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# Cardiac Troponin I Is a Heart-Specific Marker in the *Xenopus* Embryo: Expression during Abnormal Heart Morphogenesis

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**Cardiac troponin I (troponin Ic) expression is restricted to the heart at all stages of *Xenopus* development. Whole-mount *in situ* hybridization and Northern blot analysis indicates that troponin Ic is first expressed in tailbud embryos (stage 28) about the time of the first cytological heart differentiation and about 24 hr before beating tissue is observed. We have used this marker to examine abnormal heart morphogenesis in embryos treated with retinoic acid and lithium. When retinoic acid is administered to embryos prior to heart specification, heart tissue is reduced and often completely ablated. When embryos are treated after heart specification, but before the heart primordium migrates to the ventral midline, the migration is unaffected but smaller, abnormal hearts result. Lithium treatment of cleavage stage embryos causes an increase in heart tissue. In severely dorsalized embryos, heart tissue can be found around the entire embryo with the exception of a small gap at the most dorsal point. This gap indicates that migration of the heart to the ventral midline does not occur in these embryos. Later in development, a centrally located, beating heart is observed in dorsalized embryos. The timing of its appearance suggests that it is formed by movements normally associated with heart morphogenesis rather than migration.** 

## INTRODUCTION

The embryological events associated with heart development have been extensively characterized in amphibians. Before gastrulation in *Xenopus*, mesodermal tissue lies in a deep equatorial ring that has both anterior-posterior and dorsal-ventral polarity. In fate maps of this equatorial ring, heart is derived from two patches of the most anterior lateral plate mesoderm that lie on either side of the prechordal plate (Keller, 1976). During gastrulation, these patches involute at the lateral edges of the dorsal lip and move to take up positions on either side of the head. The two primordia then migrate ventrally along the body wall until they meet at the ventral midline, where they fuse to form the mature heart. The cues used by the heart primordium for this migration are not known. Embryological studies of *Xenopus* suggest that heart specification occurs during gastrulation (Sater and Jacobson, 1989) and that signals required for specification emanate from the dorsal lip of the blastopore (Sater and Jacobson, 1990a). This initial inductive interaction generates the heart field—the region of the embryo that has the potential to form heart. Shortly after induction, the heart field is larger than the region that will eventually go on to form the heart in the normal embryo (Sater and Jacobson, 1990b). However, as development proceeds, the size of the heart field decreases, probably as the result of additional cellular interactions. In urodeles, these interactions occur after neurulation and appear to arise from neural tissues themselves (Jacobson and Duncan, 1968). In *Xenopus*, the heart field is also reduced after neurulation, but in this case neural tissue is not the origin of the restrictive interaction (Sater and Jacobson, 1990b). While the source of the signal has not been identified conclusively, circumstantial evidence suggests that the third pharyngeal pouch may be involved (Sater and Jacobson, 1990b).

Further clues to the mechanisms underlying heart formation have been obtained by studying abnormal heart development in embryos treated with retinoic acid (RA). RA is present in the embryo and has been implicated in establishment of the embryonic anterior/posterior axis (Durston *et al.*, 1989), although the mechanism by which it acts in not understood. At high doses, RA is a teratogen and has profound effects on heart development in a number of organisms (Sive *et al.*, 1990; Osmond *et al.*, 1991; Stainier and Fishman, 1992; Yutzey *et al.*, 1994). In chick embryos, RA has a variety of different effects on the developing heart, depending on the time and mode of treatment. If embryos are immersed in high concentrations of RA at primitive streak stages, that is, prior to heart differentiation, beating hearts do not usually form. If, however, RA is administered directly into

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heart mesoderm, after heart specification but before migration, the effects are different. This treatment often causes a failure in migration, resulting in two distinct patches of beating heart tissue (Osmond *et al.*, 1991) and is likely accompanied by changes in the anteroposterior patterning of the heart. In zebrafish, administration of increasing concentrations of RA during gastrulation causes a loss of heart tissue in an anterior–posterior fashion (Stainier and Fishman, 1992). In this case, migration of heart tissue is apparently normal. In *Xenopus*, Sive *et al.* (1990) observed that heart and blood formation is blocked by high doses of RA when the treatment is applied during blastula or early gastrula stages, but this has not been examined in detail.

We wish to understand the reasons for the apparent absence of heart tissue in RA-treated *Xenopus* embryos. RA could be influencing heart development in several ways. First, RA may act by preventing the differentiation of heart tissue. This could occur either directly, by inhibition of cardiac gene transcription, or indirectly, by preventing inductive events required for heart formation. Second, heart tissue may differentiate in RA-treated embryos, but fail to migrate to its normal position at the ventral midline, as has been observed in the chick (Osmond *et al.*, 1991). A molecular assay would facilitate determining the role of RA because it would enable detection of small amounts of ectopic heart tissue if migration is blocked.

When embryos are treated with lithium at early stages of development, the entire mesodermal mantle is dorsalized, leading, in extreme cases, to the formation of radial embryos lacking all ventral tissues (Kao and Elinson, 1988). Later in development, a beating heart is observed to form in the middle of the abnormal embryo. This result, coupled with fate mapping (Kao and Elinson, 1988), suggests that the cardiac mesoderm of dorsalized embryos does not involute during gastrulation, but that this does not ultimately interfere with formation of a beating heart. The mechanism by which this occurs is unknown, but molecular markers for heart tissue would make it possible to locate cardiac tissue in abnormal embryos and trace the movement of this tissue as development proceeds.

To facilitate further studies of vertebrate cardiogenesis, we have isolated and characterized a marker that is heart-specific in the *Xenopus* embryo. In this report, we show that expression of cardiac troponin I is restricted to heart tissue in the *Xenopus* embryo. Although we describe cardiac troponin I as a heart-specific marker throughout the paper, it should be noted that troponin Ic is expressed only in the myocardium and the results can be applied only to the myocardium and not the endocardium. We have used this probe to investigate the effects of RA and lithium on embryonic heart development.

