Induction of cardiac muscle differentiation in isolated animal pole explants of Xenopus laevis embryos

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SUMMARY
We have isolated a cDNA fragment encoding a portion of the myosin heavy chain α-isof orm (XMHCα) in the amphibian, Xenopus laevis. The XMHCα transcript is highly enriched in adult heart RNA and is expressed exclusively in embryonic heart tissue. It therefore provides a tissue-specific marker for cardiac muscle differentiation during early embryogenesis. Using an RNAase protection assay, we can detect the onset of cardiac muscle differentiation in an anterior, ventral region of tailbud embryos, many hours before the appearance of a beating heart. Whole-mount in situ RNA hybridisation indicates that expression of the XMHCα gene is restricted to the developing heart primordium. XMHCα gene expression can also be induced in isolated animal pole explants of blastulae by treatment with the growth factor, activin A. Induction is dose-dependent, requiring high doses of the growth factor compared with that required for myotomal (skeletal) muscle differentiation. In contrast, no XMHCα transcripts are detected in explants incubated with basic FGF, despite the induction of myotomal muscle differentiation. Activin-induced explants show a similar temporal pattern of XMHCα gene expression to that found in normal embryogenesis. Furthermore, cells expressing this gene appear clustered in one or two foci within fused explant aggregates, which often show regular, spontaneous contractions after several days in culture. These results show that terminal differentiation of cardiac muscle can occur in growth factor-induced explants and may be distinguished from skeletal muscle differentiation by the dose and nature of the inducing factor.

Key words: Xenopus, mesoderm, cardiac muscle, MHC, induction

INTRODUCTION
In amphibians, the heart is formed from the embryonic mesoderm as a result of a complex series of tissue interactions (reviewed in Jacobson and Sater, 1988). Although the developing heart lies ventrally in the tadpole, it is formed from dorsal anterior mesoderm. Fate maps of Xenopus gastrulae indicate that the heart is formed from paired, dorsolateral regions of the marginal zone that lie on either side of the blastopore lip (Keller, 1976). Similar results are suggested by specification maps of urodele embryos (see Jacobson and Sater, 1988). As a result of gastrulation movements, the precardiac mesoderm lies at the anterior end of the neurula, on either side of the neural plate. The initial step in cardiogenesis may therefore be considered to be the patterning of mesoderm along the future dorsoventral and anteroposterior axes.

Subsequent steps in cardiogenesis have been defined largely by embryological studies in which the capacity of amphibian embryo fragments to form beating hearts in culture has been monitored (Jacobson and Sater, 1988 and references therein). Migration of the anterodorsal, precardiac mesoderm towards the ventral midline culminates in their fusion to form the heart primordium. In urodeles, precardiac mesoderm of the early neurula only becomes specified to form heart through an inductive interaction with underlying endoderm tissue (Jacobson and Sater, 1988; Smith and Armstrong, 1990). Furthermore, ablation experiments show that the heart can be derived from a much larger region of lateral mesoderm than participates in normal cardiogenesis and can even form from tissue derived from a single side of the embryo (Copenhaver, 1955; Wilens, 1955).

Differentiation of cardiac tissue from specified, cardiac mesoderm is delayed for many hours until long after the formation of the fused primordium, suggesting that another interaction prevents the premature differentiation of the cardiac mesoderm in neurula and early tailbud embryos. Neural tissue may be the source of such an inhibitory influence (see Jacobson and Sater, 1988) and the existence of inhibitors within the mesoderm itself has also been postulated (Smith and Armstrong, 1991). Cardiogenesis also appears to be affected by tissue interactions that have been termed ‘formative’ since they are not essential for heart formation in explants, but increase the size and rate at which beating hearts are formed in culture.
(Fullilove, 1970; Jacobson and Sater, 1988). One source of such an influence is the head endoderm which underlies the cardiac mesoderm in the neurula embryo. The c mutation (Humphrey, 1972) in the axolotl, *Ambystoma mexicanum*, appears to affect this type of interaction since the mutant embryos fail to form normal hearts but possess disorganised cardiac tissue (Lemanski, 1973).

