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Expression of the cardiac actin gene in axolotl embryos

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ABSTRACT Axolotls are an important model system for studying heart development. Patterning of the somitic mesoderm occurs in axolotls in a manner that is much more similar to the pattern observed in higher vertebrates than in *Xenopus*. For these reasons we cloned the axolotl cardiac actin gene, since this gene is expressed during the development of both somitic and cardiac muscle in other vertebrates. In this paper we characterize its expression. Expression of cardiac actin RNA is switched on during gastrula stages and appears in the somitic mesoderm when it is formed; expression is later activated in the embryonic heart. In adults the gene is expressed only in the heart. The results demonstrate that the clone encoding cardiac actin provides a useful marker for studying development of both skeletal and cardiac muscle development in axolotls.

KEY WORDS: *axolotl, urodele, Xenopus, somite, heart.*

Introduction

Animal genomes encode several different forms of actin proteins with a wide variety of functions. Among these are muscle specific forms of actin which comprise essential components of the muscular contractile apparatus. In vertebrate species at least four distinct forms of muscle specific actin are known to exist (Vandekerckhove and Weber, 1979). Of the actins found in striated muscle, one form is expressed predominantly in adult skeletal muscle, and another form is expressed mostly in the cardiac muscle of the adult heart (Vandekerckhove and Weber, 1978; 1979). The two smooth muscle actins are expressed in the vascular and intestinal tissues, respectively.

Skeletal muscle and cardiac muscle arise from different locations during vertebrate development. Skeletal muscle is formed from the somites, which are dorsally positioned along the anterior-posterior axis of the embryonic body trunk. Cardiac muscle, on the other hand, initiates development within the heart-field, derived from an anterior region of lateral plate mesoderm (Buckingham *et al.*, 1992). Amphibian embryos, both urodele and anuran, have been widely used as experimental systems to address questions concerning the mechanisms that regulate development of both cardiac and skeletal muscle. From such studies it has become clear that cardiac muscle development is regulated differently in anurans and urodeles (Jacobson and Sater, 1988). Comparisons of the mechanisms controlling skeletal muscle development, however, have lead to much less certain conclusions.

By far the most widely studied amphibian embryos are those of the anuran *Xenopus laevis*. In *Xenopus* embryos the commencement of muscle development is evident during the mid-gastrula stages (Hopwood *et al.*, 1989; Scales *et al.*, 1990), and the muscle actin genes begin to be transcribed in presumptive somitic mesoderm in late gastrulae (Mohun *et al.*, 1984). Synthesis of muscle actin proteins commences shortly thereafter (Ballantine *et al.*, 1979; Sturgess *et al.*, 1980). These data, together with results from other more classical studies (Slack and Forman, 1980), indicate that muscle specification is completed in *Xenopus* embryos by the end of the gastrula stages. By comparison, much less is known about the initiation of skeletal muscle development in urodele embryos, particularly at a molecular level. To date, available evidence suggests that muscle actin proteins do not begin to accumulate in the somitic mesoderm of axolotl embryos until about stage 25 (Mohun *et al.*, 1980; Meuler and Malacinski, 1985), the headfold stages, long after gastrulation, but at just about the same time that the somitic mesoderm becomes irreversibly committed to muscle development (Forman and Slack, 1980). From these results it is assumed that skeletal muscle specification, like other events associated with cyto-differentiation, occurs later in urodeles than in anurans (Meuler and Malacinski, 1985; Slack, 1989).

Since the emergence of *Xenopus* as the amphibian embryo of choice for molecular studies, relatively little work has been done at a molecular level with axolotl embryos. However, urodeles represent a less derived taxon than anurans (Duellman and Trueb, 1985), and therefore may provide insights into the basal processes of terrestrial vertebrate development that can not be obtained with anurans. One good example of such a process is somite patterning. In urodeles somite patterning occurs through an epithelial rosette intermediate

Abbreviations used in this paper: MBT, midblastula transition.

