28 embryonic cDNA using the following PCR primers: upstream (430-447) 5′-AGT GAC GAG GAG GTA GAC and downstream (879-860) 5′-CAA GTC GAT GAC TAA CTC CG. *XNkx-2.5* was a gift from Paul Krieg.

In situ hybridization

Whole-mount in situ hybridizations with single probes were

performed as described (Harland, 1991) and double-labeled in situ hybridizations were done according to the protocol published on the *Xenopus* Molecular Marker Resource web page (XMMR; http//:222vize.utexas.edu/), with slight modifications as follows: probes were labeled independently with either digoxigenin (for *XNkx-2.5* and *XMLC2*) or fluorescein (for *XTbx5*). The light-blue stain was developed first using BCIP (175 μg/ml) in alkaline phosphatase (AP) buffer at room temperature. Following sufficient color development, these embryos were washed 3×, 5 minutes each, in maleic acid buffer (MAB) at room temperature, and the AP enzyme was inactivated by incubating the embryos for 10 minutes in MAB + 0.1 M EDTA at 65°C. The embryos were then rinsed 3×, 5 minutes each at room temperature in MAB and incubated at room temperature for 1 hour in MAB + 2% Boehringer Mannheim Blocking reagent (BMB) containing 20% (vol/vol) goat serum. Secondary-alkaline-phosphatase-conjugated antibody (BM) was added and incubated overnight. Embryos were rinsed 5×, 1 hour each in MAB, with a final wash overnight, and the color reaction was developed using BM purple substrate (Boehringer Mannheim). This reaction was stopped by fixing the embryos in MEMFA. To clear the embryos after fixation they were dehydrated in methanol and mounted in 2:1 benzyl benzoate/benzyl alcohol (BBA) for photography. For sectioning, the embryos were embedded in Paraplast after whole-mount in situ hybridization and dehydration, and 14 μm sections were cut and mounted in Permount with no counterstain. Antisense in situ RNA probes were prepared from linearized templates using the appropriate RNA polymerase: *XTbx5* in *pBS SK* was linearized with *Bam*HI and transcribed with T7; *XMLC2* in *pCR-Script* was linearized with *Eco*RI and transcribed with T3; *XNkx-2.5* in *pGEM 3Z* was linearized with *Kpn*I and transcribed with T7.

Embryological assays

Synthetic mRNA transcripts were synthesized by SP6 in vitro transcription (mMessage machine; Ambion) of linearized *CS2+* templates containing relevant insert fragments. mRNAs were dissolved in water and injected into in vitro fertilized *Xenopus* embryos. Activation of glucocorticoid fusion proteins was achieved by incubation of embryos in 10 μM dexamethasone in 0.1× MMR. Staining of embryos for β-galactosidase activity was done using the Red-Gal substrate (Research Organic's) as described (Turner and Weintraub, 1994) prior to whole-mount in situ hybridization.

RESULTS

Isolation of *XTbx5*

A partial *Xenopus Tbx5* clone was isolated by degenerate PCR on embryonic cDNA using primers corresponding to peptide sequences that are identical in the DNA-binding (T) domains of human and mouse *Tbx5* (Fig. 1; Materials and methods). We screened a *Xenopus* stage 28 anterior library with this clone and isolated a 2973 bp cDNA that contains a 1560 bp open reading encoding a predicted protein of 520 amino acids. The T domain of this cDNA is 193 amino acids long and shares 98% identity with the T domain of hTbx5, but only 82% identity with that of mTbx4 and, because of this, we consider this cDNA an authentic *Xenopus* homologue of *Tbx5*. Alignment of the frog, human and chick Tbx5 proteins (Fig. 1A) reveals extensive amino acid identity both within and outside the T domain, with approximately 70% overall amino acid identity among all three proteins. Compared to other T domain proteins, the T domain of XTbx5 shares a high degree of amino acid identity ranging from 98% between frog and human Tbx5, to 49% between XTbx5 and Xbra (Fig. 1B).

XTbx5 is expressed in the migrating precardiac mesoderm

Although *Tbx5* has been isolated in mouse, chick and human, a detailed analysis of its expression pattern in development has not been published. Therefore, we decided to examine *XTbx5* expression in detail during early embryogenesis by whole-mount in situ hybridization. We found that *XTbx5* transcripts are first detected at mid-neurula, stage 17, in two lateral stripes on either side of the embryo, consistent with the location of the cardiac primordia (Fig. 2A arrow). By tailbud stage 20, expression of *XTbx5* within the lateral mesoderm increases and extends from just below the somites to the ventral midline (Fig. 2B); *XTbx5* mRNA is also expressed within a large domain in the eye (Fig. 2B). At tailbud tadpole stage 25, expression of *XTbx5* in the lateral mesoderm resembles a droplet, with a narrow band of expression at the dorsal side that broadens ventrally (Fig. 2C). *XTbx5* is not expressed in the most ventral region of the embryo (Fig. 2D) until stage 28 when its lateral expression domains fuse across the ventral midline (Fig. 2E,F,M). Histological cross-sections through this region confirm that *XTbx5* mRNA is localized to the mesodermal layer (Fig. 2O). By stage 30, most of *XTbx5*-expressing cells are located on the ventral side of the embryo where the heart tube is forming at this stage (Fig. 2F), while some lateral expression is detected along the posterior of the heart (Fig. 2E).