## MATERIALS AND METHODS

### *Embryo Manipulations*

*Xenopus laevis* embryos were generated as in Drysdale and Elinson (1991) except that adult females were not primed with pregnant mare serum gonadotrophin. Embryos were staged according to Nieuwkoop and Faber (1967).

Embryos were treated with various concentrations of all trans retinoic acid (Sigma) in 20% Steinberg's medium for 20 min and then transferred to a clean dish of 20% Steinberg's medium. Dimethyl sulfoxide was used as a carrier for the RA. Control embryos were immersed in 1 μl/ml dimethyl sulfoxide in 20% Steinberg's medium in the absence of RA. Lithium treatment and DAI scale assignment was carried out as described by Kao and Elinson (1988).

### *Isolation of Cardiac Troponin I cDNA Clones*

Two degenerate oligonucleotide primers (GCNGAYGCNATGATG) and (TTYTTNKNCCYTCCAT) were designed using published sequences of bovine (Leszyk *et al.*, 1988), rat (Martin and Orlowski, 1991; Murphy *et al.*, 1991), and quail (Hastings *et al.*, 1991) cardiac troponin I. The primers correspond to sequences encoding amino acids 143 to 147 and 193 to 198, respectively, of the quail sequence. PCR was used to amplify a 140-bp troponin Ic fragment from cDNA prepared from *Xenopus* adult heart RNA. No PCR products were observed when cDNA prepared from adult skeletal muscle was used as template. $^{32}$P-labeled PCR product was used to screen approximately $8 \times 10^5$ clones from a stage 28/30 *X. laevis* head cDNA library (Hemmati-Brivanlou *et al.*, 1991). Fifteen recombinants were isolated and 6 were analyzed in detail using restriction enzyme digestion. The complete sequence of a full-length clone, pXTnIc, was determined by chain-termination sequencing (Sanger *et al.*, 1977).

### *Whole Mount in Situ Hybridization*

Digoxygenin-labeled antisense cardiac troponin I probes were synthesized by *in vitro* transcription of linearized pXTnIc template. The *in situ* hybridization protocol of Harland (1991) was used with the following modifications. Tris-buffered saline (50 m*M* Tris, pH 7.4, 200 m*M* NaCl) was used throughout rather than phosphate-buffered saline. Embryos were treated with 5 μg/ml proteinase K for 15 min. Prehybridization was performed for at least 16 hr. The 0.2× SSC washes were increased to 1 hr in length, the washes after the antibody incubation were increased in frequency, and the total wash time was lengthened to 7 hr. When the staining intensity was sufficient for viewing, the embryos were rinsed in alkaline phosphatase buffer for 5 min and then

put through the following washes for 5 min each: 25% methanol, 50% methanol, 75% methanol, 100% methanol, 75% methanol, 50% methanol, 25% methanol. After the washes, the embryos were fixed in Bouin's fixative overnight to give a yellow counterstain (Harland, 1991). Embryos were cleared and photographed in benzyl benzoate:benzyl alcohol 2:1. For sections, embryos stained by whole mount *in situ* hybridization were dehydrated in ethanol and then transferred to xylene and embedded in paraffin. Ten-micrometer sections were cut on a Microm HM 325 microtome. Sections were viewed using differential interference contrast microscopy on a Zeiss Axioplan compound microscope and photographed with Kodak TMAX 100 film.

### *Northern Blot Analysis*

After fractionation on a 1.2% formaldehyde/agarose gel, poly(A)$^+$ RNA from 200 staged *Xenopus* embryos was transferred to Hybond-N+ (Amersham) in 0.05 *M* NaOH and hybridized according to the manufacturer's instructions. Radiolabeled probes were made by random priming using the 240- and 370-bp *Pst*I fragments of the cardiac troponin I cDNA. After washing in 1× SSC/0.1% SDS at 65°C, the filter was exposed to film for 3 days. The presence of equal amounts of RNA per lane was confirmed by probing with XMax-2 sequences (Tonissen and Krieg, 1994; data not shown).

## RESULTS

### *Isolation and Sequence of a Cardiac Troponin I cDNA Clone*

Complementary DNA clones containing troponin Ic sequences were isolated from a *Xenopus* tailbud tadpole head (Stage 28/30) cDNA library (Hemmati-Brivanlou *et al.*, 1990). The sequence of a full-length cDNA clone, pXTnIc, is shown in Fig. 1 together with the deduced amino acid sequence. At the amino acid level the sequence is about 76 and 64% identical to the troponin Ic sequences from bovine and avian sources, respectively (Leszyk *et al.*, 1988; Hastings *et al.*, 1991). Significantly, the *Xenopus* sequence contains a long N-terminal extension that is characteristic of troponin Ic proteins in other species. The *Xenopus* troponin Ic protein contains 243 amino acids and has a predicted molecular weight of 28.4 kDa.

### *Embryonic Expression*

Expression of troponin Ic in the *Xenopus* embryo was examined using whole mount *in situ* hybridization. Results presented in Fig. 2 show that all detectable troponin Ic mRNA is located in the heart at all stages examined. Low levels of staining are first visible in tailbud embryos (approximately stage 28) at about the time when cardiac mesoderm is beginning to form a simple tube (see Figs. 2A and 2B). The intensity of staining continues to increase as the embryo develops but remains heart-specific at all times (Figs. 2C and 2D). The appearance of troponin Ic transcripts in the tailbud embryo probably corresponds to the time at which cardiac terminal differentiation products are first expressed, since cardiac $\alpha$-actin (Logan and Mohun, 1993; T. Drysdale, unpublished observations) and XMHC$\alpha$ (Logan and Mohun, 1993) are first detected in the heart at this time. Of course, strong expression of cardiac $\alpha$-actin occurs in embryonic muscle much earlier in development (Mohun *et al.*, 1984; Hemmati-Brivanlou *et al.*, 1990).