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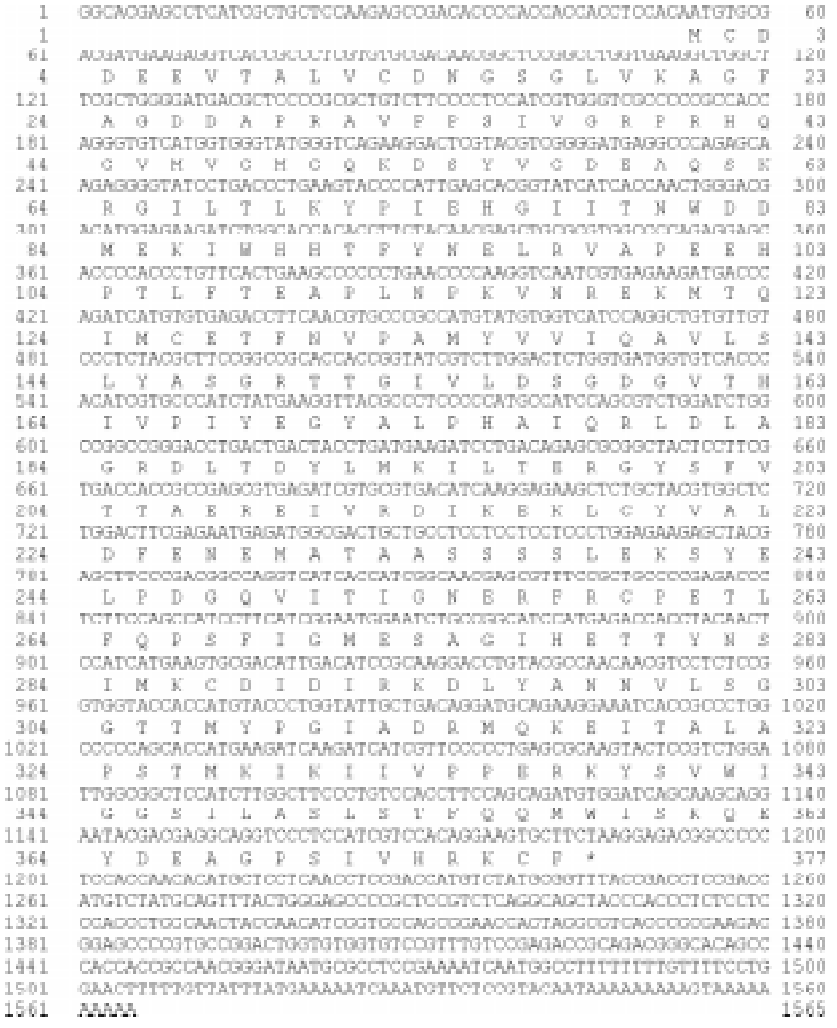

Fig. 1. Sequence of an axolotl alpha-actin cDNA. *Nucleotide and amino acid sequence of the transcript encoded by Ax-α-actin (Genbank Accession # AF276076). The protein sequence is 98% identical to Xenopus cardiac actin.*

(Youn and Malacinski, 1981b) as it does in all other terrestrial vertebrates, but somite patterning in anurans occurs through a mechanism that appears to be unique (Youn and Malacinski, 1981a). For this reason urodele embryos, such as those of the axolotl, might provide a better model system for understanding the mechanisms that govern somite patterning in higher vertebrates.

As a first step towards understanding the molecular mechanisms governing the development of somitic mesoderm in axolotl embryos we isolated a clone encoding cardiac actin from an embryonic cDNA library. In other species that have been tested the cardiac α–actin gene is first expressed in the developing somites, where it is co-expressed with skeletal α–actin. Later, in adults, its expression becomes restricted to heart muscle (Mohun *et al.*, 1984; Paterson and Eldridge, 1984; Lyons *et al.*, 1991). For this reason the expression of the cardiac α–actin gene has been widely used to designate the differentiation of skeletal muscle during *Xenopus* development (Gurdon *et al.*, 1989).