The molecular basis for the tissue interactions in vertebrate cardiogenesis is unknown. Such studies are hampered by the absence of molecular markers for any early stages in cardiac differentiation and the coexpression of many terminal differentiation products in both skeletal and cardiac muscle. For these reasons, differentiation of cardiac tissue has generally been scored by the appearance of a beating heart and this assay is of limited use in the study of early cardiogenesis. Furthermore, in amphibians, most studies of heart formation have been based on the relatively slow developing urodele embryos, in which the inductive interaction between pharyngeal endoderm and precardiac mesoderm can clearly be demonstrated. However, almost all molecular studies of development have used the anuran, *Xenopus laevis*. In this species, specification of the cardiac mesoderm occurs in the earliest stages of gastrulation and is not amenable to embryological study (Sater and Jacobson, 1989).

Here we present a model system to study the molecular events of cardiogenesis in *Xenopus laevis*. We describe the characterisation of a sensitive molecular marker for cardiac muscle in *Xenopus* embryos that detects terminal differentiation in the heart primordium many hours before the appearance of a beating heart. Using this marker, we demonstrate that cardiac muscle differentiation can be induced in isolated animal pole explants of blastulae by treatment with activin A. The use of such a system to investigate the earliest molecular events of cardiogenesis is discussed.

**MATERIALS AND METHODS**

**Isolation of RNA**

Total RNA from adult *Xenopus* tissues was isolated by a modification of the AGPC method. Each tissue was powdered in liquid nitrogen and dissolved in guanidine isothiocyanate buffer (10 M LiCl) overnight. After centrifugation (10 K, 15 minutes, 4°C), the RNA was washed twice with 70% ethanol, dissolved in water and reprecipitated. Total nucleic acid was extracted from embryos and cultured explants as previously described (Mohun et al., 1984). Poly(A)+ RNA was selected from adult heart RNA using the PolyA Tract system (Promega).

**RT-PCR**

Alignment of vertebrate MHC sequences from the EMBL database suggested the following two MHC-specific primers to use in a 3′ RACE procedure:

**MHC-1:** CTGTCCAAGTCCGCAAGGTGCAG
**MHC-2:** CAACAAGCTTGCGGCGCAAGCCG

These were used with oligo(dT)-adapter (GACTCGAGTCTGACATCG) and adapter (GACTCGAGTCTGACATCG) primers previously described (Frohman et al., 1988) to amplify a 3′ fragment of the *Xenopus* MHCα cDNA.

Heart cDNA was synthesised using poly(A)+ RNA selected from 50 µg of total heart RNA using MMLV reverse transcriptase (Gibco BRL) as described by the manufacturers. Synthesis was primed using the oligo(dT)-adapter primer (25 µg/ml) in a reaction volume of 20 µl and the product used directly for PCR amplification. Reactions were carried out in 50 µl (Saiki et al., 1989) using primers at a concentration of 2 µM.

In the first round of amplification, 1-5% of the cDNA reaction was used with the MHC-1 and oligo(dT)-adapter primers. In the first cycle, samples were denatured at 95°C (5 minutes), annealed at 55°C (60 seconds) and extended at 72°C (10 minutes). In the subsequent 29 cycles, the duration of each step was reduced as follows: denaturation (40 seconds), annealing (60 seconds), extension (150 seconds). 1% of the reaction was used for a second round of amplification with the nested MHC-2 and adapter primers under similar conditions, except that the annealing temperature was increased to 58°C.

The final PCR product was phenol extracted, precipitated with ethanol. After digestion with *XhoI* and *HindIII*, the amplified DNA was cloned into the phagemid pKS(+). Inserts were sequenced by the dideoxy method, using single-stranded phage DNA recovered from the clones. The *Xenopus* MHCα plasmid obtained in this manner was designated pXMHCα.