While the whole mount *in situ* hybridization procedure described above provides excellent information concerning the spatial distribution of RNA, the method is not particularly sensitive to transcripts that are broadly expressed in the embryo and are therefore present at a low concentration. In order to determine the temporal regulation of troponin Ic we have performed RNA blot experiments using RNA from staged *Xenopus* embryos (Fig. 3). Troponin Ic RNA is first detected at stage 27, in precise agreement with the *in situ* hybridization results. The broad band, approximately 1 kb in length, may in fact represent two RNAs of similar size resulting from transcription of the two cardiac troponin I genes present in the *Xenopus* pseudotetraploid genome (Kobel and Du Pasquier, 1986). Importantly, while the expression of skeletal muscle structural genes commences at about stage 13 in *Xenopus* development (Mohun *et al.*, 1984), no transcription of troponin Ic is detectable until stage 28. In addition, RNase-protection experiments using RNA from dissected tailbud stage embryos shows that no troponin Ic expression is detectable in skeletal muscle tissue (manuscript in preparation). We conclude that troponin Ic expression is heart-specific at all stages of embryonic development.

### *Effects of Retinoic Acid on Heart Development*

Treatment of embryos with RA often results in reduction or elimination of visible heart tissue (Sive *et al.*, 1990; Osmond *et al.*, 1991; Stainier and Fishman, 1992). Is this effect of RA due to a failure of cardiac tissue to differentiate or a failure of cardiac tissue to migrate to the appropriate position in the embryo? Our first experiments involved RA treatment during early gastrulation, prior to commitment of heart tissue. As reported previously (Durston *et al.*, 1989; Sive *et al.*, 1990; Drysdale and Elinson, 1991) this treatment results in a dose-related loss of many anterior structures. As the RA dose increases, hatching gland, cement gland, olfactory pit, and eye all disappear or are reduced in size. The troponin Ic marker shows that this treatment also results in a loss of heart tissue. With 100 $\mu M$ RA treatment there is

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  1  GAATTATCCA GAAAACAGCT AAAGAGCCTG GACAAGATGTCTGATGAGGA AGAGGTAACC
                                          M  S  D  E  E  E  V  T

 61  TATGAAGAAG AGGAGGAAGA ATATGTTGAA GAAGAAGAAG AAGAAGTTGT GGCTCCTGAG
  9   Y  E  E  E  E  E  E  Y  V  E  E  E  E  E  V  V  A  P  E

121  CCACCAAAAC CTGCTCCTCC TCCTGCAGCT CCTCCACCTC TCATCCGCCG GCGCTCCTCG
 29   P  P  K  P  A  P  P  P  A  A  P  P  P  L  I  R  R  R  S  S

181  GCTAACTATC GGTCCTATGC CACAGAACCA CAGGTTAAGA GAAAACCCAA GATTTCTGCA
 49   A  N  Y  R  S  Y  A  T  E  P  Q  V  K  R  K  P  K  I  S  A

241  TCACGGAAGC TCCAACTGAA CACTATGATG TTGCAAATTG CTAAGGCAGA AATGGAACGT
 69   S  R  K  L  Q  L  N  T  M  M  L  Q  I  A  K  A  E  M  E  R

301  GAGGAAGAGG AGAGAGCTCG CGAGAAGGAG AGATACCTTG CAGAACACTG TCAGCCTCTG
 89   E  E  E  E  R  A  R  E  K  E  R  Y  L  A  E  H  C  Q  P  L

361  CAACTGTCTG GCTTGTCCCG ATCTGAACTG CAGGACCTGT GTCAGGAGCT TCATGCAAGA
109   Q  L  S  G  L  S  R  S  E  L  Q  D  L  C  Q  E  L  H  A  R

421  ATCGATGTAG TAGATGAAGA AAGATATGAC ATGGAAGCCA AAGTAAACAA AAACATAACT
129   I  D  V  V  D  E  E  R  Y  D  M  E  A  K  V  N  K  N  I  T

481  GAGCTCGAAG ACTTGAACCA AAAGATCTTT GACCTCCGTG GCAAGTTCAA AAAGCCAAAC
149   E  L  E  D  L  N  Q  K  I  F  D  L  R  G  K  F  K  K  P  N

541  CTCAGGAGGG TGCGTCTCTC TGCCGACGCC ATGATGATGG CGCTGTTGGG CACCAAGCAC
169   L  R  R  V  R  L  S  A  D  A  M  M  M  A  L  L  G  T  K  H

601  AAAGTCTCCA TGGATCTACG GGCCAATCTC AAACAGGTCA AGCAAACCAA AAAGGACGAT
189   K  V  S  M  D  L  R  A  N  L  K  Q  V  K  Q  T  K  K  D  D

661  GCAGACAAGG ATATAAGAGA AGTAGGTGAC TGGAGGAAGA ATGTGGATGC CCTGAGTGGT
209   A  D  K  D  I  R  E  V  G  D  W  R  K  N  V  D  A  L  S  G

721  ATGGAAGGCA GAAAGAAGAA GTTTGAATCC ACTGGGGCTG CTGCAGTTTA AGAAGGGGAG
229   M  E  G  R  K  K  K  F  E  S  T  G  A  A  A  V  *