Here we report the expression profile of the axolotl cardiac actin gene during embryogenesis. We show that the gene is first expressed in the somites, and then later expression is observed in the embryonic heart. Significantly, we show that the gene becomes transcriptionally activated during the gastrula stages, which is when the cardiac actin gene is first expressed in other vertebrates. These results demonstrate the usefulness of this sequence as a molecular marker for studying the development of the skeletal and cardiac muscle in axolotl embryos. Our results, moreover, challenge previous conclusions that urodele (e.g., axolotl) embryos exhibit delayed cardiac actin expression compared with *Xenopus*: its mRNA appears to be expressed relatively early, rather than late, as previous reports have emphasized.

Results

Isolation of pAx–α–actin

From screening of 10^6 independent plaques with *Xenopus* cardiac actin cDNA a single cDNA clone containing an insert of 1565bp was sequenced to completion (Fig. 1). Because the clone length closely matches the size of mRNA detected on Northern blots (1.8kb), and due to the length of 5' and 3' untranslated regions, we presume that the clone represents a full length mRNA. The sequence of the clone, Ax–α–actin, encodes a predicted protein of about 41kd that is 98% identical to *Xenopus* cardiac actin (Stutz and Spohr, 1986).

Adult expression of Ax–α–actin in the heart

During vertebrate embryogenesis skeletal and cardiac muscle actin genes are co-expressed first in the developing somites, then later in the embryonic heart. In adults, however, cardiac actin is the predominant form in heart, and skeletal actin is the most abundant form in the skeletal muscle (Mohun *et al.*, 1984; Paterson and Eldridge, 1984; Sassoon *et al.*, 1988). Thus, expression in adult tissue has been used diagnostically to determine the identity of the two highly related actin isoforms (Mohun *et al.*, 1984).

To determine how the transcript encoded by Ax–α–actin is expressed in adults, and therefore reveal its identity relative to the muscle specific actin isoforms, we prepared a Northern blot containing RNA from various adult tissues and reacted it with Ax–α–actin probe. A strong signal of about 1.8 kb is observed in the lane corresponding to RNA from the heart that is not found in the RNA from any other tissue (Fig. 2A). A higher molecular weight transcript appears in most of the tissues tested (indicated by arrow), which we presume to represent cross-reaction with RNA encoding cytoskeletal actin. As a positive control the filter was stripped and rehybridized with a probe corresponding to the axolotl EF-1α cDNA, which is expressed in all tissues (Fig. 2B). Heart specific adult expression indicates that Ax–α–actin encodes the cardiac α-actin isoform, not the product of the skeletal α-actin gene, and this is consistent with the degree of homology that the transcript encoded by Ax–α–actin shares with the respective *Xenopus* genes.

Cardiac actin expression commences during the gastrula stage

In *Xenopus*, transcription of both skeletal and cardiac α–actin genes begins at a low level in the dorsal mesoderm during gastrula stages, prior to the overt differentiation of the somitic mesoderm (Mohun *et al.*, 1984), and earlier work suggests that muscle actin protein is produced in early gastrulae (Ballantine *et al.*, 1979; Sturgess *et al.*, 1980). This is quite different than the results obtained with axolotls. Existing evidence shows that in axolotls α–actin proteins are not detectable until the tailbud stage of embryogenesis (stage 26 Bordzilovskaya *et al.*, 1989), when they are produced in the somites (Mohun *et al.,*1980; Meuler and Malacinski, 1985). This suggests that skeletal muscle development is considerably delayed in axolotls relative to *Xenopus*. However, it is impossible to determine how muscle development is regulated without first knowing when the α–actin genes become transcriptionally active.

