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Expression of a cardiac myosin gene in non-heart tissues of developing frogs

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Abstract

Direct developing frogs, like *Eleutherodactylus coqui*, provide opportunities to investigate limb early development in anuran amphibians that are less available in species with tadpoles. We have found that *myh6*, a myosin gene usually considered heart-specific in *Xenopus* and other animals, is expressed in limbs of *E. coqui* embryos. The gene for *microRNA-208* is contained in an intron of the *E. coqui myh6* gene as in mammals, and *miR-208* was detected as a microRNA, more highly expressed in a microarray of *E. coqui* limb buds, compared to *Xenopus laevis* limb buds. *Myh6* is also expressed in several muscles of tadpoles and froglets of *Xenopus tropicalis*. These connections raise the possibility of an involvement of *myh6* and *miR-208* in the thyroid dependent metamorphosis of anurans.

Keywords

Direct development; myosin gene family; thyroid hormone; metamorphosis; frog

INTRODUCTION

Investigations using the amphibian model, *Xenopus laevis*, have contributed greatly to the understanding of early development, including axis determination, germ layer specification, and organ initiation. With a few exceptions (Abu-Daya et al. 2010) however, *X. laevis* and other anuran amphibians have not been useful in the analysis of limb development. As with many other anurans, limb buds are delayed in initiation in *X. laevis*, and they are small and inconspicuous (Tarin and Sturdee 1971; Abu-Daya et al. 2010). In contrast, some anurans develop directly to a morphology of a frog without an intervening larval tadpole stage, and these species usually have large limb buds that arise early (Elinson and del Pino 2012). These direct developers provide opportunities to examine limb development, and the Puerto Rican tree frog, *Eleutherodactylus coqui* has proven to be useful in this regard.

In *E. coqui*, a number of genes involved in limb patterning are expressed in familiar tetrapod patterns. These include *Shh*, *Ptch1*, *Hand2*, *Fgf8*, *Wnt5a*, and *BMP4* (Gross et al. 2011). A molecular manifestation of the apical ectodermal ridge (AER) is the expression of both *Dlx* and *Fgf8* in a narrow strip along the edge of the *E. coqui* limb bud, even though the AER morphology is lacking (Fang and Elinson 1996; Gross et al. 2011). *Lbx1* expressing cells from the somite enter the limb bud, and likely establish limb muscle lineage (Sabo et al. 2009), and retinoic acid is required for initiation of forelimb but not hindlimb (Elinson et al. 2008).

In a continuing examination of limb development in *E. coqui*, we have detected the expression of myosin heavy chain 6 (*myh6*), also known as cardiac myosin heavy chain alpha (α MHC), in developing limbs and other tissues. This was unexpected, since a careful analysis by Garriock et al (2005) showed expression only in heart, jaw muscles, and lymph heart of *X. laevis* embryos.

MATERIALS AND METHODS

Animals and embryos

Eleutherodactylus coqui were collected on the island of Hawaii under Injurious Wildlife Export permits issued by the Department of Land and Natural Resources, Hawaii. Adults were maintained in the laboratory as breeding pairs in terraria. Pairs mated naturally, and clutches of embryos were collected from their guarding father. The embryos were staged according to Townsend and Stewart (1985), designated as TS1 (cleaving embryo) through TS15 (hatched froglet). They were usually cultured in Petri dishes on filter paper, wetted with 20% Steinberg's solution. *Xenopus laevis* were maintained as a breeding colony, and embryos and tadpoles were obtained by in vitro fertilization, according to standard procedures. Embryos, tadpoles, and froglets of *Xenopus (Silurana) tropicalis* were obtained from Takuya Nakayama (University of Virginia) and Xenopus I (Dexter, MI). *X. laevis* and *X. tropicalis* tadpoles were staged according to the standard Nieuwkoop - Faber (NF) table. Use of *E. coqui*, *X. laevis*, and *X. tropicalis* in this research was carried out under protocols approved by the Duquesne University Institutional Animal Care and Use Committee (IACUC).