781  ATTTCTGATG GATGAAGTGC AATTACTTCT GGACTGAAAT ATGAAAATTC TTCTTCGTTC

841  CAAGGCAGTT AATCTGAAAT GGTTAGAGCT GAATCCCCAA TAAAGCACCT TGACAGAGAA

901  TAGCAAAAAA AAAAAAAAAA AAAAAAA
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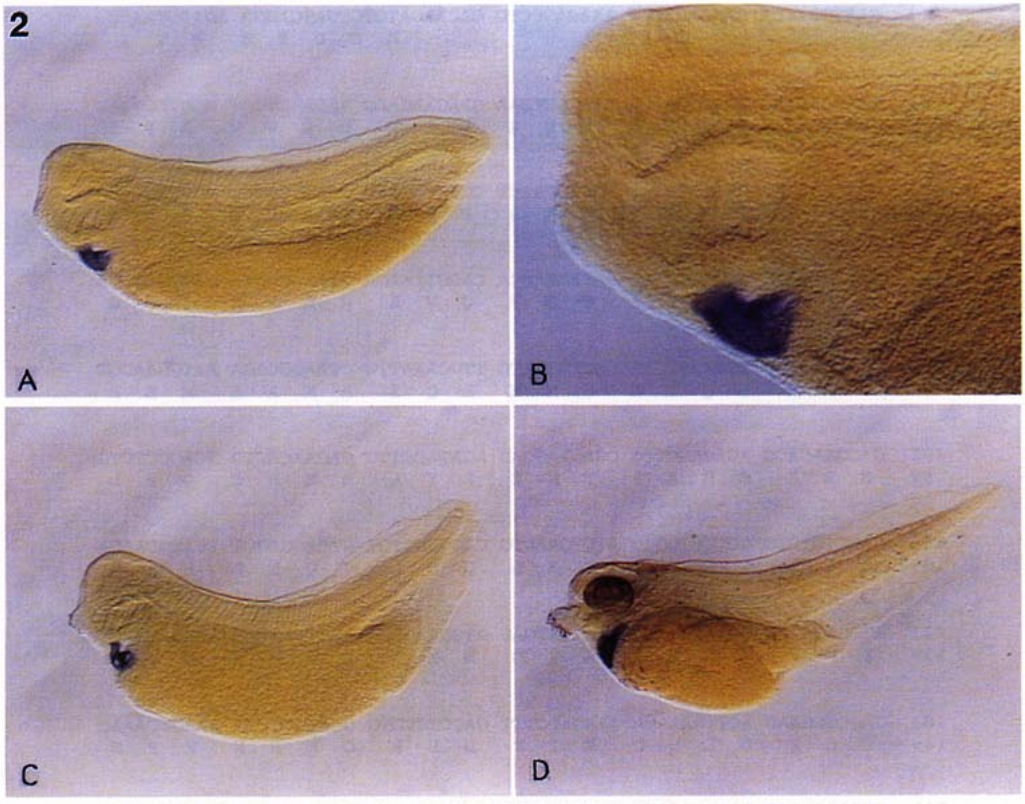
FIG. 1. The nucleotide sequence and derived amino acid sequence of pXTnIc, a *Xenopus* cardiac troponin I cDNA clone. The sequence is 904 bp long, not including the poly(A) tail. The nucleotide and amino acid residue numbers are indicated at the left of the figure. A consensus polyadenylation signal is underlined. The GenBank Accession No. for the sequence is L25721.

almost never any detectable heart tissue. With 10 $\mu M$ RA treatment some heart tissue appears to be present (Figs. 4B and 4E). It is important to note, however, that all cardiac tissue appears to successfully migrate to the ventral midline, even when only small amounts of heart tissue are present.