To determine if the axolotl cardiac α–actin gene begins transcription at around stage 25, when α–actin protein is first detected, or if transcription begins at an earlier stage, as it does in *Xenopus* and other species, RNA from staged embryos was used to prepare a Northern blot, and this was hybridized with cardiac actin probe. Figure 3A shows that the first cardiac actin transcripts appear at a low level during the early gastrula stage, stage 10 (Bordzilovskaya *et al.*, 1989), well before synthesis of the newly made protein has been detected. The blot was later reacted with a probe corresponding to EF-1α (Fig. 3B), which is switched on during the midblastula transition (MBT), and therefore is among the earliest genes to be transcribed from the zygotic genome (Krieg *et al.*, 1989). The results show that zygotic EF-1α transcripts begin accumulating at about stage 9, several hours prior to the commencement of gastrulation. Thus, expression of the cardiac actin gene does not result from the global transcriptional activation that occurs during the MBT, but instead is probably triggered by cell specification events that are initiated during gastrulation. In this way the timing of cardiac actin gene expression in axolotls is in accord with *Xenopus* (Mohun *et al.*, 1984) and other vertebrates (Hayward and Schwartz, 1986; Buckingham *et al.*, 1992).

Cardiac actin transcription begins in the somites and later appears in the heart

In vivo radiolabeling experiments with axolotl embryos first detect α-actin protein at about stage 26, and its expression is restricted to the somites (Mohun *et al.*, 1980; Meuler and Malacinski, 1985). However, our results show that at least one of the messages that encode α-actin protein begins to be transcribed at stage 10, more than a day earlier. Our clone encodes the cardiac α-actin isoform, which in adults is found only in the heart, presenting the possibility that in axolotls cardiac α-actin is not expressed in somites, as it is in other vertebrates. To test this we dissected stage 22 embryos into various parts, some of which contain the somites, as indicated in Fig. 4A. RNA from the various sections was used to prepare Northern blots, that were probed to detect cardiac actin mRNA. The results (Fig. 4B) show that by as early as stage 22, shortly after the somites begin to segment (Bordzilovskaya *et al.*, 1989), cardiac actin RNA can be detected in those regions of the embryo that contain somites, but it is not found in ventral regions, or in the endoderm, regions that do not contribute to the development of striated muscle. Moreover, the results show a gradient of cardiac actin mRNA, with high levels in anterior sections and much lower levels in posterior sections, indicative of the normal anterior to posterior sequence of events that normally govern somitic development (Hopwood *et al.*, 1989).

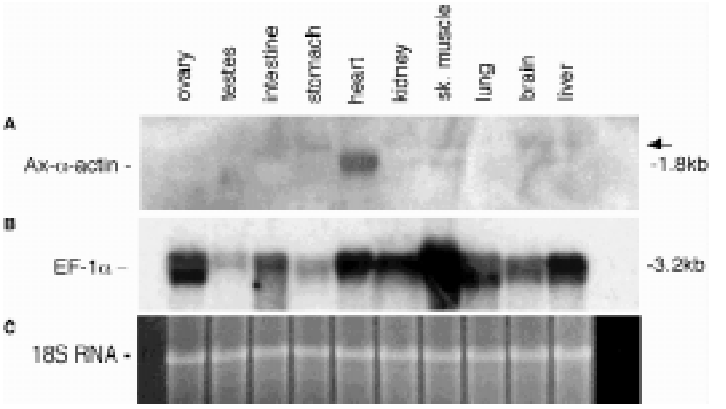

Fig. 2. Ax-α-actin encodes a heart-specific transcript in adults. *10μg of total RNA from the indicated tissues was Northern blotted.* **(A)** *Filter hybridized with Ax-α-actin probe. Ax-α-actin RNA is indicated by 1.8 kb band. Higher molecular weight species that we presume to encode cytoskeletal actin are indicated by an arrow.* **(B)** *The same blot as in A was stripped and reprobed with axolotl EF-1α sequences. EF-1α RNA is indicated by a 3.2 kb band.* **(C)** *18S ribosomal RNA from each tissue stained with ethidium bromide.*