XTbx5 is expressed in most of the heart tube during morphogenesis

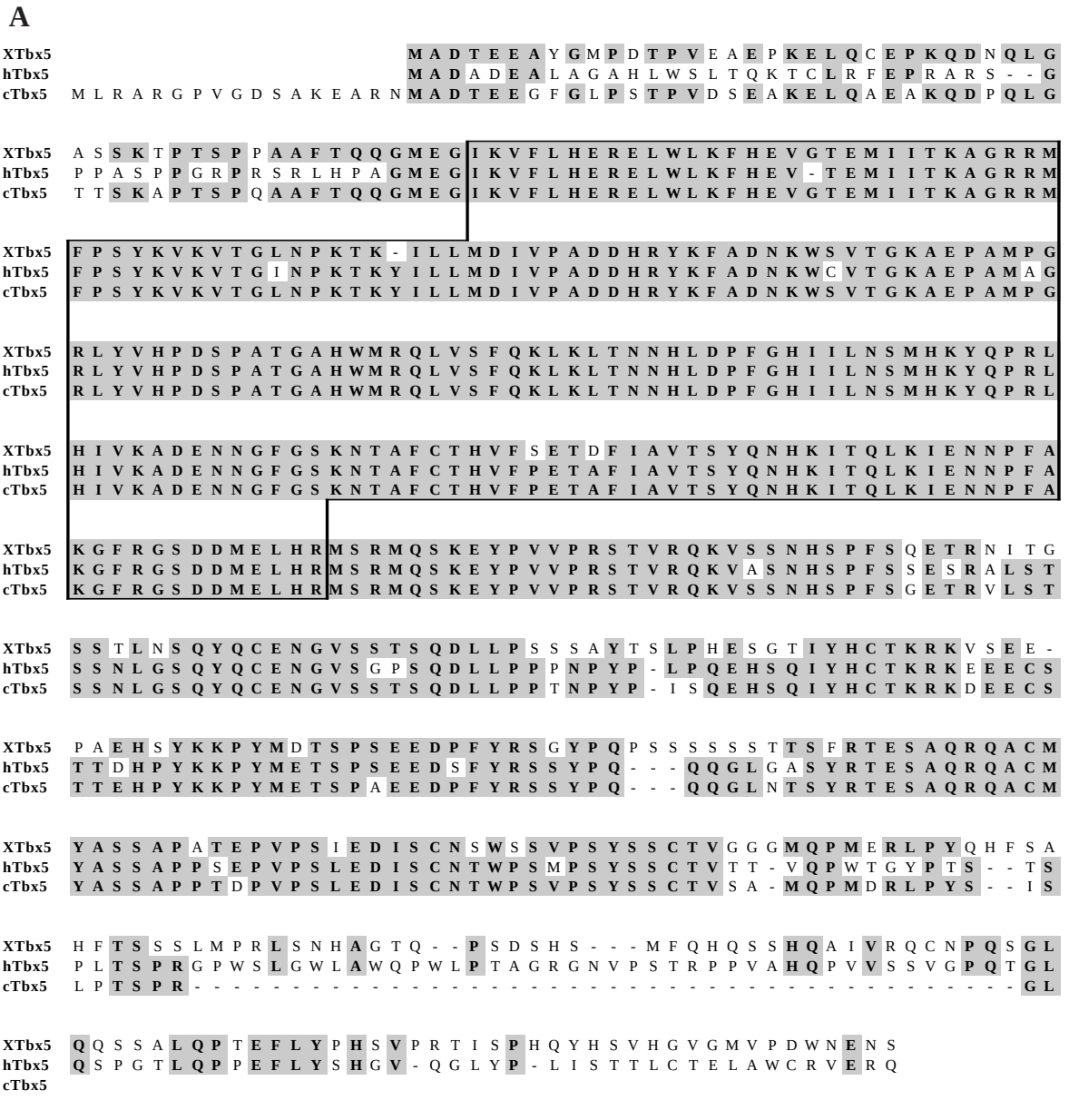
The heart tube begins looping and morphogenesis in the tadpole at approximately stage 35. In the heart tube, the principle chambers are oriented from anterior to posterior as follows: bulbus cordis, ventricle, atrium and sinus venosus. At the posterior end of the heart, the sinus venosus branches into a right and left horn, each of which connects to a cardinal vein on either side of the embryo at the dorsal edge of the lateral plate. At tadpole stage 35, whole-mount staining showed that *XTbx5* is expressed throughout most of the heart tube, and extending from the ventral part of the pericardial cavity into the lateral mesoderm (Fig. 2G) where *XTbx5*-positive cells condense into a 'wishbone' shape, as seen in a dorsolateral view of a cleared embryo (Fig. 2H). At stage 40, this lateral domain of expression extends dorsally and marks the right and left branches of the sinus venosus (sv) (Fig. 2I,J), which form the inflow tract of the heart that leads into a common atrium at this stage (atrial septation occurs later in development) (Niewkoop and Faber, 1967). Within the heart tube at stage 40, expression of *XTbx5* persists in the atrium (a) and ventricle (v) but is absent from the bulbus cordis (bc) located at the anterior end of the pericardial cavity (Fig. 2J).

We confirmed that the ventral expression domain of *XTbx5* in the tadpole corresponds to cardiac tissues by comparing *XTbx5* expression to a heart-specific marker of terminal differentiation, *Xenopus myosin light chain 2 (XMLC2)* (Chambers et al., 1994). At tadpole stage 30, expression of *XTbx5* and *XMLC2* partially overlap within the developing heart (Fig. 2F,K). The anterior boundary of *XMLC2* expression extends beyond that of *XTbx5*, but their posterior boundaries approximately coincide (Fig. 2F,K). In contrast to *XTbx5*, *XMLC2* expression is not detected in the lateral

mesoderm. These differences in *XTbx5* and *XMLC2* expression become more apparent at tadpole stage 40 and later, as *XMLC2* is strongly expressed in tissues within the ventral part of the pericardial cavity (containing the ventricle and atrium), but it is absent in the lateral sinus venosus (Fig. 2L).

To precisely determine the localization of *XTbx5* during heart morphogenesis, we examined histological sections of stage 35 and 40 embryos that had been stained for *XTbx5* mRNA by whole-mount in situ hybridization. Fig. 3A,B display two sections through an early developing heart of a stage 35 embryo. At this stage, the heart tube has formed, but looping and subdivision of the chambers have not occurred to any substantial degree. A cross section through the anterior heart tube at the level of the ventricle shows abundant *XTbx5* expression in both the endocardium and the myocardium, with lower levels of expression also detected in the underlying epicardium (Fig. 3A). Within the sinus venosus, *XTbx5* transcripts are localized to the ventral layer, although there is faint expression surrounding its dorsal and lateral portions as well (Fig. 3B).

The expression of *XTbx5* in the heart tube of an older tadpole (stage 40), in which the heart is undergoing looping and structural subdivision, is displayed in serial section proceeding from anterior to posterior (Fig. 3C-K). At the anterior end, no expression of XTb*x5* is detected in the bulbus cordis (bc) (Fig. 3C), consistent with the lack of anterior expression in whole-mount in situ (Fig. 2H-J). In a slightly more posterior section, at the junction of the bulbus cordis and ventricle, *XTbx5* expression is only detected in the ventricular tissue (v) located adjacent to the bulbus cordis (Fig. 3D). Moving posteriorly, sections through the ventricle show that *XTbx5* is expressed in all tissue layers including what we identify as epicardium (ep) (Fig. 3E) (Hiruma and



Hirakow, 1989; Ho and Shimada, 1978; Viragh and Challice, 1973, 1981). *XTbx5* is also detected throughout the adjacent atrium (Fig. 3F,G). Note that, at its anterior end, the atrium directly abuts the dorsal endoderm (Fig. 3F), but as it projects toward the posterior the atrial tube rises dorsally within the pericardial cavity (Fig. 3G,H) and splits into two lateral horns (Fig. 3I-L) that we provisionally identify as extentions of the sinus venosus, which form the inflow tract of the heart and that link to the posterior cardinal veins. *XTbx5* expression in these regions is strongest ventrally. Serial sections through the left and right horns of the sinus venosus reveal the dorsalward extension of the branches into the lateral sides of the embryo. The expression of *XTbx5* in the sinus venosus terminates on each side of the embryo in an area of intermediate mesoderm where expression of several vascular specific markers, such as *Xmsr*, *Xflk-1* and *XHex* are expressed (Cleaver et al., 1997; Devic et al., 1996; Newman et al., 1997) and we presume that this boundary is where the sinus venosus joins the cardinal veins. Thus in both early (stage 25) and late (stage 28-40) phases of heart tube morphogenesis *XTbx5* is expressed in all but the most anterior portion of the heart, the bulbus cordis.

XTbx5 and *XNkx2.5* are expressed in distinct and overlapping domains

Some of the earliest markers of cardiac specification in vertebrates are the Nkx (or tinman) set of homeodomain transcription factors (Biben and Harvey, 1997; Chen and Fishman, 1996; Cleaver et al., 1996; Evans et al., 1995; Harvey, 1996; Komuro and Izumo, 1993; Lints et al., 1993; Lyons et al., 1995; Sebedzija et al., 1998; Tonissen et al., 1994). In *Xenopus* embryos, both *XNkx2.3* and *XNkx2.5* are expressed within the migrating cardiac mesoderm at neurula stages, coincident with the timing of *XTbx5* expression (Evans et al., 1995; Tonissen et al., 1994). Subsequent to the appearance of these and other early markers (such as *GATA-4*, *GATA-5* and *GATA-6*) (Gove et al., 1997; Jiang and Evans, 1996), the terminal differentiation marker *XMLC2* (Chambers et al., 1994) is expressed throughout the heart tube. To better understand the relationship between the expression of XTb*x5* and some of these early markers of cardiac specification and differentiation, we compared their expression patterns by double-labeled, whole-mount in situ hybridization.