MicroRNA microarray

E. coqui TS4 embryos were anesthetized and killed in 0.1% Tricaine methane sulfonate (MS222) (Sigma-Aldrich, St. Louis, MO), made to pH 7.4 by addition of Na₂HPO₄. Fore- and hindlimb buds were collected separately and were put into a small drop of 100% Steinberg's solution on a piece of Parafilm, taking care not to allow the bud to come in contact with air. After ~14 limb buds were collected, the excess 100% Steinberg's solution was pipetted off, and the piece of Parafilm was placed on dry ice. Using forceps and a microfuge tube chilled on dry ice, the drop containing the frozen limb buds was placed in the microfuge tube and stored at -80C until RNA extractions were carried out. Hindlimb buds were collected similarly from *X. laevis* NF49/50 tadpoles.

Total RNA was extracted using the miRCURY™ RNA Isolation Kit - Cell & Plant (Exiqon Inc., Woburn, MA), a kit designed to preserve microRNAs (miR) for analysis. Three *X. laevis* hindlimb RNA samples, three *E. coqui* hindlimb RNA samples, and three *E. coqui* forelimb RNA samples were submitted to Exiqon for microarray analysis. Each of the samples had been prepared from approximately 14 limb buds and contained 2.3 – 6.6 mg RNA. As determined by Exiqon, all of the samples had an RNA Integrity Number (RIN) of 9.5–10, where an RIN>7 is a good sample for microarray. Each of our nine limb samples was run against a pooled common reference sample on the miRCURY™ LNA Array (v.11.0 *other species*). The results were presented in terms of *Xenopus tropicalis* miRBase annotations. Any miRs with p-values less than 0.001 were considered to have differential expression across the various limb tissue groups.

Cloning *E. coqui* myosins

To clone the *E. coqui* orthologue of a myosin heavy chain expressed in the heart (*myh6*), RNA was extracted from *E. coqui* TS7 embryos with Trizol (Invitrogen, Carlsbad, CA). cDNA was synthesized using M-MLV reverse transcriptase, and the cDNA was used as a template for PCR amplification with degenerate primers, which were designed based on *M.*

musculus, *Danio rerio*, *X. laevis*, and *X. tropicalis*. The first fragment cloned was near the 5' end of the ORF, and cloning proceeded iteratively towards the 3' end. With each cloned fragment, PCR was carried out using an exact primer within the fragment and a degenerate primer more 3', eventually yielding 5841 nts of *myh6* sequence.

In the course of cloning *myh6*, two different sequences were obtained after about 1kb. The second sequence was used as the start for cloning a second *myh*, which appears most similar to *myh6.2* in *X. tropicalis* (Abu-Daya et al. 2009).

In situ hybridization

In situ hybridization was carried out on whole *E. coqui* embryos as described previously (Elinson et al. 2008). *E. coqui* embryos were anesthetized in 0.05–0.1% MS222 pH7.4 and fixed for 3–4 hours at room temperature in freshly prepared MEMFA (1M MOPS, 2mM EGTA, 1mM MgSO₄, 3.7% formaldehyde, pH 7.4). They were washed in ethanol and stored at –20C. The template for sense and antisense probes was a 1.5 kb piece of the *E. coqui myh6* cDNA, cloned into the TEasy Vector. Digoxigenin-labeled probes were prepared with T7 and SP6 RNA polymerases.

RT-PCR for gene expression

To obtain RNA from different embryo parts, hearts, forelimbs, hindlimbs, and somite-containing pieces of 6–10 *E. coqui* embryos were isolated and frozen as described above for limb buds. Isolated hearts continued to beat in 100% Steinberg's solution. In isolating limb buds, care was exercised to avoid including adjacent somitic tissue. The somite-containing pieces consisted of the dorsal axial trunk region between the limbs. The pieces included somites, spinal cord, and notochord. RNA was extracted with Trizol, and cDNA was synthesized for PCR. PCR was carried out with gene-specific primers, using *EcL8*, a ribosomal protein gene, as a loading control. The primer set for detection of *E. coqui myh6*, which produced a 505 nt amplicon, was:

Forward: 5' CAG TGG TGG CAA AGG CAA AGA AG-3'

Reverse: 5' CAT CAA TAA TCC TCG AGA TTG T-3'