**RNAase protection assay**

In order to obtain a cDNA fragment suitable for use as an RNAase protection assay probe, heart cDNA was amplified using the PCR primer MHC-1 and third MHC-specific primer (MHC-3: GCCATTCACTGTGTGCTTCTGTGG) suggested from *Xenopus* MHCα DNA sequence. The PCR product was subcloned directly into a the Eco RV site of the phagemid, Bluescript KS(+), which had been tailed with dideoxy TTP (Holton and Graham, 1991) to generate the plasmid pXMHCα1/3. This contains a 149 nucleotide fragment of the *Xenopus* MHCα cDNA (nucleotides 1-149; Fig. 1A) attached to primer MHC1. An RNA probe was synthesised using *HindIII*-linearised pXMHCα1/3 as template and T7 RNA polymerase (Melton et al., 1985). The cardiac actin (Mohun et al., 1988) and XMMyoD (Hopwood et al., 1989) probes have been described previously. A second XMMyoD probe, pSP73-5bGXMMyoD (R. Wilson, unpublished) was used for the assays shown in Fig. 3B. An EF1α (Sargent and Bennett, 1990) probe synthesised at one fifth of the usual specific activity was used to provide an internal control for XMHCα assays. RNAase protection assays were performed under standard conditions (Zinn et al., 1989). Products of the T7 RNAase digestion reaction were resolved on 6% polyacrylamide denaturing gels using a radiolabelled HindII digest of pBR322 to provide approximate size markers.

**Whole-mount RNA in situ hybridisation**

Whole-mount RNA in situ hybridisations were performed exactly as described previously (Harland, 1991) using digoxigenin-labelleled RNA probes. These were derived from a subclone of pXMHCα in which the poly(A) tail had been removed by digestion with HindIII and *XhoI*, followed by fill-in and religation. The resulting plasmid, pXMHCα3, contains a 115 nucleotide fragment of the XMHCα cDNA (nucleotides 55-169; Fig. 1A) including 4 mismatches introduced by the PCR primer MHC-2. Antisense probe was synthesised using T3 RNA polymerase from pXMHCα3 linearised with HindIII. For controls, sense orientation probe was synthesised using T7 RNA polymerase and *KpnI*-digested template, which had been treated with T4 DNA polymerase.

**Embryo and explant culture**

Embryos were cultured at 18-21°C and staged according to
RESULTS

Cloning of Xenopus MHCα cDNA

A survey of muscle-specific proteins characterised in vertebrate skeletal and cardiac muscle confirmed that many muscle-specific gene products, such as the sarcomeric actins, are coexpressed in both skeletal and cardiac muscle tissue of embryos (see Bandman, 1992 for review). One candidate heart-specific marker was a myosin heavy chain α-isofrom. In chick embryos, transcripts of a heart-specific myosin heavy chain gene (VMHC1) have been detected by polypeptide and the entire 3′ tested, employing the unit definition described previously (Green et al., 1990). In early mouse embryos, the MHC gene is expressed exclusively in the cardiac tube (Lyons et al., 1990).

Using PCR primers derived from a comparison of vertebrate MHC sequences in the EMBL database, we amplified a 222 nucleotide fragment from adult heart cDNA, using the 3′ RACE procedure (Frohman et al., 1988). Sequencing revealed that this fragment comprises the final 37 carboxy-terminal residues of an MHC polypeptide and the entire 3′ untranslated region of the transcript including the poly(A) signal site (Fig. 1A). A similar cDNA fragment was also isolated by the same PCR strategy from embryonic heart RNA and sequencing confirmed that this was identical to the PCR fragment derived from adult heart RNA. The predicted amino acid sequence shows 81% identity to the corresponding carboxy terminal region of other vertebrate MHCα proteins and slightly less similarity (77%) to MHCβ isoforms. Alignment of the sequences reveals that the α- and β-isofroms are most divergent in the final seven residues (Fig. 1B). Over this region, the frog sequence, like that of the chick VMHC1, most closely resembles the MHCα rather than MHCβ sequences. The only other amphibian MHC sequence available is that derived from a newt cDNA clone, MHCc/s (Casimir et al., 1988), which shows only restricted similarity to the frog sequence and is most similar to the β-isofroms on the basis of it carboxy-terminal residues (Fig. 1B). We conclude that the Xenopus clone encodes the carboxy terminal fragment of an MHCα-like protein. Although this clone is a PCR-derived fragment rather than a cDNA library isolate, its independent isolation from different RNA sources, the conservation of appropriate sequence features (termination codon, poly(A) addition signal) and the high degree of similarity of its putative translation product to mammalian MHCs all indicate that the DNA sequence is authentic.