At the onset of *XTbx5* expression in the neurula, it was difficult to determine whether its expression overlapped with

B

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XTbx5   I K V F L H E R E L W L K F H E V G T E M I I T K A G R R M F P S Y K V K V T G L N P K T K Y I L L
hTbx5   I K V F L H E R E L W L K F H E V G T E M I I T K A G R R M F P S Y K V K V T G L N P K T K Y I L L
mTbx5               L W L K F R S G V T E M I I T K A G G R M F P S Y K V K V T G L N P K T K Y I L L
cTbx5   I K V F L H E R E L W L K F H E V G T E M I I T K A G R R M F P S Y K V K V T G L N P K T K Y I L L
mTbx4                                 M I I T K A G R R M F P S Y K V K V T G M N P K T K Y I L L
Brat    V G A S L E D Q D L W S Q F H Q E G T E M I I T K S G R R M F P Q C K I R L F G L H P Y A K Y M L L
eom     A Q V F L C N R P L W L K F H R H Q T E M I I T K Q G R R M F P F L S F N I T G L N P T A H Y N V F
Xbra    L K V S L E E R D L W T R F K E L T N E M I V T K N G R R M F P V L K V S M S G L D P N A M Y T V L

XTbx5   M D I V P A D D H R Y K F A D N K W S V T G K A E P A M P G - R L Y Y V H P D S P A T G A H W M R Q L
hTbx5   M D I V P A D D H R Y K F A D N K W S V T G K A E P A M P G - R L Y Y V H P D S P A T G A H W M R Q L
mTbx5   M D I V P A D D H R Y K F A D N K W S V T G K A E P A M P G - R L Y Y V H P D S P A T G A H W M R Q L
cTbx5   M D I V P A D D H R Y K F A D N K W S V T G K A E P A M P G - R L Y Y V H P D S P A T G A H W M R Q L
mTbx4   I D I V P A D D H R Y K F C D N K W M V A G K A E P - M P G - R L Y Y V H P D S P A T G A H W M R Q L
Brat    V D F V P L D N F R Y K W N K N Q W E A A G K A E P H P P C - R T Y Y V H P D S P A P G A H W M K D P
eom     V E V V L A D P N H W R F Q G G K W V T C G K A D N N M Q G N K V Y Y V H P E S P N T G A H W M R Q E
Xbra    L D F V A A D N H R W K Y V N G E W V P G G K P E P Q A P S - C V Y I H P D S P N F G A H W M K D P

XTbx5   V S F Q K L K L T N N - - - H L D P F G H I I L N S M H K Y Q P R L H I V K A D E N - N G F G S K N
hTbx5   V S F Q K L K L T N N - - - H L D P F G H I I L N S M H K Y Q P R L H I V K A D E N - N G F G S K N
mTbx5   V S F Q K L K L T N N - - - H L D P F G H I I L N S M H K Y Q P R L H I V K A D E N - N G F G S K N
cTbx5   V S F Q K L K L T N N - - - H L D P F G H I I L N S M H K Y Q P R L H I V K A D E N - N G F G S K N
mTbx4   V S F Q K L K L T N N - - - H L D P F G H I I L N S M H K Y Q P R L H I V K A D E N - N A F G S K N
Brat    I C F Q K L K L T N N - - - T L D Q Q G H I I L H S M H R Y K P R F H V V Q S D D - - - M Y N S P W
eom     I S F G K L K L T N N K G A N N N S T Q M I V L Q S L H K Y Q P R L H I V E V S E D G V E D L N D S
Xbra    V S F S K V K L T N K - - - - M N G G G Q I M L N S L H K Y E P R I H I V R V G G - - - - - - - T Q

XTbx5   T A F C T H V F S E T D F I A V T S Y Q N H K I T Q L K I E N N P F A K G F R G S D D M E L H R
hTbx5   T A F C T H V F P E T A F I A V T S Y Q N H K I T Q L K I E N N P F A K G F R G S D D M E L H R
mTbx5   T A F C T H V F P E T A F I A V T S Y Q N H K I T Q L K I E N N P F A K G F R G S D D L E L H R
cTbx5   T A F C T H V F P E T A F I A V T S Y Q N H K I T Q L K I E N N P F A K G F R G S D D M E L H R
mTbx4   T A F C T H V F P E T S F I S V T S Y Q N H K I T Q L K I E N N P F A K G F R G S D D S D L R V A
Brat    G L V Q V F S F P E T E F T S V T A Y Q N E K I T K L K I N H N P F A K G F R E Q E R S H K R D
eom     A K N Q T F T F P E N Q F I A V T A Y Q N T D I T Q L K I D H N P F A K G F R D N Y D S M Y T A
Xbra    R M I T S H S F P E T Q F I A V T A Y Q N E E I T A L K I K H N P F A K A F L D A K E R N D Y K
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Fig. 1. XTbx5 protein comparison. (A) A comparison of the predicted amino acid sequence of *Xenopus*, human and chick Tbx5. XTbx5 and hTbx5 share 67% amino acid identity overall, while XTbx5 and cTbx5 share 70% amino acid identity. The greatest degree of amino acid identity is found within the T domain, which is boxed. (B) Amino acid sequence alignment of the T domain of XTbx5 with other T domain proteins. The overall amino acid identity of each with the T domain of XTbx5 is: 98% human Tbx5, 91% mouse Tbx5, 98% chick Tbx5, 82% mouse Tbx4, 54% Brat, 53% Eom, 49% Xbra. Protein analysis was done using MacVector's Clustal protein alignment software. Identical amino acids are shaded and gaps are represented as dashes. The amino acid sequences corresponding to the degenerate primers are indicated by arrows. GenBank accession number for Tbx5 clone is AF133036.