By stage 35 cardiac actin mRNA levels have increased substantially, and at about this time, differentiation of the embryonic heart becomes discernible. To examine the distribution of cardiac actin mRNA in these later-stage embryos we performed whole-mount in-situ analysis (Harland, 1991). Figure 5A shows that cardiac actin is detectable only in the somites (arrows) at stage 35 by this method, and Figure 5B, performed with a sense strand control, demonstrates the specificity of hybridization in these experiments. We next used in-situ hybridization to sectioned embryos expecting to increase sensitivity and allow detection of the transcripts in the developing heart tube. Figure 5C shows an anterior section through a stage 35 embryo that was reacted with cardiac actin probe. The results show that the probe reacts specifically with the heart tube (arrowhead). A more posterior section (Fig. 5D), that includes anterior somites, demonstrates hybridization with both the heart tube (arrowhead), and somites (arrow). As a control we hybridized sections with probe encoding the axolotl wnt-8 transcript (Fig. 5E), which should not react with the heart, to demonstrate the specificity of detection methods. The results we present here show that the axolotl cardiac actin gene is expressed first in the somites, and later in the heart, a sequence of events that appears to be conserved in all vertebrate embryos.

Discussion

In this paper we present the sequence and expression profile of the cardiac α–actin gene from axolotls. As in other vertebrate embryos (Mohun *et al.*, 1984; Hayward and Schwartz, 1986; Sassoon *et al.*, 1988), axolotl cardiac actin is expressed early in somites, and then later it is also expressed in the developing heart. Ultimately expression becomes restricted to the adult heart, and not the skeletal muscle. Axolotls are an important model system for studying heart development in vertebrate embryos. They offer some features that *Xenopus* embryos do not such as the ability to induce beating heart tissue in an in-vitro system (Muslin and

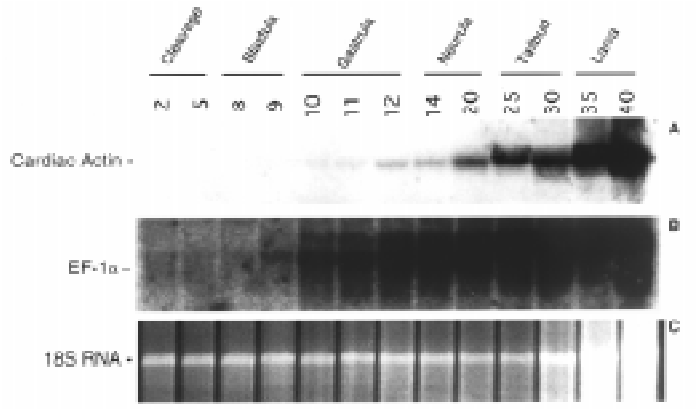

Fig. 3. The cardiac actin gene begins transcription during the early gastrula stages. *Single embryo equivalents of RNA from embryos of the indicated stages were Northern blotted.* **(A)** *Blot hybridized with probe from Ax-α-actin (cardiac actin cDNA).* **(B)** *The blot shown in A was stripped and reprobed using the EF-1α probe.* **(C)** *18S ribosomal RNA from each embryo stage stained with ethidium bromide.*

Williams, 1991; Ariizumi *et al.*, 1996), as well as heart specific genetic mutants (Luque *et al.,*1997; Zajdel *et al.*, 1998). The cardiac actin probe described here can be added to a short list of cloned heart specific probes that are available for axolotls (Ward *et al.*, 1995, 1996; Luque *et al.*, 1997). These can be used to uncover the molecular mechanisms that govern cardiac specification in axolotls, which occurs differently than it does in *Xenopus* (Neff *et al.*, 1996; Jacobson and Sater, 1988). Among other things, this would improve our understanding of how the embryological mechanisms that underlie the divergence of these related species evolved.

Actin is a component of striated muscle, and in *Xenopus* expression of the muscle actin genes is considered a reliable marker for muscle development during embryogenesis (Gurdon *et al.,* 1989). We show here that the axolotl cardiac actin gene becomes transcriptionally active by stage 10, and its expression is detected very early in the somitic mesoderm. Under normal circumstances transcription of a tissue specific structural gene is considered indicative of terminal differentiation, but this typically assumes that transcribed messages are translated almost immediately thereafter. However, in the case of axolotl embryos previous work demonstrates that muscle actin protein synthesis is not detectable until about stage 26 (Mohun *et al.*, 1980; Meuler and Malacinski, 1985), long after the message has been transcribed. This brings into question the timing of the specification and differentiation of the cells that will form skeletal muscle in axolotl embryos.