Expression of Xenopus MHCα during development

In order to determine the tissue specificity of XMHCα gene expression, we first examined the distribution of XMHCα transcripts in adult tissues using an RNAase protection assay. Transcripts corresponding to full-length protection of the Xenopus clone are highly abundant in adult heart RNA but undetectable in RNA from stomach, oviduct and liver (Fig. 2A, lanes 2, 3, 5 and 7). A low level is evident in lung RNA (lane 4) and also in skeletal muscle after prolonged exposure of the autoradiogram (lane 6). When the same tissue RNAs were tested for the presence of cardiac actin transcripts, the highest levels were detected as expected in adult heart (Fig. 2B, lane 7). In addition, low levels were detected in stomach and skeletal muscle samples (lanes 2 and 6, respectively), consistent with minor expression of this gene in adult striated and smooth muscle types (T. Mohun and E. Gionti, unpublished data). A low level was also detected in lung RNA (lane 4) suggesting the presence of contaminating muscle tissue (presumably from blood vessels) in the sample.
From Fig. 2A, it is evident that the MHCα transcripts can be detected in stage 42 tadpoles (lane 9), by which stage a beating heart has formed. We therefore examined total RNA from a range of developmental stages from neurula stage onwards to assess when the MHCα gene was activated during embryogenesis. Since the developing heart of the tadpole comprises only a small percentage of tissue when compared with the axial muscle of the myotomes, we dissected ventral and dorsal pieces from the anterior end of the embryo and compared their expression of cardiac actin, XMyoD and XMHCα genes (Fig. 3). Ventral pieces encompassed tissue immediately behind the cement gland, which includes the heart primordium (Sater and Jacobson, 1990a). When cultured until sibling embryos reached stage 42, such explants invariably differentiated to include beating hearts (data not shown). Equivalent-sized dorsal pieces were composed largely of myotomal muscle tissue (see Fig. 3D).

In Xenopus, as in other vertebrates, the cardiac actin gene is expressed in both skeletal and cardiac muscle in embryonic stages of development (Mohan et al., 1984). Cardiac actin transcripts were therefore highly abundant in dorsal pieces at all stages of development (Fig. 3A) since...
much of the tissue in these fragments comprises differentiating axial muscle. Similarly, XMyoD transcripts, which are expressed in skeletal but not cardiac muscle, were detected only in dorsal fragments, and were most abundant in the earlier staged embryos (Fig. 3B). This is consistent with the progressive decline in XMyoD gene expression reported for the myotomes of tailbud and tadpole embryos (Hopwood et al., 1989). In contrast, XMHCα transcripts were undetected in dorsal pieces at any stage of development (Fig. 3C). Expression was detected, however, in ventral pieces from late tailbud embryos (stage 28) and accumulated in this region during subsequent development (Fig. 3C, lanes 5, 7, 9 and 11). Cardiac actin transcripts were also detected in ventral pieces of late tailbud embryos (Fig. 3A, lanes 7, 9 and 11), and prolonged exposure of the autoradiograms indicated that they too could be detected as early as stage 28. This suggests that the XMHCα and cardiac actin genes are coexpressed during terminal differentiation of embryonic cardiac tissue.

From whole-mount in situ hybridisation studies (Hemmati-Brivanlou et al., 1990; data not shown), it is unlikely that the presence of cardiac actin transcripts in the ventral pieces indicates contamination with myotomal tissue. In pieces from neurula and early tailbud, XMyoD expression provides a reasonably sensitive marker for the presence of myotomal muscle and no transcripts are detected in the ventral pieces (Fig. 3B, compare lanes 2, 4, 6, 8 and 10 with lanes 3, 5, 7, 9, and 11, respectively). In older embryos, a partial protection product obtained with the XMHCα probe appears to provide a very sensitive marker for myotomal tissue (see Fig. 3C). We presume that this is obtained by cross-hybridisation of a skeletal muscle-specific MHC transcript. Whatever its origins, this protection product is not found with any ventral fragment RNA, despite its high levels in assays of dorsal, myotomal tissue (Fig. 3C, lanes 2, 4, 6, 8 and 10). Together, these results exclude the possibility that ventral pieces are contaminated with myotome and indicate that the *Xenopus* XMHCα cDNA encodes an isoform that is restricted to cardiac muscle in the early embryo.