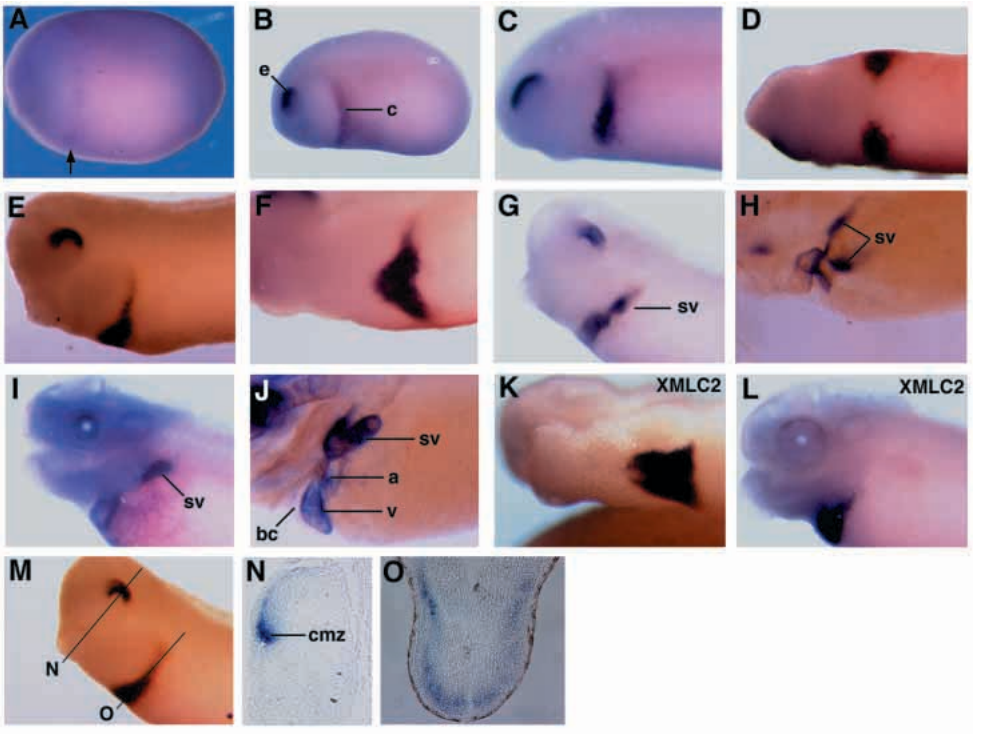

Fig. 2. Expression of *XTbx5* mRNA during organogenesis. (A) Stage 17 embryo, lateral view. The arrow shows faint expression of *XTbx5* mRNA in the lateral mesoderm. (B) Stage 20 embryo, lateral view. Expression is detected in the eye (e) and the cardiac field (c). (C) Stage 25 embryo, lateral view. Expression of *XTbx5* in the lateral mesoderm is greatest near the ventral midline and tapers off dorsally. (D) Ventral view of the same embryo in C. *XTbx5* mRNA is not expressed across the ventral midline. (E) Stage 30 embryo, lateral view. Expression has moved further ventrally and has fused across the ventral midline, but some expression persists in lateral mesoderm. (F) Ventral view of the same embryo in E. Notice the heart-shaped expression along the ventral midline. (G) Stage 35 embryo. The expression of *XTbx5* condenses in the lateral mesoderm, marking the sinus venosus (sv) (Nieuwkoop and Faber, 1967). (H) Dorsal-lateral view of a stage 35 embryo cleared in benzyl benzoate/benzyl alcohol (BBA). Notice that the expression of *XTbx5* leaving the atrium branches onto either side of the embryos marking the developing sinus venosus. (I) Stage 40 embryo. The sinus venosus (sv) has moved further dorsally to lie just below the somites. Notice the lack of *XTbx5* expression in the anterior portion of the pericardial cavity. (J) Stage 40 embryo in BBA. Notice the horseshoe-shaped expression marking the sinus venosus (sv). (K) *XMLC2* expression, stage 30 embryo ventral view. Expression is seen further anteriorly than *XTbx5* along the ventral midline and does not resemble the heart-shaped *XTbx5* expression. (L) *XMLC2* expression at stage 40. In contrast to *XTbx5*, *XMLC2* is detected throughout the pericardial cavity. Abbreviations used in the figure are: atrium (a), bulbus cordis (bc), sinus venosus (sv) and ventricle (v). (M) Stage 28 embryo used to show the positions of the sections shown in N and O. (N) A section through the eye shows that *XTbx5* is expressed only in the outer edge of the ciliary marginal zone (cmz), marking the stem cell population. There is also expression along the dorsal surface of the retina. (O) A section through the developing heart region shows that *XTbx5* is expressed in the lateral and ventral mesoderm.

XNkx2.5 due to the weak Tbx5 expression signal. However, in the tailbud tadpole, the expression of *XNkx2.5* and *XTbx5* do not overlap within the lateral, migrating mesoderm (stage 25) (Fig. 4A and histological sections not shown). This is somewhat surprising because *XNkx2.5* has been considered as a general marker of early cardiac commitment. The adjacent expression of *XNkx2.5* and *XTbx5* in the early tadpole thus provides evidence that the cardiac mesoderm becomes regionalized relatively soon after its specification and prior to heart tube formation. In older, stage 30 tadpoles, the lateral expression domains of these genes do not overlap but, in the developing heart tube, expression of *XTbx5* and *XNkx2.5* overlaps within the ventral-anterior midline (Fig. 4B). We also compared the expression of *XTbx5* with *XMLC2*, which is expressed in the heart tube beginning at stage 28 (Chambers et al., 1994). Consistent with the patterns shown in Fig. 2K,L, *XMLC2* expression at stage 30 partially overlaps with cells that express *XTbx5* in the posterior of the heart (Fig. 4C,D). *XMLC2* is not however, co-expressed with *XTbx5* in the lateral cardiac mesoderm where the sinus venosus is derived (Fig. 4D).

Expression of *XTbx5* in the eye

In addition to its expression in the heart, *XTbx5* is also expressed in the developing eye. A broad arc of expression is first noted at stage 20 in the dorsal half of the eye (Fig. 2B) and this domain becomes refined during tadpole stages 25-40 until it is expressed exclusively in a subset of dorsal cells (compare Fig. 2B,C,E and G). Sections through the eye of a stage 28 embryo (Fig. 2N) reveal that the dorsal localization of *XTbx5* transcripts is within retinal cells of the ciliary marginal zone (CMZ). The CMZ is composed of three layers containing stem cells, dividing retinoblasts and differentiating cells, and each group is localized to distinct regions of the CMZ (Dorsky et al., 1995; Wetts et al., 1989). In its early phase of expression (from stage 20 to 25), *XTbx5* mRNA is detected throughout the CMZ in all cell layers (data not shown), but by stage 30 *XTbx5* is expressed exclusively in the stem cell population at the outer edge of the CMZ (Fig. 2N). Localization of *XTbx5* to the CMZ is similar to the expression of two other eye transcription factors: *ET*, a T-box gene, and *Xbr-1*, a homeobox gene (Li et al., 1997a; Papalopulu and Kintner, 1996) and suggests that it plays a role in neural cell commitment to retinal fates (Wetts and Fraser, 1988).

Construction of a hormone-inducible repressor form of *XTbx5*

The *XTbx5* expression data that we have just presented and the similar patterns of Tbx5 expression in chicken, mouse and human embryos (Chapman et al., 1996; Gibson-Brown et al.,