When *Xenopus* embryos were treated with RA at the time of neural fold closure (stage 15), after heart commitment has occurred (Sater and Jacobson, 1989), we observed no significant loss of anterior structures, although some minor alterations in morphology did occur. Alterations included an apparent shift of the cement gland to a position on top of the heart, rather than rostral to the heart, and abnormalities in the formation of the ventral half of the eye. Examination of troponin Ic expression in these embryos (Figs. 4C and 4F) showed that 10 $\mu M$ RA treatment caused a reduction in the size of the heart, although much less dramatic than treatments at stage 10, and that the hearts had abnormal morphology (Fig. 5). No variations in RA dose or time of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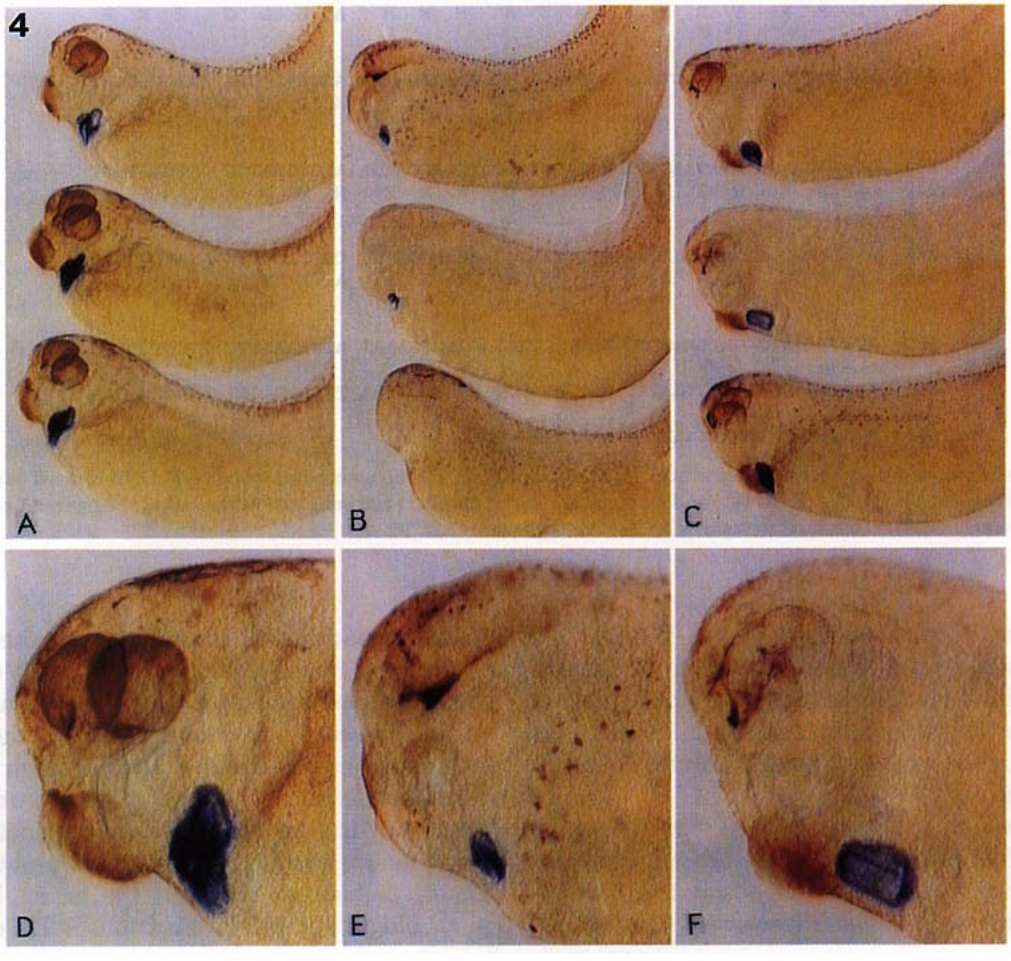
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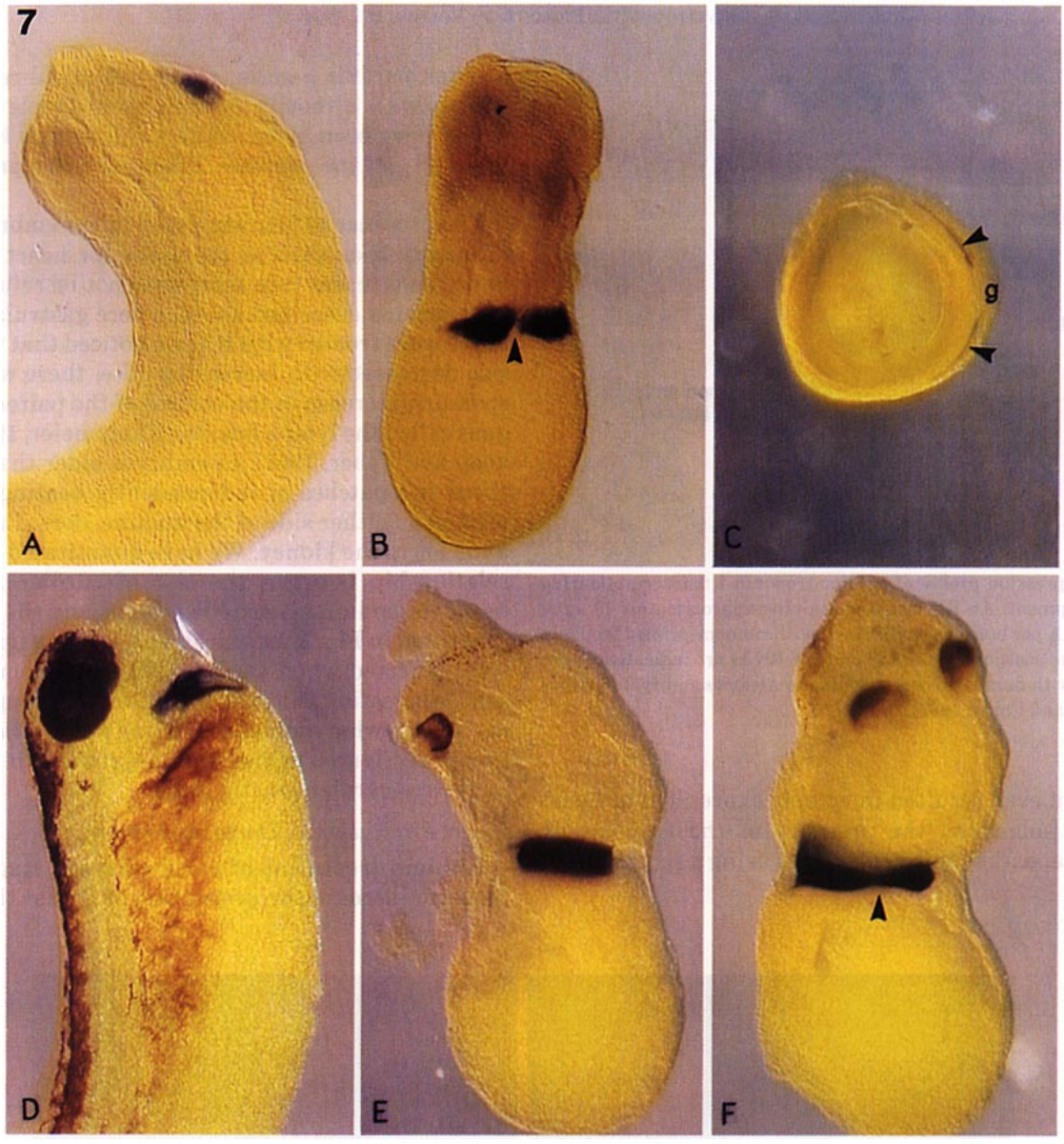


FIG. 7. Expression of troponin Ic in lithium-treated embryos. (A) The heart in a stage 28/30 control embryo. Note that it has already formed a simple tube at the ventral midline. (B) A DAI 8 embryo that has an enlarged heart region. Cardiac tissue is absent on the dorsal side of the embryo and has not fused at the ventral midline. (C) A top view of a DAI 9 embryo showing the distribution of heart tissue (arrowheads). Although not visible in its entirety in this view, the heart forms a ring that could be detected around the entire circumference with the exception of a gap (g) on the dorsal side. The heart tissue is just beneath the surface, indicating that it has not involuted. (D) A stage 38 control embryo with the heart undergoing looping and deformations that result in the final morphology. (E) A DAI 9 embryo viewed from the side. The heart now appears radial and is moving to the final location at the center of the embryo. The timing of this movement corresponds to the normal looping and folding events, rather than migration to the ventral midline. (F) A stage 38 DAI 8 embryo with an almost radial heart. The heart has begun to move to the center of the embryo but the most dorsal side of the embryo (arrow) is still thin.