To verify this, we examined the distribution of the XMHCα transcripts throughout the entire embryo, using whole-mount in situ hybridisation. No staining was obtained with a sense-orientation XMHCα probe at any stage of development (data not shown). However, with an antisense probe, we detected XMHCα mRNA in the stage 28/9 tadpole (Fig. 4A), indicating that the whole-mount in situ procedure was virtually as sensitive as the RNAase protection assay (cf. Fig. 3C, lane 5). The signal was localised beneath the ventral ectoderm, immediately behind the cement gland of the tadpole and increased in intensity during subsequent development. This region corresponds to the location of the developing heart primordium and also stains for the presence of cardiac actin transcripts (Hemmati-Brivanlou et al., 1990; data not shown). No XMHCα expression was detected in any of the head or body musculature and by stage 35, signal was clearly restricted to the developing heart anlage (Fig. 4B). These results confirm that the XMHCα transcript provides a specific molecular marker for differentiation of embryonic cardiac muscle tissue.
Induction of MHC\(\alpha\) expression in cultured animal pole explants

Cells from the animal pole of blastula-stage embryos normally form ectoderm and neural tissues (Keller, 1975). Their developmental fate can be changed by exposure to a variety of growth factors that induce the differentiation of mesodermal derivatives, including muscle (reviewed in Smith, 1989; Whitman and Melton, 1989; Dawid and Sargent, 1990; Sieve, 1993). The onset of muscle-specific gene expression following growth factor treatment approximates to the time course of myotomal muscle differentiation in the normal embryo but the actual character of the induced muscle has not been investigated.

To assess whether cardiac-type muscle could be induced to differentiate from animal pole blastomeres, we tested the ability of the potent mesoderm-inducing factor, activin A, to activate expression of the *Xenopus* MHC\(\alpha\) gene in cultured explants. A member of the TGF\(\beta\) superfamily, activin A is capable of inducing a broad spectrum of mesodermal derivatives, including those that are considered anterodorsal and others that are ventral-posterior in character (Green et al., 1992). Isolated animal caps were dissected from blastula (stage 8) embryos and cultured in the presence of recombinant human activin A (80 units/ml; see Materials and Methods). During culture, the majority of explants fused into aggregates (see below). The equivalent of 10-20 explants were harvested at various times up to four days after dissection, and analysed for the presence of cardiac actin, XMyoD and XMHC\(\alpha\) transcripts.

Cardiac actin mRNA was highly abundant in all the induced samples, indicating that extensive muscle differentiation had occurred in response to activin A treatment (Fig. 5A, lanes 2-8). The presence of XMyoD mRNA in all the samples (Fig. 5B) demonstrated that at least some of the induced muscle was of myotomal type (Fig. 5B). We detected much less XMyoD expression in explants harvested later than the equivalent of stage 22 (Fig. 5B, lanes 4-8) consistent with the decline in MyoD expression during normal development. However, all but the youngest explants showed the myotome-specific protected band when assayed for XMHC\(\alpha\) transcripts (Fig. 5C, lanes 3-8), consistent with the presence of abundant myotomal-type muscle in all the induced explants (cf. Fig. 2B).

In addition to inducing the differentiation of myotomal-like muscle, activin A also induced expression of the XMHC\(\alpha\) gene in the treated explants (Fig. 5C, lanes 5-8). Transcripts were evident after culture to the equivalent of stage 30 and longer exposures detect a low level of expression coincident with activation of the gene in the heart primordium of control tadpoles (data not shown). The level of expression increased during culture of the explants and after two days it was approximately 10-25% as abundant in explants as it was in equivalent-staged control embryos.

**Induction of cardiac muscle by activin A is dose-dependent**

Previous studies have shown that the range of mesodermal cell types that are induced in animal blastomeres by activin A treatment is affected in a dose-dependent manner, suggesting that gradients of inducing signal(s) may underlie orderly patterning of the mesoderm during normal develop-

![Fig. 5. Induction of XMHC\(\alpha\) expression in animal pole explants.](image)

**Fig. 5.** Induction of XMHC\(\alpha\) expression in animal pole explants. Activin-induced animal pole explants were assayed for the presence of cardiac actin (A), XMyoD (B) and XMHC\(\alpha\) transcripts (C) using an RNAase protection assay. P, undigested probe; lane 1, 10 \(\mu\)g tRNA; lanes 2-8, total RNA from induced explants cultured until sibling embryos reached stages 18, 22, 26, 30, 34, 38 and 42, respectively; lane 9, 3 \(\mu\)g of total tadpole RNA (stage 42). Full-length, protected fragments for each probe are indicated, as is the myotome-specific band (open triangle) obtained with the XMHC\(\alpha\) probe. The equivalent of four explants were analysed in each assay and contained similar amounts of total RNA, as judged by the level of EF1a transcripts (C).
activity (see Materials and Methods). These results suggest that cardiac muscle is only induced by relatively high concentrations of the growth factor compared with those required for differentiation of myotomal-like muscle.