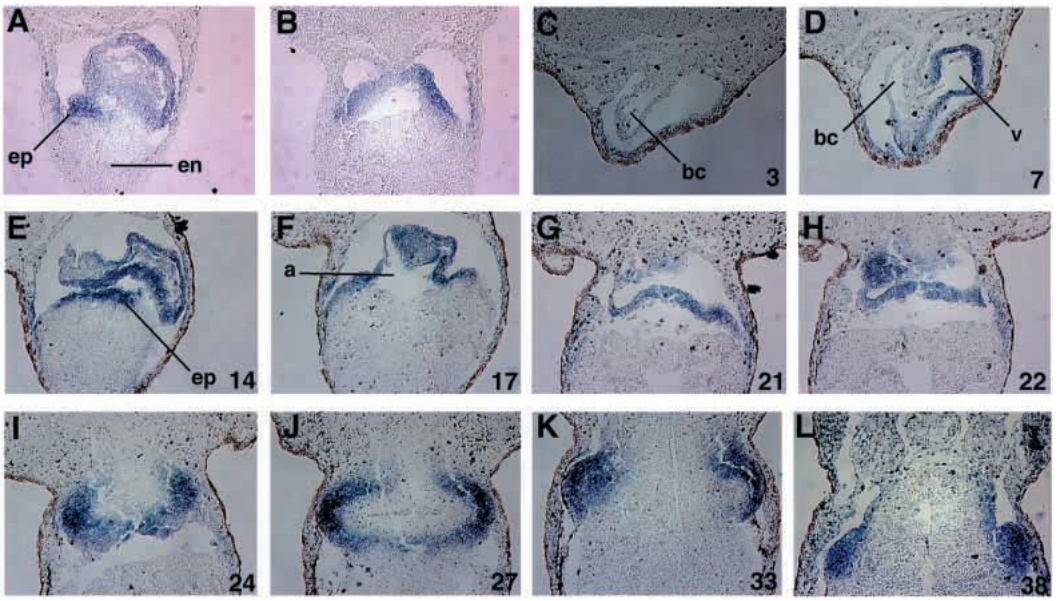

Fig. 3. Serial sections through the heart of a stage 35 (A,B) and a stage 40 (C-L) embryo stained for *XTbx5* mRNA expression. (A) Section through the ventricle of a stage 35 embryo. Expression is detected in both the endocardium and myocardium as well as in the tissue we identify as epicardium (ep), overlying the endoderm (en). (B) Section through the sinus venosus slightly posterior to the section in A. *XTbx5* mRNA is detected in the ventral part of the sinus venosus. (C-L) Selected serial sections through the heart of a stage 40 embryo proceeding from anterior to posterior. (C) Bulbus cordis (bc) (D) Bulbus cordis (bc) and ventricle (v). *XTbx5* is expressed only in the ventricle. (E) Ventricle. Expression is also detected in the underlying epicardium (ep). (F,G) Sections through the atrium (a) as it turns dorsally. (H-L) Sections through the developing sinus venosus. The sections through the heart in C-L have been numbered going from anterior to posterior, beginning with the bulbus cordis and ending with the sinus venosus. The corresponding number for each section is printed in the lower right-hand corner of each panel. Each section is 14 μm thick. The bulbus cordis encompasses sections 1-8 (112 μm), the ventricle sections 5-16 (168 μm), the atrium sections 17-22 (84 μm) and the sinus venosus sections 23-46 (336 μm).

1998b; Li et al., 1997b) suggest that this gene plays an important role in early heart development. More directly, mutations in human *Tbx5* cause Holt-Oram Syndrome, which is characterized by congenital heart malformations affecting primarily septum formation (Basson et al., 1997; Li et al., 1997b). The HOS patients studied thus far, however, have been heterozygous for *Tbx5* mutations, rather than fully defective for *Tbx5*, so the extent to which *Tbx5* is required for heart development has not been well established.

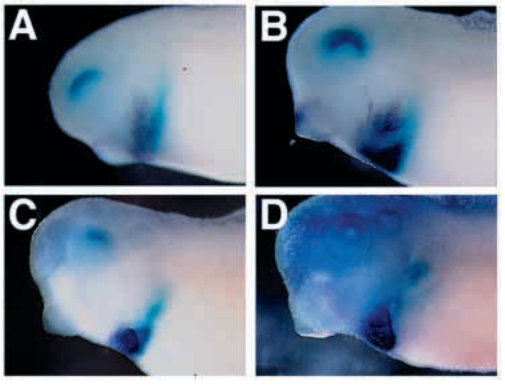

Fig. 4. Double-labeled whole-mount in situ hybridization. (A,B) Expression of *XTbx5* (blue) and *XNkx-2.5* (purple). (A) At tailbud tadpole stage 25, cells expressing *XNkx-2.5* are anterior to those expressing *XTbx5*. (B) At stage 30, expression of both overlaps in the developing heart in ventral, but not lateral domains. (C,D) Expression of *XTbx5* (blue) relative to *XMLC2* (purple). (C) Stage 30 embryo. Expression of both genes overlaps in the heart, while only *XTbx5* expression is seen in the lateral mesoderm. (D) Stage 40 embryo. No *XMLC2* expression is detected in the dorsal sinus venosus.

We sought to test the importance of Tbx*5* in vertebrate heart development by inhibiting its function in the *Xenopus* embryo using a dominant-negative strategy that has proven effective at inhibiting other T domain proteins (Conlon et al., 1996; Horb and Thomsen, 1997; Ryan et al., 1996). Our approach was to block endogenous Tbx5 activity in embryos by expressing a transcriptional repressor form of the protein. We created a repressor version of XTbx5 by removing its C-terminal region, which encodes a transcriptional activation domain in other T domain proteins (Conlon et al., 1996; Kispert, 1995; Zhang and King, 1996), and replacing it with a transcriptional repressor domain of Drosophila Engrailed (Badiani et al., 1994; Han and Manley, 1993). This construct, *XTbx5-En*^{R}, is shown in Fig. 5A. In preliminary tests injection of synthetic *XTbx5-En*^{R} mRNA into the dorsal marginal zone (DMZ) of early cleavage embryos resulted in significant defects in gastrulation and body patterning (Fig. 5C). Since *XTbx5* is not expressed until after gastrulation, we interpret these effects to be a consequence of expressing *XTbx5-En*^{R} protein in the blastula and gastrula, which perhaps interferes with other T domain proteins expressed endogenously in the blastula and gastrula, such as Xbra, Eom and Brat (also known as VegT, Xombi and Antipodean) (Horb and Thomsen, 1997; Lustig et al., 1996; Ryan et al., 1996; Smith et al., 1991; Stennard et al., 1996; Zhang and King, 1996). To test this possibility, we injected mRNA for *XTbx5-En*^{R} (1.0 ng) together with a ten-fold lower amount (0.1 ng) of Tbx5, Xbra or Brat mRNA and found that gastrulation defects were rescued at a greater than 50% frequency with Xbra (50%, n=26) and Brat (65%, n=23), but only 8% (n=26) with Tbx5 (data not shown). These results are consistent with cross-inhibition of early embryonic T-box proteins by dominant-negative *XTbx5-En*^{R}.

Since ectopic expression of *XTbx5-En*^{R} during early stages interferes with early developmental processes, this posed a

significant obstacle to our analysis of *Tbx5* function in heart formation. To circumvent this problem, we adapted a hormone-inducible, fusion protein method that has been employed to regulate the activity of nuclear and cytoplasmic factors in a variety of systems (Gammill and Sive, 1997; Kolm and Sive, 1995; Mattioni et al., 1994; Tada et al., 1997). This method is based on the principle that a hormone-binding domain of steroid hormone receptors can be used to regulate the activity of heterologous proteins to which it is fused (Danielian et al., 1992; Smith and Toft, 1993; Tsai and O'Malley, 1994). This technique was initially used in *Xenopus* embryos, with the glucocorticoid receptor hormone-binding domain (GR), by Sive and colleagues to regulate the activity of *XMyoD* and *Otx2* (Gammill and Sive, 1997; Kolm and Sive, 1995). It has also been shown effective in regulating the activity of *Xenopus Brachyury (Xbra),* a T-box gene (Tada et al., 1997).