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FIG. 2. Expression of troponin Ic (blue stain) is limited to the heart in *Xenopus* embryos. Troponin Ic expression was visualized by whole mount *in situ* hybridization using a digoxygenin-labeled antisense troponin Ic RNA. (A) A stage 28–30 *Xenopus* embryo showing that troponin Ic is expressed when the heart is still a simple tube. (B) Magnified view of (A) showing that expression is limited to the heart. (C) A stage 36 *Xenopus* embryo showing expression when the heart has the characteristic S shape as a result of looping. (D) Troponin Ic expression in a differentiated heart (stage 41).

FIG. 4. Heart morphology in embryos treated with retinoic acid. The heart morphology of control embryos at stage 38 is seen in (A), with an enlarged view of a control heart in (D). At the equivalent stage, embryos that were treated with 10 $\mu M$ RA at stage 10.5 (B) have hearts which are extremely reduced in size or absent (bottom embryo). An enlarged view (E) shows the significant reduction in heart size in RA-treated embryos. Note that many other anterior structures are also absent. (C) Embryos at the equivalent of stage 38 that were treated with 10 $\mu M$ RA at stage 15. These show almost normal morphology of anterior structures but the hearts are smaller than those in control embryos. The enlarged view (F) shows a failure of the heart to separate into distinct chambers. In all RA treatments, the heart tissue migrated to the ventral midline.

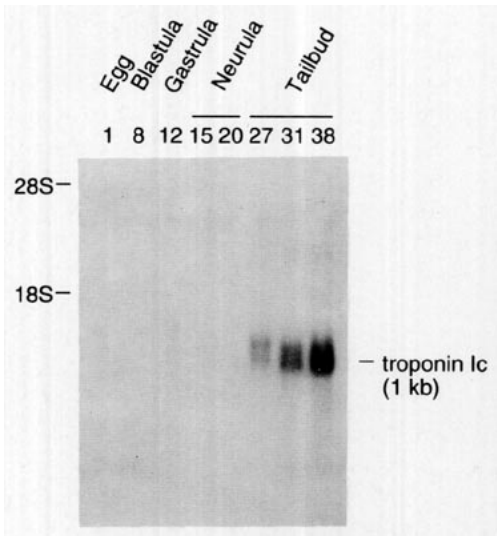


FIG. 3. Expression profile of cardiac troponin I transcripts during early development. An RNA blot, containing approximately 10 μg of poly(A)$^+$ RNA per lane, was hybridized with random-primed troponin Ic probes. The positions of the 18S and 28S RNAs are indicated. Lanes are labeled with numbers corresponding to *Xenopus* embryonic stages (Nieuwkoop and Faber, 1967).

application ever resulted in ectopic expression of heart tissue as judged by the presence of the troponin Ic marker. Repeats of this experiment using a second heart marker, cardiac α-actin, yielded identical results (data not shown), although detection of ectopic expression would have been more difficult due to high levels of expression in the somites (Hemmati-Brivanlou *et al.*, 1990).

The presence of beating tissue in the embryo has traditionally been used as the assay for heart formation, but in some cases this assay may not be reliable. In the experiments described above, where gastrula stage embryos were treated with RA, we noticed that while heart size decreased with increasing dose, there was a corresponding increase in the volume of the paired dorsal organs called the lymph hearts, (Kampmeier, 1922; Nieuwkoop and Faber, 1967). In embryos older than stage 40, these two patches of independently beating tissue are located on either side of the midline, dorsal and slightly anterior to the kidney. We have quantitated the inverse relationship between the size of cardiac and lymph heart tissue in RA-treated embryos and the results are presented in Fig. 6. In some cases, beating lymph hearts were detected when cardiac tissue was completely absent, suggesting that the traditional beating tissue assay is somewhat ambiguous. Using the *in situ* hybridization assay, lymph hearts were negative with both troponin Ic and cardiac α-actin probes.

*Heart Formation in Dorsalized Embryos*

Lithium treatment of early cleavage stage embryos tends to increase the proportion of dorsal tissues (Kao