One possibility is that actin mRNA is transcribed in the presumptive somites, beginning at stage 10 (gastrula stage) and then remains untranslated until just before the head fold stage, stage 26, when α-actin protein synthesis can be detected. However, studies in *Xenopus* show that α–actin protein begins to be produced by about stage 11 (Ballantine *et al.*, 1979; Sturgess *et al.*, 1980), almost immediately after synthesis of the mRNA (Mohun *et al.*, 1984), so there appears to be little if any lag between the appearance of α–actin mRNA and its translation in *Xenopus* embryos. Studies in mouse embryos have reached an identical conclusion (Lyons *et al.*, 1991). Thus it seems unlikely that delayed α–actin translation would have evolved independently in axolotls. An alternative explanation is that axolotl embryos, with a volume of about 6 to 8 fold greater than *Xenopus*, are not as amenable to in-vivo protein labeling, making early low-level α-actin protein synthesis difficult to detect. In this regard cardiac actin mRNA increases by about 10 fold during the period from gastrulation to stage 26, which could account for detectability of the protein in later embryos. We consider it likely, therefore, that actin protein begins to accumulate at low levels earlier than previous studies have suggested, probably during or shortly after stage 10. If so, then does muscle actin expression signify the onset of muscle differentiation in axolotls?

Forman and Slack (1980) showed conclusively that presumptive somitic mesoderm explanted from axolotl embryos at the neurula stage differentiates into muscle (indicating specification), but the same tissue can be redirected toward an alternate fate when disaggregated and transplanted into ectopic sites (indicating that it has not completed determination). Since actin mRNA begins to be transcribed during the gastrula stages, hours before neurulation, then the most direct interpretation of these results is that low level muscle actin expression indicates muscle specification, but not acquisition of the determined state or differentiation, as studies with *Xenopus* embryos would suggest. Instead, in axolotls, somite determination is not complete until about stage 25 (Forman and Slack, 1980), at about the time that muscle proteins are abundant enough to begin organizing into myofibrils (Mohun *et al.,* 1980). An interesting possibility is that the cardiac actin gene may become transcriptionally active during the gastrula stages as a passive consequence of the expression of those genes involved in the process of muscle specification, with robust actin expression, indicating cell differentiation, being actively induced later. Such a model would be supported by the data we report in this paper.

Because the early embryos of anurans and urodeles look quite similar it has been assumed they develop in almost identical ways. However, this view is clearly inaccurate given that embryology