The GR fusion protein method just outlined has not been employed to regulate an artificial transcriptional repressor, such as *XTbx5-En^R*. Nevertheless, we attempted to create an inducible repressor version of *XTbx5* by fusing the GR hormone-binding domain onto the C terminus of *XTbx5-En^R*, creating *XTbx5-En^R-GR* (Fig. 5A). To establish whether or not we had conferred hormone-inducibility onto *XTbx5-En^R*, we injected various doses of *XTbx5-En^R-GR* mRNA into the DMZ of 4- to 8-cell embryos, added dexamethasone at various developmental time points to activate the fusion protein, and then monitored the resulting phenotype of the embryos and their hearts in particular. Embryos treated with dexamethasone alone developed normally (Fig. 5B). Embryos that were injected with *XTbx5-En^R-GR* mRNA, but not treated with dexamethasone, also developed normally (Fig. 5D). This demonstrates that overexpression of XTbx5-En^R-GR protein without activation has no effect on development and contrasts the deleterious effects observed when the constitutively active protein, XTbx5-En^R, is expressed in embryos (Fig. 5C).

When embryos were injected with *XTbx5-En^R-GR* mRNA and immediately treated with dexamethasone severe gastrulation defects resulted (Fig. 5E), and those defects were essentially identical to those caused by the non-inducible (constitutively active) XTbx5-En^R protein. Addition of dexamethasone to *XTbx5-En^R-GR*-injected embryos at any stage prior to, or during, gastrulation also produced similar defects (data not shown). In contrast, when dexamethasone was added to *XTbx5-En^R-GR*-injected embryos after gastrulation was finished (stages 14-15), the general appearance of the embryos was normal (Fig. 5F), but severe defects were manifested only in the heart (Fig. 6). We also sometimes noted a slight reduction in the size of the eye. We observe the most severe heart defects when *XTbx5-En^R-GR* activity is induced at stage 15, which is the time in development just preceding detectable *XTbx5* expression at stage 17. When dexamethasone was added at stages later than 15, heart development was only mildly affected (data not shown). Overexpression of *XTbx5-En^R-GR* in tissues outside of the precardiac mesoderm, had no effect on their development (data not shown, and results below).

Inhibition of *XTbx5* by *XTbx5-EnR-GR* blocks heart formation

The defects that we observed in embryos affected by induction of *XTbx5-En^R-GR* (Fig. 6) were grouped into two major categories: reduced heart and heartless. In the reduced heart phenotype, a heart is present, but it is much smaller than a wild-type heart and displays an abnormal morphology (Fig. 6B,C arrows). These reduced hearts pulsate, but no circulating blood cells are seen in either the heart or the blood vessels; instead, the blood pools around the developing gut (Fig. 6B,E arrowhead). In the heartless phenotype, no heart forms and the pericardial cavity is empty (Fig. 6D-F), although sometimes a beating nub of tissue is visible in the dorsal portion of the pericardial cavity. Heart defects caused by *XTbx5-En^R-GR* are sometimes associated with a bloated pericardial cavity, and a partially malformed intestinal tract, observed at late stages of development. This might be caused by secondary endodermal defects due to loss of cardiac tissue, since precardiac mesoderm participates in liver induction (Zaret, 1996; Fukuda-Taira, 1981). Swelling might also be caused by osmotic imbalance due to a lack of a heart, and thus proper circulation. It is also possible that *XTbx5-En^R-GR* interferes with uncharacterized T-box genes involved in endoderm formation (perhaps even endodermal Brat/VgT protein encoded by maternal mRNA). Table 1 shows that the effects of *XTbx5-En^R-GR* on heart formation are dose-dependent. When 250 pg of XTb*x5-En^R-GR* mRNA was injected, 64% of the embryos had heart defects. This frequency rose to 87% when 500 pg was injected and, at the highest dose, 1 ng, 96% of the embryos displayed heart defects. In our assays, we injected 1 ng of *XTbx5-En^R-GR* (0.5 ng per blastomere) into the dorsal marginal zone (DMZ) at the 4- to 8-cell stage and added dexamethasone at stage 15, unless specified otherwise.

To determine whether interference with *XTbx5* perturbs early and/or late steps in cardiac differentiation, we examined the expression of heart-specific markers, *XNkx2.5*, *XMLC2* and endogenous *XTbx5* itself in swimming tadpole stage embryos injected with *XTbx5-En^R-GR* mRNA. Tissues targeted with *XTbx5-En^R-GR* were lineage traced with co-injected β-gal mRNA (250 pg) and embryos were processed at stage 35 for β-gal activity (using a red substrate) and by whole-mount in situ hybridization. In embryos properly targeted to the heart field, we observed a significant reduction (or absence) of *XMLC2*, *XNkx2.5* and endogenous *XTbx5* expression (Fig. 7). In these experiments, *XTbx5-En^R-GR* and the β-gal lineage tracer mRNAs were injected into a variety of anterior and ventral tissues nearby the heart, as shown by the red stain. Unlike the heart, and perhaps the eye, these tissues developed normally, illustrating that the dominant-negative Tbx5 protein does not generally interfere with tissue differentiation and morphology. Since the dominant negative protein was most effective at blocking these cardiac markers and overall heart formation when induced at stages 14-15, we conclude that *XTbx5* is required from the onset of its expression for a normal program of cardiac differentiation and morphogenesis. We note, however, that, since the dominant-negative Tbx5 protein appears to interfere with other T-box genes at gastrulation, it is formally possible that other, as yet unidentified, Tbx family members expressed in the developing heart are also blocked by *XTbx5-En^R-GR*.

XTbx5-GR rescues the heartless phenotype

To determine whether the defects caused by *XTbx5-EnR-GR*

Table 1. Overexpression of XTbx5-En^R-GR affects heart formation

Dose XTbx5-En^R-GR	Phenotype			Total
	Normal heart	Reduced heart	Heartless	
0.25 ng	19 (36%)	28 (53%)	6 (11%)	53
0.50 ng	7 (13%)	27 (50%)	20 (37%)	54
1.0 ng	6 (4%)	55 (41%)	74 (55%)	135

are directly attributable to inhibition of endogenous *XTbx5*, we attempted to rescue the heartless phenotype by co-expressing a hormone-inducible version of wild-type *Tbx5*, named *XTbx5-GR* (Fig. 5A). The results of rescue experiments are summarized in Table 2. Injection of our standard 1 ng of *XTbx5-En^R-GR* mRNA by itself caused heart defects in 75% of the embryos (26% had reduced hearts; 49% were heartless). Coinjection of 10 pg of *XTbx5-GR* resulted in 51% normal embryos, and decreased the number of embryos in the reduced heart (9%) and heartless (40%) categories. Coinjection of 50 pg of *XTbx5-GR* resulted in 49% normal embryos, and 21% heartless embryos and 30% embryos with reduced hearts. The greatest degree of rescue was achieved when 100 pg of *XTbx5-GR* was coinjected: 73% of the embryos had normal hearts, 12% had reduced hearts and only 15% were heartless. Co-injection of more than 100 pg of *XTbx5-GR* was less effective at rescuing the effects of *XTbx5-En^R-GR*. This may reflect a gain-of-function effect of *XTbx5-En^R-GR* since injection of 100 pg *XTbx5-GR* mRNA alone also causes heart defects (data not shown). We are presently characterizing the gain-of-function phenotype in more detail. These results verify that *XTbx5-En^R-GR* acts as a dominant-negative inhibitor of *Tbx5* activity, since the defects it causes can be rescued by wild-type *XTbx5*. Most importantly, all of our dominant-negative results demonstrate that *XTbx5* activity is required for normal development of essentially the entire heart.