**bFGF does not induce expression of MHCα in animal pole explants**

We next tested whether bFGF could also induce MHCα expression in the explant assay. Members of the FGF family, like those of the TGFβ superfamily, also possess the ability to induce mesodermal derivatives from animal pole explants (Kimelman and Kirschner, 1987; Slack et al., 1987). The inducing activity of bFGF is distinguished from activin A by the absence of notochord tissue in cultured explants and a relative reduction in the proportions of other dorsal mesodermal cell types. Consistent with this, we found that *Xenopus* bFGF failed to induce any MHCα expression in explants treated with a range of concentrations, from 10 to 120 units/ml despite the induction of the cardiac actin gene at all doses (compare Fig. 6A and 6B, lanes 3-7). Taken together, our results demonstrate that the induction of cardiac and myotomal muscle differentiation in animal pole explants can be distinguished by both the dosage and the nature of the mesoderm-inducing factor.

From Fig. 6, it is evident that there is a non-linear relationship between the level of cardiac actin mRNA detected and the dose of bFGF used to induce the explants. After normalisation with respect to the EF1α signal, direct quantitation confirms that higher doses of bFGF reduce the level of the actin mRNA (data not shown) although the precise shape of the curve varies considerably between experiments. This is consistent with previously published studies. A bell-shaped dose-response relationship has been described for disaggregated animal pole cells (Green et al., 1992), whilst the results for isolated explants are quite variable (compare table 2 with fig. 3 in Green et al., 1990).

### Cardiac muscle cells are clustered in cultured explants

Histological studies demonstrate that whilst induced animal pole explants comprise several distinct cell types, they often show some degree of cellular organisation (for example Sokol and Melton, 1991). Induced muscle cells are frequently clustered in a single or several blocks and notochord-like structures have also been described. The ability of induced explants to acquire cellular pattern is an intriguing phenomenon that has important implications for any model of mesoderm induction during normal development. It may, for example, arise from the varying doses of inducing factor received by cells in different locations within the explant. It may also reflect a prepattern within the explant (Sokol and Melton, 1991; Bolce et al., 1992) or cooperative interactions between adjacent cells (Gurdon, 1988). Finally, it is possible that signalling events that are proposed to be necessary for patterning of the mesoderm during normal development (Slack and Forman, 1980) also occur within the induced explant.

We have examined the distribution of XMHCα-staining cells within induced explants using RNA whole-mount in situ hybridisation. Interestingly, we found that staining was only detected in explants that had fused into aggregates during the course of the culture period. Isolated explants showed no detectable staining, suggesting that fusion was in some way necessary for MHC expression (compare Fig. 7A with 7B). Similar results were obtained when single and fused explants were tested by RNAase protection assay (data not shown). Within aggregates, staining for the MHCα transcript was invariably restricted to one or two foci, indicating that the cardiac muscle cells were clustered together (see Fig. 7A). In no case was the staining distributed throughout the explant, although we cannot entirely exclude the possibility that individual MHCα-positive cells would be undetected by this assay.

![Fig. 6. XMHCα expression in explants is induced by activin A but not by bFGF. Blastula animal pole explants were induced with bFGF or activin A and cultured until control embryos reached stage 42. Total RNA was assayed for cardiac actin (A) and XMHCα mRNA (B) by RNAase protection. P. Undigested probe; lane 1, 10 µg tRNA; lane 2, uninduced explants (control for bFGF); lanes 3-7, explants induced with 10, 20, 40, 80 and 120 units/ml of *Xenopus* recombinant bFGF; lane 8, uninduced explants (control for activin); lanes 9-11, explants induced with 8, 32 and 80 units/ml of human recombinant activin A. The equivalent of four explants were analysed in each assay. The level of cardiac actin mRNA detected should be normalised by reference to the signal obtained for the cross-hybridising cytoskeletal actin transcripts (A). In the XMHCα assays (B), the EF1α probe was included to monitor the relative amounts of explant RNA.](image-url)
DISCUSSION