To obtain RNA from *X. tropicalis* muscle, tadpoles and froglets were anesthetized and killed in 0.1% Tricaine methane sulfonate (MS222) pH 7.4. Tissues were dissected in 200% Steinberg's solution, collected into Eppendorf tubes, and frozen on dry ice. From tadpoles, the abdominal body wall was the tissue overlying the intestines and included skin, abdominal muscle, and peritoneum. Tissue overlying the heart was carefully excluded. The hindlimbs included skin, muscle, cartilage, and bone, with tail carefully excluded. The tail included muscle, spinal cord, and notochord. Liver was collected as a negative control tissue. From feeding froglets, the abdominal body wall was the same tissue as from tadpoles, but with no skin. Only muscle was isolated from hindlimbs, with no skin or bone. The back muscle was muscle on either side of the dorsal midline, with no skin. RNA was extracted with Trizol, and cDNA was synthesized for PCR. PCR was carried out with gene-specific primers, using *Ef1a* as a loading control. The primer set for detection of *X. tropicalis myh6*, which produced a 1094 nt amplicon, was:

Forward: 5' CAG ATT GTT AAA TCC AAG GCT-3'

Reverse: 5' AAC TGG GTT TGA AGT GCA TCC-3'

RESULTS and DISCUSSION

Differences in microRNA expression in limb buds of *X. laevis* and *E. coqui*

As part of an investigation of anuran limb development, we compared expression of miRs in *E. coqui* TS4 limb buds to those in *X. laevis* NF 49/50 limb buds with an miR microarray. At these stages, the limb buds of both species are hemispherical, with an *E. coqui* limb bud having about four times the volume of a *X. laevis* limb bud. The miRs used in the microarray were mainly from *X. tropicalis*, for which the greatest number of potential anuran amphibian miRs is known from its genome project. Most of the 30 *xtr-miRs* showing significant differences can be grouped into three categories (Table 1). One of those expressed more in hindlimb buds of both *E. coqui* and *X. laevis*, as compared to forelimb buds of *E. coqui*, is *xtr-miR-196a*. This result corresponds to the important role of *miR-196* in mouse and chick hindlimb buds compared to forelimb buds (Hornstein et al. 2005) and provides an internal control of our microarray.

The miR microarray identified *xtr-miR-208* as one of the miRs, preferentially expressed in *E. coqui* TS4 limb buds compared to *X. laevis* NF 49/50 hindlimb buds. This possibility was particularly interesting, because *miR-208* mediates thyroid hormone dependent heart muscle growth in mouse (van Rooij et al. 2007), and thyroid hormone plays an essential role in frog metamorphosis, including a cryptic metamorphosis in *E. coqui* (Callery and Elinson 2000).

Cloning *E. coqui* myosin genes

Attempts to confirm the presence of *miR-208* in limb buds by in situ hybridization were inconclusive, so we took advantage of the fact that *miR-208* is encoded in an intron of the gene for myosin heavy chain 6 (*myh6*) (van Rooij et al. 2007). Consequently, we cloned *myh6* from *E. coqui* and examined its expression.

Myh6 in several species is a large protein translated from an open reading frame (ORF) of more than 5800 nts. To clone *E. coqui myh6*, we began with degenerate primers near the 5' end of the ORF and progressively cloned fragments in a 3' direction using an exact primer within the cloned sequence and a degenerate primer more downstream. Our *E. coqui myh6* cDNA clone (Genbank JQ700060) is 5760 nt of the ORF plus a 3' UTR of 81 nts, and has high identity to *myh6* of *X. laevis*, *X. tropicalis*, mouse and other vertebrates (Table 2). To confirm its identity as the *E. coqui* orthologue of *myh6*, we took advantage of the fact that *miR-208* is in an intron of *myh6* in *X. tropicalis* as well as in mouse and human. The sequence for the precursor *pri-miR-208* is present in intron 28 at nt 4200 of *X. tropicalis myh6*. We cloned through this region of *E. coqui myh6* cDNA, designed exact primers on either side of the putative intron site, and used them to obtain a genomic sequence (GenBank JQ700066). The *pri-miR-208* (GenBank JQ700065) is present within this *E. coqui* intron as expected. The *E. coqui miR-208* is identical to that of *X. tropicalis miR-208* (AUAAGACGAGCAUAAAGCUUGU) and differs by one nucleotide from that of mouse and human (AUAAGACGAGCAAAAAGCUUGU).