DISCUSSION

Members of the T-box gene family of transcription factors have emerged recently as key players in embryonic patterning, tissue differentiation and morphogenesis, particularly in vertebrates (Papaioannou and Silver, 1998; Smith, 1997). We have used the *Xenopus* embryo to study one member of the family, *Tbx5*, a gene implicated in normal heart development and the etiology of human Holt-Oram Syndrome (Basson et al., 1997; Li et al., 1997b), a condition manifested by congenital heart and limb defects (Basson et al., 1994; Holt and Oram, 1960; Hurst et al., 1991; Newbury-Ecob et al., 1996; Smith et al., 1979). *Xenopus Tbx5* is one of the earliest markers of cardiac specification, being expressed in the heart field as it is established and, as the heart develops, *XTbx5* is expressed in all but the most anterior portion of the heart tube (the bulbus cordis). We have demonstrated that *XTbx5* is critical for normal heart development by overexpressing a dominant-negative version of the protein, which ablates nearly all of the heart. We conclude that *Tbx5* is an essential factor in early cardiac specification and morphogenesis of the vertebrate heart. Furthermore, our findings may be relevant to the mechanism of abnormal heart development associated with Holt-Oram syndrome.

Table 2. XTbx5-GR rescues the heartless phenotype caused by XTbx5-En^R-GR

Dose XTbx5-GR*	Phenotype			Total
	Normal heart	Reduced heart	Heartless	
–	17 (25%)	18 (26%)	34 (49%)	69
10 pg	33 (51%)	6 (9%)	26 (40%)	65
50 pg	48 (49%)	29 (30%)	20 (21%)	97
100 pg	105 (73%)	17 (12%)	22 (15%)	144

*The dose of XTbx5-En^R-GR used in these injections was 1 ng.

Expression of *Tbx5* and other cardiogenic factors in heart development

XTbx5 is first expressed in the *Xenopus* neurula in the anterior lateral mesoderm on both sides of the embryo. This region corresponds to the migrating precardiac mesoderm, and it is within this area that other transcription factors associated with early cardiogenesis are also expressed, including *Xenopus GATA-4*, *GATA-5* and *GATA-6* (Jiang and Evans, 1996), as well as *XNkx2.3* and *XNkx2.5* (Evans et al., 1995; Tonissen et al., 1994). The *GATA* factors are the first of these to be expressed, encompassing a wide domain that includes the heart field at the beginning of neurulation. *XTbx5*, *XNkx2.3* and *XNkx2.5* are expressed slightly later, within a subset of *GATA*-expressing cells in the cardiac field. *XTbx5* along with the *XNkx* factors thus appear to act subsequently to *GATA* factors in the commitment of mesoderm to a cardiac fate. Presently we do not understand regulatory relationships, if any, between Tbx-5 and other heart-specific transcription factors such as the GATA and Nkx genes. As neurulation is completed and the embryo progresses into early tadpole stages, cells that express *XNkx2.5* and *XTbx5* occupy distinct bands within the lateral cardiac mesoderm, with *XNkx2.5* positioned anterior to *XTbx5*. These different expression domains suggest that two distinct populations of precardiac cells exist within the lateral mesoderm, perhaps reflecting the initial acquisition of anterior-posterior polarity by the developing cardiac tissue. In both chicken and zebrafish, ventricular and atrial cell lineages become established in the anterior and posterior precardiac mesoderm, respectively, during their migration into lateral positions, but prior to ventral fusion of cardiac primordia (DeHaan, 1965; Stainier et al., 1993; Yutzey et al., 1995). Those movements of chicken and fish cardiac mesoderm are analogous to those observed in *Xenopus* embryos, so the differences in *Tbx5* and *Nkx2.5* expression may reflect early aspects of atrial and ventricular specification.

From tailbud tadpole (stage 25) through early swimming tadpole (stage 40), the heart tube condenses and undergoes looping. During this period *XTbx5* and *XNkx2.5* expression domains partially overlap in the posterior of the heart tube, coincident with cells expressing the *XMLC2* gene, which marks the bulbus cordis, ventricle and the atrium. Unlike *XNkx2.5* or *XMLC2*, however, *XTbx5* expression extends from the ventricle to the most posterior extent of the heart tube, the sinus venosus. *XTbx5* is expressed in the endocardial and epicardial layers of the heart tube, with a notable dorsal-ventral gradient of expression (highest levels ventrally) in some areas such as the ventricle and the sinus venosus. The very early expression of *XTbx5* in the migrating cardiac

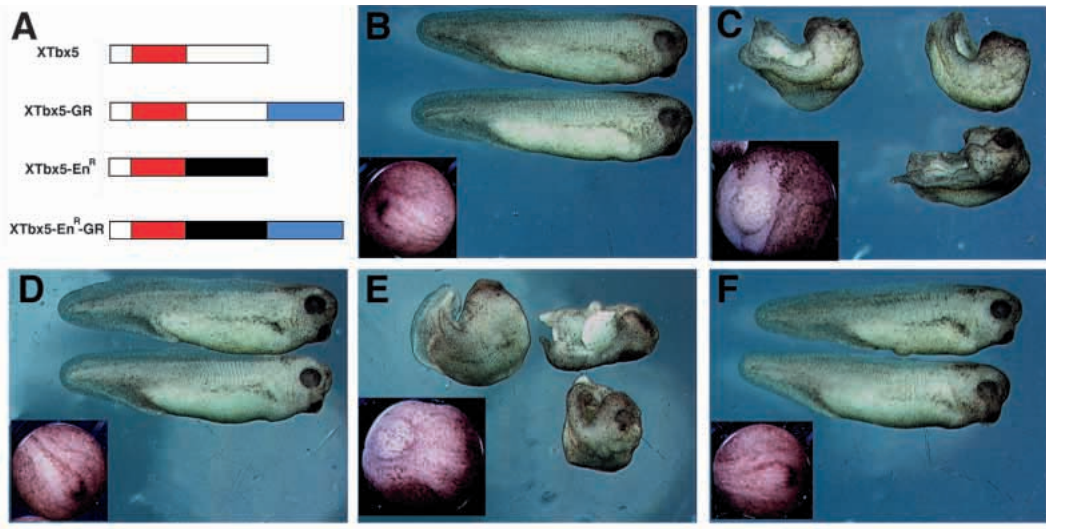

Fig. 5. Construction and effectiveness of a hormone-inducible XTbx5 protein. (A) Schematic diagram of wild-type and dominant negative *XTbx5* constructs. The T domain is in red, the engrailed repressor domain is depicted in black and the GR domain is in blue. (B) Wild-type stage 35 embryo with dexamethasone added at the 4 cell stage. (C) Stage 35 embryo injected with 1 ng of *XTbx5-En^R* mRNA into the dorsal marginal zone at the 4-cell stage. (D) Stage 35 embryo injected with 1 ng of *XTbx5-En^R-GR* mRNA and no dexamethasone added. (E) Stage 35 embryo injected with 1 ng of *XTbx5-En^R-GR* mRNA and dexamethasone added immediately after injections. (F) Stage 35 embryo injected with 1 ng of *XTbx5-En^R-GR* mRNA and dexamethasone added at stage 14/15. In B-F, the inset in the lower left corner shows a single example of a stage 17 embryo.

mesoderm, and its widespread later expression in all but the anterior (bulbus cordis) portion of the developing heart tube, suggest that *XTbx5* plays an important role in heart development.