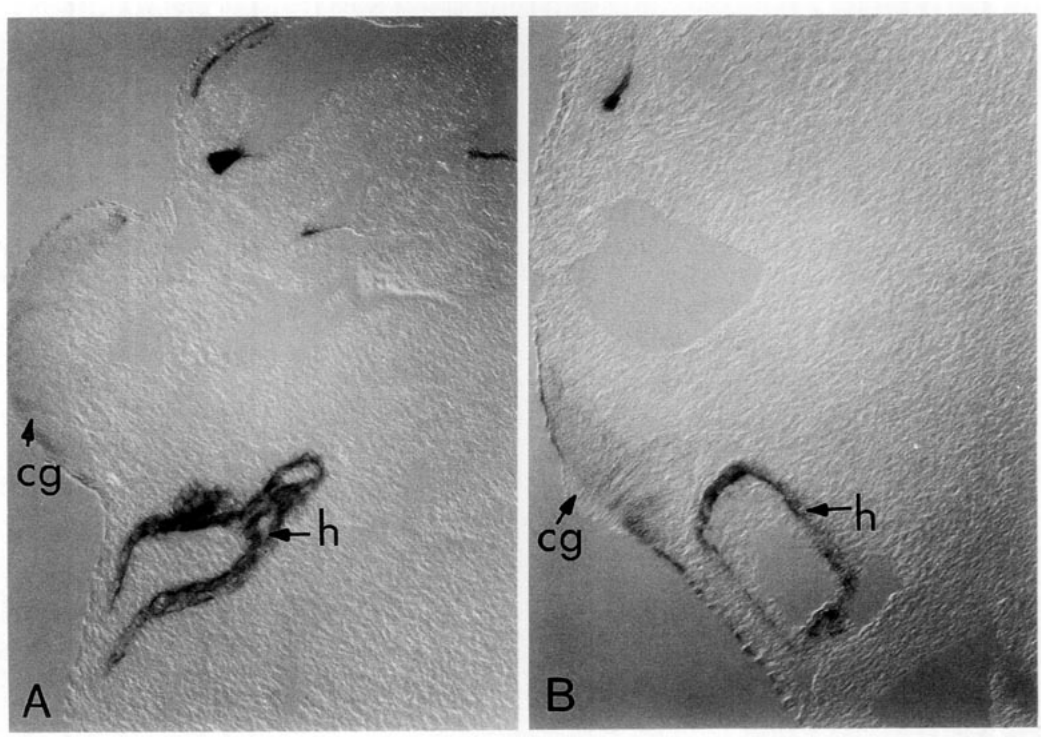


FIG. 5. Retinoic acid-treated embryos have abnormal hearts. The heart (h) of a normal stage 38 embryo in longitudinal section (A) is multichambered and is flattened against the gut (see Fig. 6). In (B), the heart (h) of the retinoic acid-treated embryo has a single large chamber that does not appear to have any of the normal constrictions or looping associated with normal cardiac morphogenesis. In both embryos anterior is to the left as indicated by the position of the cement gland (cg).

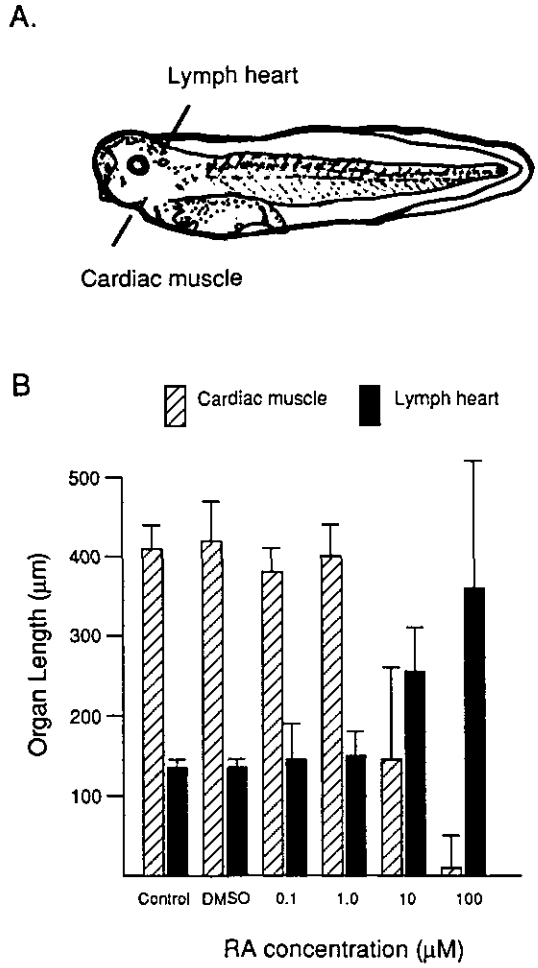


FIG. 6. Retinoic acid effects upon cardiac and lymph heart size. (A) The location of the cardiac muscle and the lymph heart in a normal embryo. (B) With increasing doses of retinoic acid, the length of the cardiac muscle diminishes and the length of the lymph heart increases. Error bars represent ±1 SD.

and Elinson, 1988). In extreme cases, lithium-treated embryos are radially symmetrical and completely lack detectable ventral structures. Since heart is derived from dorsal mesoderm, an increase in the size of the heart is expected in lithium-treated embryos. The results of experiments using troponin Ic probe to detect heart tissue in dorsalized embryos are presented in Fig. 7. In all cases the region of the embryo expressing the heart marker is enlarged and is distributed in a thin band almost completely surrounding the embryo. However, even in extreme dorsalized embryos (DAI = 9), a small region did not express the heart marker (Fig. 7C). This gap corresponds to the position of the cement gland, suggesting that it is the most dorsal region in these embryos. This region is probably derived from prechordal plate, which separates the heart primordia in normal embryos (Keller, 1976). Later in development, at the equivalent of stage 38–40, when the heart tube in normal embryos is bending and looping to take up its final form, the band of heart tissue in lithium-treated embryos begins to thicken and contracts toward the center of the embryo, (Figs. 7E and 7F). Despite this unusual series of morphogenic movements, a beating heart eventually forms in lithium-treated embryos.

## DISCUSSION

The amphibian embryo has traditionally been used for the study of heart development because of its large size and ease of manipulation (Copenhaver, 1926; Jacobson and Duncan, 1968; Sater and Jacobson, 1989, 1990a,b). Since heart tissue is of mesodermal origin, recent advances in understanding the induction and patterning of mesoderm (Smith, 1989; Kimelman *et al.*, 1992) make *Xenopus* an ideal organism for exploring the molecular basis of vertebrate heart specification. The cloning and characterization of markers specific for heart tissue in the *Xenopus* embryo (Logan and Mohun, 1993; Kelley *et al.*, 1993, and this study) will facilitate these molecular studies.