Myosin heavy chains from different genes can be very similar to each other. In the course of cloning *E. coqui myh6* with degenerate primers, we cloned 4041 nt of another *myh* gene, which we will provisionally call *E. coqui myh6.2-like* (GenBank JQ700061). The difficulty in naming this gene comes from the situation where both *myh6.2* and *myh7* in Xenbase (www.xenbase.org) lead to *myh7* in GenBank (XM_002939015); yet there does not seem to be an *myh7* in *X. tropicalis* on the basis of synteny (Garriock et al. 2005).

Expression of *myh6* in *E. coqui* development

Myh6 is expressed in embryonic heart of mouse, chick, zebrafish, and both *X. laevis* and *X. tropicalis* (Garriock et al. 2005 and references therein; Abu-Daya et al. 2009), and *E. coqui myh6* was likewise expressed strongly in heart at TS4 (Fig. 1). Somites at TS4 were weakly stained in *in situ*, and staining increased at TS5-8 (Fig. 1). Staining was particularly strong in the tail. Somite staining was not expected, since *myh6* staining in embryos of *X. laevis* was exclusive to the heart, jaw muscles, and lymph heart (Garriock et al 2005). There was little or no staining of limb buds, so *myh6 in situ* did not provide evidence of expression of *miR-208* in the developing limbs.

To clarify the results, we isolated by dissection limb buds, heart, and dorsal axial pieces of trunk that included somites, and examined expression by RT-PCR. We used primers that amplified *myh6* and did not amplify *myh6.2-like*. *Myh6* was strongly expressed in heart from TS5 through TS10/11 as expected (Fig. 2). There was little or no expression in early forelimbs, but *myh6* was expressed at low levels in hindlimbs at TS 5 and TS7 (Fig. 2). *Myh6* was up-regulated at TS 8 in both forelimbs and hindlimbs (Fig. 2). *Myh6* was also expressed in pieces of trunk that included somites at TS5, confirming the *in situ* results.

Because the expression of *myh6* in the limbs and trunk was not expected, we sequenced a selection of amplicons from each tissue and confirmed their identity as *myh6*. Although we do not know the complete *myh* family in *E. coqui*, the use of *myh6*-specific primers and amplicon sequencing make it likely that *myh6* is expressed in tissues other than heart in *E. coqui* embryos.

Expression of *myh6* in *Xenopus tropicalis* muscle

Detection of *myh6* expression in non-heart tissues in *E. coqui* led us to examine *myh6* expression in *X. tropicalis*. The genome of *X. tropicalis* is the best characterized among amphibians. That would give higher confidence that we were detecting expression of *myh6* and not that of another *myh*.

We examined by RT-PCR RNA from muscles of pre-metamorphic tadpoles (NF 54-56), tadpoles close to metamorphosis (NF 57-58), and froglets that had completed metamorphosis and had begun feeding. The primer set was designed to distinguish maximally *myh6* from *X. tropicalis myh4-like*, *myh7*, and *myh7-like*. *Myh6* was strongly expressed in all samples of heart from tadpoles and froglets, as expected. *Myh6* expression was also detected in tail, with a low level in hindlimbs and abdominal body wall, of NF54-56 tadpoles (Fig. 3). In older NF57-58 tadpoles, *myh6* was expressed in tail (2/3 samples) and abdominal body wall (1/3 samples), besides the heart (Fig. 3). In froglets, expression of *myh6* was detected in muscle of the abdominal body wall (2/4 froglets) and of the back (3/4 froglets), and at a very low level in hindlimbs (2/4 froglets) (Fig. 3). Sequencing of amplicons from each tissue series confirmed their identities as *myh6*.