Tbx5 is essential for heart development

The prediction that *XTbx5* provides an important function in heart development is borne out by our demonstration that an inducible, dominant negative version of *XTbx5* severely interferes with heart development. The hormone-inducible version of this inhibitor, *XTbx5-En^R-GR*, blocked formation of nearly the whole heart but, when it was expressed in other regions of the embryo, it had no effect, illustrating its specificity for development of cardiac tissues. Furthermore, the effects of this inhibitor can be rescued by co-expression of wild-type *XTbx5* (as GR fusion protein), demonstrating that it acts as a dominant-negative inhibitor specific for *XTbx5* (and perhaps closely related genes with similar function). Over half of the embryos (55%) had no recognizable heart at the gross phenotypic level and when scored by the expression of early *(XNkx2.5)* and late *(XMLC2)* cardiac markers. In many cases, a small vestige of cardiac tissue was present as a 'beating nub' that pulsated within an otherwise empty cavity where the heart would normally reside. Perhaps this residual tissue corresponds to the bulbus cordis, which does not express *XTbx5* and which therefore may develop independent of its activity.

Our dominant-negative experiments also reveal that Tbx5 activity at the neurula stage is critical for heart development. By activating the glucocorticoid-inducible version of XTbx5-EnR at various times in development, we found that the most severe heart phenotypes resulted from dexamethasone treatment at the beginning of neurulation, coincident with the onset of endogenous *XTbx5* expression. Activation of the repressor at later stages was not nearly as effective at inhibiting formation of the heart tube. We conclude that *Tbx5* function is necessary for the initiation and general development of the heart.

Tbx5 and Holt-Oram Syndrome

Our demonstration that *Tbx5* plays an important role in heart development supports recent findings that mutations in *hTbx5* are the genetic cause of the upper limb and cardiac defects seen in Holt-Oram Syndrome (HOS) (Basson et al., 1997; Li et al., 1997b). The phenotypes displayed by HOS patients, however, do not likely reveal the full degree to which *Tbx5* functions in heart development. All patients described thus far have been

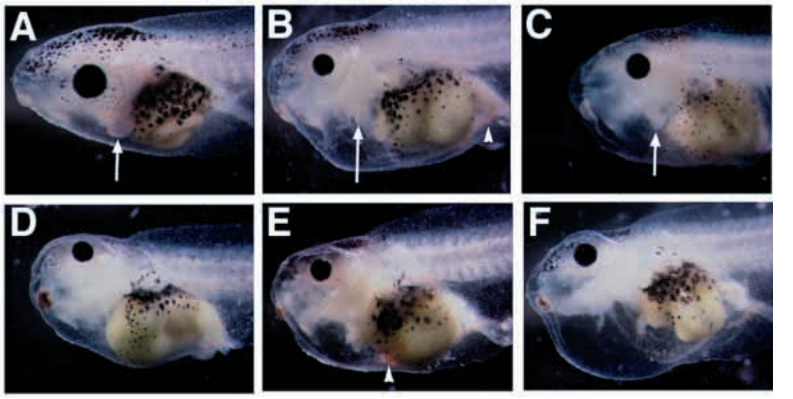

Fig. 6. Embryonic phenotypes generated by a dominant negative XTbx5 protein. (A) Wild-type stage 45 embryo. Arrow points to the heart. Notice how the heart extends to the edge of the pericardial cavity and is near the ventral surface of the gut. (B-F) Typical phenotypes caused by *XTbx5-En^R-GR*. In each case, embryos were injected dorsally at the 4-cell stage with 1 ng of mRNA and treated with dexamethasone at stage 14/15. (B,C) Examples of the reduced heart phenotype. Arrow points to the small heart present in this class of embryo. (D-F) Examples of the beating nub or heartless phenotype. This phenotype is typified by the absence of a morphologically recognized heart. Sometimes, some small amount of beating tissue is present in the dorsal portion of the pericardial cavity. In B-F, notice the bloated pericardial cavity and improper gut formation in the injected embryos. In addition, the eyes are smaller in size. Arrowhead in B and E points to regions where blood cells have pooled.

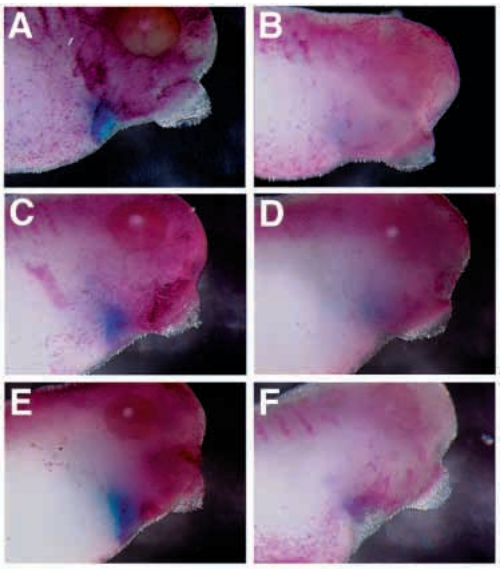

Fig. 7. Expression of heart markers in heartless embryos. (A,C,E) Stage 35 control embryos injected with 250 pg *lacZ* mRNA in the DMZ. (B,D,E) Stage 35 embryos injected with 1 ng of *XTbx5-En^R-GR* mRNA and 250 pg *lacZ* mRNA in the DMZ at the 4-cell stage and treated with dexamethasone at stage 14/15. (A,B) *XMLC2* expression. (C,D) *XNkx-2.5* expression. (E,F) Endogenous *XTbx5* expression. Expression was scored at stage 35 in embryos injected with *lacZ* alone (A,C,E) or with *lacZ* + *XTbx5-En^R-GR* (B,D,F).

heterozygous for a variety of *Tbx5* mutations, suggesting that the condition is caused by a partial loss of *Tbx5* function. All but one of the *Tbx5* mutations described in HOS encode proteins truncated within the DNA-binding domain or C-terminal transcriptional activation domain. HOS phenotypes vary in their severity, and perhaps different Tbx5 mutants have different levels of activity that affect the range of phenotypes seen in HOS patients. By creating a potent dominant negative protein, XTbx5-En^R-GR, we have been able to antagonize wild-type XTbx5 activity, which results in heartless *Xenopus* embryos. We suggest that homozygous mutations in Tbx5 associated with HOS might result in a similar heartless, lethal phenotype.

The conclusion that mutations in human *Tbx5* are the genetic cause of HOS is consistent with our data showing *XTbx5* expression within the epicardium, the outermost layer of the heart. Epicardial cells originate from the mesothelium between the sinus venosus and the endoderm, and from there the epicardial cells migrate to envelope the exterior of the entire heart (Hiruma and Hirakow, 1989; Ho and Shimada, 1978; Viragh and Challice, 1973, 1981). Epicardial cells also participate in the formation of the endocardial cushion and coronary blood vessels (Markwald et al., 1996). The expression of *XTbx5* in the *Xenopus* epicardium is consistent with the hypothesis that *Tbx5* is necessary for proper septal formation, which is the most commonly encountered abnormal feature of cardiac development in HOS patients.

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