### *Embryonic Expression of Troponin Ic*

In both mammals and birds, troponin Ic expression is limited to heart tissue (Martin and Orlowski, 1991; Murphy *et al.*, 1991; Hastings *et al.*, 1991). Our experiments with *Xenopus* show that expression of troponin Ic is also heart-specific in the amphibian embryo. The specificity of expression contrasts with that of cardiac α-actin which is transcribed at high levels in the somites and other body musculature during early *Xenopus* development (Hemmati-Brivanlou *et al.*, 1990; Logan and Mohun, 1993).

Troponin Ic, cardiac myosin heavy chain (Logan and Mohun, 1993), and cardiac α-actin transcripts (Logan and Mohun, 1993) all appear in the heart in tailbud embryos at about stage 28, the time when the differentiation of the heart primordium begins (Nieuwkoop and Faber, 1967). This coordinate expression is in apparent contrast to the situation in mammals and birds, where cardiac muscle markers appear over an extended period (Lyons, 1994). Unlike many cardiac muscle genes (Olson, 1993), expression of troponin Ic is heart-specific at all stages of development. At present, the only troponin Ic promoter sequence to be reported is from the mouse (Ausoni *et al.*, 1994). Comparison of the *Xenopus* and mouse troponin Ic promoter sequences could be very useful for the identification of evolutionarily conserved DNA sequences involved in cardiac-specific gene expression.

### *Retinoic Acid Does Not Block Migration of Heart Primordia*

In chick embryos, a variety of RA-induced heart defects are observed depending on the timing and the dose

of RA administered (Osmond *et al.*, 1991). If the primitive streak chick embryo is treated with high doses of RA, no heart forms. At later stages, if RA is administered locally, near the determined cardiac mesoderm, migration of the cardiac mesoderm is prevented. This results in the formation of two patches of beating tissue located on either side of the dorsal midline behind the head. Our results with *Xenopus* show that RA does not effect the ability of cardiac mesoderm to migrate, even when the treatment is applied after heart specification (stage 15). When small hearts differentiate, they are always located in the normal midline site (Fig. 4).

Two patches of beating tissue, the lymph hearts, were observed on either side of the head in RA-treated embryos. Lymph hearts are contractile organs which pump lymph into the venous system and are found in nonmammalian vertebrates (Satoh and Nitatori, 1980). As shown in Fig. 6, there is an inverse relationship between the size of the lymph heart and the true heart in RA-treated embryos. Initially, it was tempting to speculate that the lymph hearts were derived from nonmigrating cardiac mesoderm, but we failed to detect expression of either troponin Ic or cardiac $\alpha$-actin in the lymph heart at any developmental stage. We conclude that the lymph heart is not closely related to cardiac tissue. It remains to be determined whether enlargement of these organs results from an increase in the amount of tissue specified to become lymph heart or is a response to the physiological stress of the RA phenotype.

### *RA-Induced Defects in Heart Formation*

When embryos are treated with RA early in development, prior to heart determination, the amount of tissue forming heart is sharply reduced (Fig. 4). At these early stages, it is not clear if RA is having a specific effect on heart formation or if the absence of heart tissue is a consequence of the general ablation of anterior structures. Our experiments show that heart formation is also affected in *Xenopus* embryos that are treated later in development, during early neurula stage, after heart is specified (Sater and Jacobson, 1989). At stage 15, a heart field exists in the *Xenopus* embryo. The heart field defines a region that has the potential to form heart; however, it is important to note that not all of this field will contribute to the heart itself. As development proceeds, tissue interactions restrict the size of the heart field (Sater and Jacobson, 1990b). This restriction is complete by late tailbud (stage 28) just before heart differentiation is first observed (Sater and Jacobson, 1990b). It is possible that exogenous RA exerts its effect by mimicking natural signals that reduce the size of the heart field. Treatment of embryos with excess RA would result in a smaller heart field and ultimately a reduction in heart size. Although RA is not generally thought to act by killing presumptive cardiac cells (Stainier and Fishman, 1992), it remains a possibility that small hearts result from the specific killing of cardiac mesoderm cells. Retinoic acid does not appear to interfere with the fusion of the two heart primordia as it does in chick (Osmond *et al.*, 1991).

### *Heart Development in Lithium-Treated Embryos*

Treatment of *Xenopus* embryos with lithium at the 32-cell stage, causes a dorsalization of the mesoderm (Kao and Elinson, 1988). Fate mapping of lithium-treated embryos suggests that cardiac mesoderm is still formed in radially dorsal embryos, but because of the excess dorsal tissue, gastrulation is inhibited. This inhibition prevents the presumptive cardiac mesoderm from involuting during gastrulation. Using the cardiac troponin I marker, we are able to confirm and extend these observations. In radially dorsal embryos, the heart tissue lies in a thin band immediately below the surface, indicating that it has not involuted (see Figs. 7B and 7C). The absence of cardiac troponin I expression in the most dorsal part of the DAI 9 embryo suggests that the two heart primordia do not migrate to the midline as they would in a normal embryo (Figs. 7B and 7C). This lack of migration could be due to the lack of ventral mesoderm which may provide migrational cues. Alternatively, since the mesoderm does not involute, it never contacts the substrates required for migration, presumably the blastocoel roof (Yost, 1992). The heart does move to the center of the lithium-treated embryo later in development. This occurs at the time when the heart tube of normal embryos is undergoing the complex looping and deformations which result in the mature heart. The movement of heart tissue toward the center thus appears to be a consequence of morphogenic movements and is probably not equivalent to migration of the cardiac mesoderm.

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