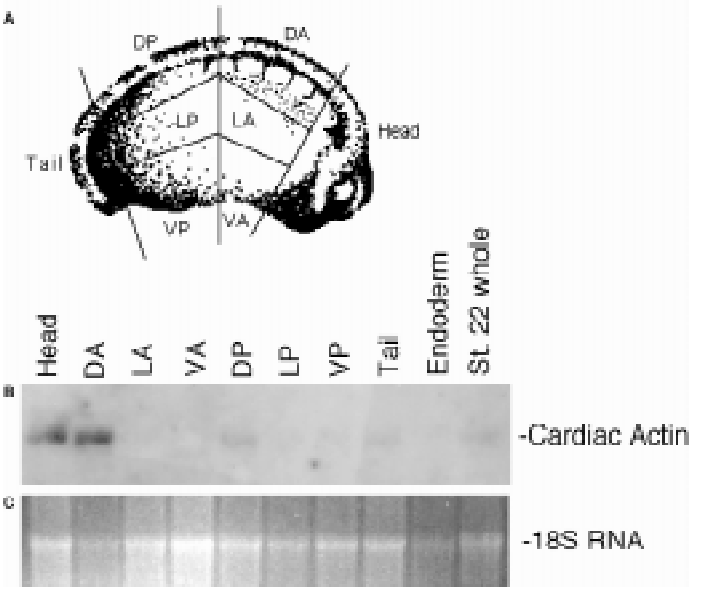

Fig. 4. Cardiac actin expression in stage 22 embryos is restricted to those regions that contain somite material. **(A)** *Mesodermal and ectodermal layers from stage 22 embryos were dissected from the various regions as indicated. In addition, the endoderm, comprising the yolky cells in the interior of the embryo, was also dissected and used for analysis.* **(B)** *Northern blot containing RNA from the indicated sections was reacted to detect cardiac actin RNA.* **(C)** *Ethidium bromide staining of the 18S RNA species, showing loading in each lane.*

must account for the vastly different morphology of the adult animals. Indeed, Hanken (1986) has detailed a number of ways in which the embryology of urodeles and anurans differ, one striking example being the radically different mechanisms through which the somites develop in *Xenopus* and axolotls. *Xenopus* has evolved a mechanism that is unlike any non-anuran tetrapod. In *Xenopus* the somites develop as bars of paraxial mesoderm that are at first perpendicular to the axis of the notochord, and then rotate into an axis that is parallel to the notochord (Youn and Malacinski, 1981a). In axolotls, on the other hand, paraxial mesoderm is first modeled into an epithelial rosette structure typical of those found in the embryos of all other terrestrial vertebrates. Later the rosettes undergo further differentiation into somites (Youn and Malacinski, 1981b). Because axolotls and higher vertebrates share a common pattern of somite development, while somite patterning in *Xenopus* appears to be highly derived, axolotl embryos might be a more useful amphibian model for studying somite patterning in higher animals than *Xenopus* embryos.

When molecular biology was first used a tool to study development it was important to limit the number of experimental systems that were studied in order to maximize the conceptual knowledge that could be derived from major organismic groups. Molecular methods are now more routine, and it is now possible to carefully select additional species for investigation so that they can offer specific insights into aspects of development that can not be obtained from existing systems. Urodeles have retained the basal tetrapod morphology from which all extant terrestrial vertebrates evolved (Duellman and Trueb, 1985), and can therefore offer insights into mammalian patterning that *Xenopus*, given its derived morphology (Cannatella and De Sa, 1993), can not. With this in mind, we are currently developing the tools that will allow experiments aimed at understanding axolotl development to be carried out at a molecular level.