These results demonstrate that *myh6* is expressed in tissues other than the heart in *X. tropicalis* as in *E. coqui*. The difference between our results in *Xenopus* and previous results is most likely due to the stages examined. Heart-specific expression of *myh6* in *X. laevis* was found in embryos prior to feeding (<stage NF 46) (Garriock et al 2005), while we examined late feeding tadpoles prior to (NF55, 56) and at (NF58) the onset of metamorphosis.

In addition to our results on *myh6* expression in anurans, *myh6* expression has been detected in mammalian jaw, diaphragm, and extraocular muscles (Mu et al. 2004, and references therein). Of particular note is the transient expression of *myh6* in leg muscle of neonatal pigs (Lefaucheur et al. 1997). Lefaucheur et al. (1997) speculate that since *myh6* expression is

sensitive to thyroid hormone, its transient presence may be connected to the high level of thyroid hormone in pigs in the first week following birth. Thyroid hormone is the key regulator of frog metamorphosis, even in the direct developing *E. coqui* (Callery and Elinson 2000). Thyroid hormone is up-regulated around TS10 in *E. coqui* and NF58 in *X. tropicalis*, so *myh6* expression in various muscles in these two species occurs before up-regulation. This raises the possibility that the expression of *myh6* and its intronic *miR-208* could be involved in the responses of different muscles to thyroid hormone, and this possibility should be explored in the future.

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Figure 1. Expression of *myh6* in *E. coqui* embryos

There is high expression in the heart of a TS4 embryo and low expression in somites. At TS5, *myh6* is expressed in somites, including those of the tail, and expression continues in the tail at TS8.

Figure 2. RT-PCR of *myh6* in *E. coqui* embryos

(A) *Myh6* expression is high in heart at all stages. There is no expression in forelimb buds at TS5 or TS7, but *myh6* is expressed at TS8-11. There is a low level of expression in hindlimb buds at TS5 and TS7, with higher expression at TS8-11. (B) An example of the controls at TS10 is shown. For samples at each stage, loading was equalized for a tissue using *EcL8* expression, and no bands were present in samples which were not reverse transcribed (–).

Figure 3. RT-PCR of *myh6* in *X. tropicalis* tadpoles and froglets

Myh6 expression is high in heart at all stages. It is detectable in tail and abdominal body wall (BW) of tadpoles at NF56 and NF58, and in hindlimb (HL) at NF56. It is not expressed in liver as expected. In a froglet, *myh6* is expressed in back and body wall muscle and at a very low level in hindlimb. Note that 'Back' muscle was run for froglet and 'Tail' was run for tadpoles (NF56, NF58). *Ef1a* is the loading control, and '-' is the negative control without reverse transcription.

Table 1

Comparative expression of miRs between limb buds of *X. laevis* (NF49, 50) and *E. coqui* (TS4).

<u><i>E. coqui</i> and <i>X. laevis</i> hindlimb buds > <i>E. coqui</i> forelimb buds.</u>
<i>xtr-miR-10c, 15a, 196a.</i>
<u><i>X. laevis</i> hindlimb buds > <i>E. coqui</i> forelimb and/or hindlimb buds.</u>
<i>xtr-miR- 23a, 23b, 24a, 24b, 25, 26, 27a, 27c, 30b, 30e, 100, 125a, 125b, 130a, 135, 142-5p, 196b, 199a, 363-3p, 451, let-7a.</i>
<u><i>E. coqui</i> forelimb and/or hindlimb buds > <i>X. laevis</i> hindlimb buds.</u>
<i>xtr-miR – 18b, 208, 363-5p.</i>

Table 2

Characteristics of *E. coqui myh6* and *myh6.2-like* clones.

	<i>myh6</i> clone	<i>myh6.2-like</i> clone
Length (nt)	5841	4041
Length (aa)	1919	1347
Position relative to XI/Xt orthologs	~50 nts from XI ORF Start	~1200 nts from Xt ORF Start
nt identity to <i>X. tropicalis myh6</i>	82%	79%
aa identity to <i>X. tropicalis myh6</i>	92%	89%
nt identity to <i>X. tropicalis</i> “myh7-like”	78%	81%
aa identity to <i>X. tropicalis</i> “myh7-like”	89%	91%