We have isolated a fragment of the Xenopus MHC cDNA that encodes the carboxy terminal portion of the \( \alpha \)-cardiac isoform. Although the cDNA fragment was obtained by PCR, its sequence appears authentic since an identical sequence was obtained from both adult and embryonic heart RNA and the fragment is fully protected in RNAase protection assays. Like its avian (Bisaha and Bader, 1991) and murine (Lyons et al., 1990) counterparts, the XMHC\( \alpha \) transcript is highly abundant in adult cardiac muscle. We have detected no expression of the XMHC\( \alpha \) gene in skeletal muscle from the leg of adult frogs but we have not established whether expression is detectable in any other skeletal muscles. In Xenopus embryos, the XMHC\( \alpha \) gene is expressed exclusively in the developing embryonic heart and provides the first molecular marker for terminal differentiation of cardiac rather than skeletal muscle. We have detected the onset of XMHC\( \alpha \) gene expression in an anterior, ventral region of the tailbud embryo, many hours before the morphological appearance of the embryonic heart tube. Using this marker, we have been able to distinguish between the induction of cardiac and skeletal-type muscle differentiation in growth factor-treated explants from blastula embryos.

A threshold response to activin

Our main finding is that cardiac muscle differentiation can be induced in isolated animal pole explants by exposure to activin A but not bFGF. This is consistent with the observation that the heart is derived from anterodorsal mesodermal rudiments. bFGF induces mesoderm of a more ventral and posterior character than activin A, exposure to which can induce a complete spectrum of mesodermal derivatives. Dose-response experiments using cultured explants are difficult to interpret since the exposure of different cells within the tissue fragment to the inducing factor presumably varies. Nevertheless, an increasing proportion of dorsoanterior tissue types are induced as the concentration of activin A is increased (Green et al., 1990) and disaggregation experiments have shown a precise dose-response of individual animal blastomeres (Green and Smith, 1990; Green et al., 1992). These findings may account for our observation that expression of the cardiac muscle-specific MHC\( \alpha \) gene is only induced by high concentrations of activin A. Several other factors (Wnt1, Wnt8 and noggin) have been described that can rescue dorsal mesodermal derivatives in ventralised Xenopus embryos (Smith and Harland, 1991, 1992; Sokol et al., 1991). It will be interesting to determine whether these factors, alone or in combination with bFGF, will induce MHC\( \alpha \) gene expression in blastula explants.

Dispersed animal pole cells show distinct thresholds in their response to small differences in activin and FGF concentrations, suggesting that complementary gradients of these factors could underly mesodermal patterning in normal development (Green et al., 1992). Interestingly, cardiac actin gene expression was only induced by a narrow range of concentrations in these experiments and was not detected with high doses of activin. From the present results, we would predict that this gene in fact shows a biphasic pattern of induction. At lower doses, expression would result from the differentiation of skeletal (myotomal) muscle and this would be detected within hours of treatment. At higher doses, prolonged culture should result in a second window of cardiac actin gene transcription due to the induction of cardiac muscle differentiation. This would not have been
detected in the study of Green et al. (1992) since the induced cells were harvested at the neurula stage, long before the onset of cardiac differentiation.

**Cardiogenic differentiation in animal pole explants**

Embryological studies have distinguished successive steps in the formation of the amphibian heart from mesodermal tissue. In *Xenopus*, specification of the cardiac mesoderm depends on the presence of the blastopore dorsal lip, which can also repattern other regions of lateral mesoderm to form heart tissue (Sater and Jacobson, 1990b). This indicates that a ‘dorsalisising’ signal (Slack and Forman, 1980; Lettice and Slack, 1993; Smith et al., 1993) is necessary for establishment of heart mesoderm. In urodeles, specification of a cardiogenic fate also requires an inductive interaction between cells of the dorsolateral mesoderm and the underlying pharyngeal endoderm. In the anuran, *Xenopus laevis*, it has proved impossible to test whether this is also necessary, since the precardiac mesoderm becomes specified early in gastrulation before it is feasible to distinguish the target tissue from the proposed inductive source (Sater and Jacobson, 1989).