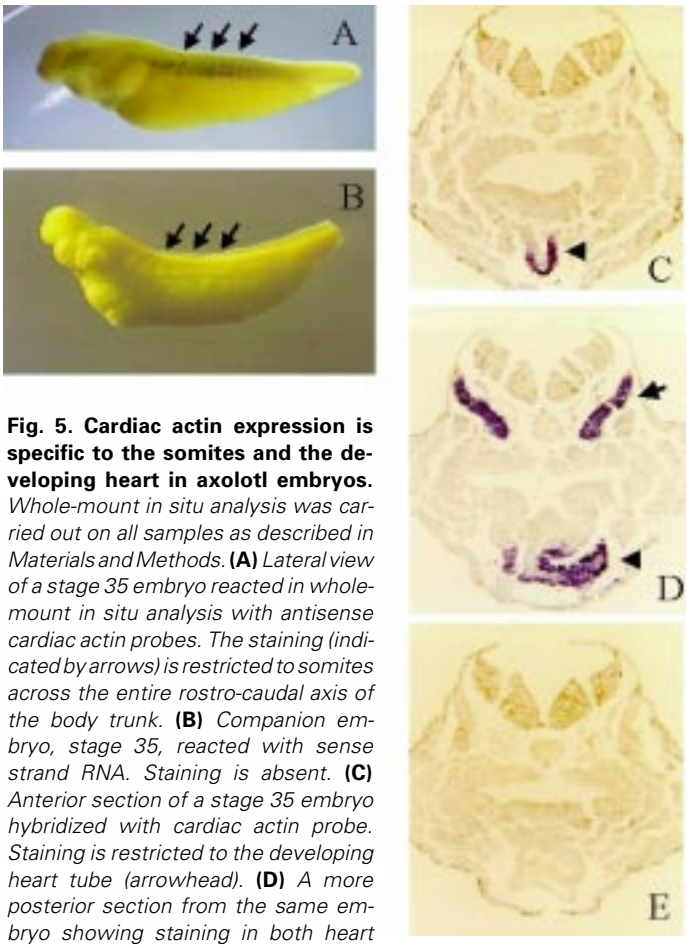

Fig. 5. Cardiac actin expression is specific to the somites and the developing heart in axolotl embryos. *Whole-mount in situ analysis was carried out on all samples as described in Materials and Methods.* **(A)** *Lateral view of a stage 35 embryo reacted in whole-mount in situ analysis with antisense cardiac actin probes. The staining (indicated by arrows) is restricted to somites across the entire rostro-caudal axis of the body trunk.* **(B)** *Companion embryo, stage 35, reacted with sense strand RNA. Staining is absent.* **(C)** *Anterior section of a stage 35 embryo hybridized with cardiac actin probe. Staining is restricted to the developing heart tube (arrowhead).* **(D)** *A more posterior section from the same embryo showing staining in both heart tube (arrowhead) and somites (arrow).* **(E)** *Section at level equivalent to D hybridized with control axolotl wnt-8 sense strand probe.*

Materials and Methods

Cloning

Probe corresponding to the protein coding region of *Xenopus* cardiac actin cDNA was prepared by random priming and hybridized to duplicate filters containing 10^6 plaques from an axolotl larval stage cDNA library (Stratagene) under moderately stringent conditions (Sambrook *et al.*, 1989). From over one hundred potential positive clones 10 were carried through additional screening procedures and plasmid DNA was prepared from several of these. Those with the largest inserts were subjected to automated sequencing for identification.

Embryos and tissues

All embryos used in this study were either obtained from the Axolotl Colony at Indiana University, or through natural spawnings from our own colony at FSU. Adult animals were raised in our laboratory from embryos.

RNA extraction

Embryos from specific stages were harvested in groups of 10 and RNA was extracted using Trizol (Life Technologies) according to the manufacturer's recommendations. After initial isolation the RNA was further purified from polysaccharide contaminants by selective precipitation overnight at 4°C in 4 M LiCl. RNA was extracted from adult tissues by the LiCl/Urea method (Auffray and Rougeon, 1980).

Northern Blotting

For each lane 5 to 10 μg of total RNA was separated on formaldehyde-agarose gels according to standard methods (Sambrooke *et al.*, 1989). The RNA was blotted to Nylon filters (MSI), and UV cross-linked. To prepare probe Ax-α-actin was linearized with BamH1 and transcribed with T7 polymerase. For EF-1α controls, a fragment of the axolotl EF-1α cDNA was amplified by RT-PCR using degenerate primers, and the resulting fragment was cloned into pBluescript SK (A.D.J., unpublished). ^{32}P-labeled riboprobes (actin 1.5 kb, EF-1α 0.5 kb) were prepared and used to probe filters under high stringency conditions (Krieg and Melton, 1987). Filters were analyzed by autoradiography.

In situ *analysis*

Digoxigenin labeled probes were prepared from linearized pAx–α–actin using the Genius system (Boehringer), and the transcripts were purified by precipitation in 4 M LiCl. For whole-mount analysis wild-type embryos were bleached in 5% formamide 0.5X SSC 10% H_2O_2 (Bertwistle *et al.*, 1996) then treated exactly as described by Harland (1991). For hybridization to sections, embryos were collected fixed in MEMFA (Harland, 1991), embedded in paraffin, and sectioned at 15 microns. The procedure of Henrique *et al.* (1995) for whole-mount in-situ hybridization was adapted for use on sections, and carried out essentially as described except that BM-purple (Boehringer) was used for color detection.

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