Are similar interactions responsible for MHCα gene expression in activin-treated caps or does the inducing factor act by directly initiating terminal differentiation of cardiac tissue? We consider it unlikely that activin acts by short-circuiting the normal steps of cardiogenesis for several reasons. Direct activation of the cardiac myogenic programme by activin might be expected to result in precocious expression of the MHCα gene and the early detection of spontaneous contractile activity. In contrast, we have found that MHCα gene expression in activin-induced explants follows a similar time course to that detected for the gene in normal development. Furthermore, contractile activity is only detected after prolonged culture and mirrors the appearance of beating in cultured heart explants. In normal development, terminal differentiation of cardiac muscle is likewise delayed many hours after specification in both urodeles and anurans and the activin-induced explants appear to recapitulate this process. It is noteworthy that, in axolotl embryos, treatment of cardiac mesoderm explants from neurulae with murine activin A prior to their specification has no stimulatory effect on heart formation in contrast to the effects of several other growth factors (Muslin and Williams, 1991). Activin does not therefore directly activate the cardiogenic programme in this species. Finally, the acquisition of limited, albeit disorganised, patterning of cell types within induced *Xenopus* animal pole explants indicates that differentiation of individual blastomeres is indeed influenced by subsequent cellular interactions. It is therefore conceivable that at least some of the cell signalling events required for heart differentiation in normal development (such as dorsalisation) could also occur within induced explants.

We suggest that activin induction of animal pole explants effectively reproduces most of the essential interactions of normal cardiogenesis and provides a useful model system for their investigation. Although the role of pharyngeal endoderm in the induction of cardiogenesis cannot be demonstrated in *Xenopus*, we note that this tissue, like the mesoderm itself, is a product of animal hemisphere cells, in response to a vegetal inductive influence (Nieuwkoop et al., 1985). Such tissue may also therefore be induced by activin treatment and play an instructive role in the formation of cardiac muscle tissue. This proposition cannot be tested directly until endoderm-specific markers become available. However, it may be possible to examine whether individual induced blastomeres are capable of cardiac muscle differentiation in the absence of cell-cell interactions (Godsave and Slack, 1991).

Induced explants not only activate MHCα gene transcription in discrete foci within the fused aggregates, they also frequently form spontaneously contracting tissue (M. L. and T. M., unpublished observations) indicating that relatively complete cardiac muscle differentiation has occurred. Similar foci of cardiac muscle have been described in cultured explants of specified cardiac mesoderm from axolotl embryos (Muslin and Williams, 1991). Interestingly, the presence of underlying endoderm results in more extensive differentiation and the formation of a heart loop in the axolotl explants, indicating a continued role for endodermal influences in cardiogenesis. We have never observed similarly organised heart structures in induced animal pole tissue, suggesting that the ‘formative’ interactions necessary for heart morphogenesis are absent from *Xenopus* explants.

It is intriguing that we have only detected cardiac muscle differentiation in aggregates of explants that have fused during the course of culture. We presume that at least one of the necessary steps in the induction of cardiogenesis requires a minimum of tissue, although we have no indication of which step this might be. The precise number of explants required to fuse before MHCα could be detected is difficult to assess due to inevitable variation in the exact size of explants dissected, the loss of some material during prolonged culture and the extensive morphological changes that occur. We have observed MHCα expression in aggregates comprising apparently two or three explants (see for example Fig. 7A). A comparison of cardiac induction in differently sized explants and in cultures in which fusion is delayed will help to define further this phenomenon.

**Molecular analysis of cardiogenesis**

Despite extensive embryological characterisation, remarkably little is known about the molecular basis for the regulation of cardiogenesis. A major obstacle has been the dearth of useful model systems that facilitate genetic and biochemical analysis of cardiac muscle differentiation. Furthermore, despite the similarities of their genetic programs, no cardiac equivalents of the myogenic determination genes have so far been identified. The ability to induce cardiac muscle differentiation in *Xenopus* animal pole explants is of practical significance since relatively large amounts of induced tissue can readily be obtained and the type of muscle differentiation that occurs can be regulated by the dose of inducing factor. It therefore provides a model system for cardiogenesis which is amenable to molecular study.

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