Cloning and characterization of zebrafish (*Danio rerio*) RyR orthologs and analysis of expression in developing fish

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Abstract

Ryanodine receptors are calcium channels responsible for the controlled release of intracellular calcium from the sarcoplasmic reticulum. Three ryanodine receptor genes have been identified in mammals, RyR1, RyR2 and RyR3. RyR1 is predominantly expressed in skeletal muscle, while RyR2 is expressed in cardiac muscle, and RyR3 appears to be ubiquitously expressed, and may have a role in development. Fish express two RyR1 isoforms that are discretely expressed in the slow and fast-twitch muscle fibres (RyR1-slow and RyR1-fast). We have cloned two partial sequences of the isoforms believed to be the RyR1 and RyR3 paralogs in zebrafish. Alignments with known RyR sequences and tBLASTn searches performed in Genbank identify the sequences as RyR1 and RyR3. In situ hybridization to developing zebrafish embryos from immediately post-fertilization to five days post-fertilization reveals primary expression in the developing somites. Interestingly, the RyR1 message becomes predominantly expressed in the swimbladder muscle of the 5 day old embryo. Phylogenetic analyses cluster the RyR1 sequence with published RyR1 sequences and the RyR3 sequence with published RyR3 sequences with bootstrap values of 80 and 75 respectively. The results indicate that two isoforms of ryanodine receptors, with distinct expression patterns in embryos, have been identified in zebrafish.

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1. Introduction

1.1 Ryanodine Receptors: Structure and Function

Excitation-contraction coupling is the process that pairs nerve impulses with muscle contraction. The cytoplasmic concentration of calcium in a resting cell is very low relative to the extracellular concentration of calcium. When a nerve impulse reaches a cell membrane, the cell is depolarized and an influx in calcium results in contraction of muscle fibers. A calcium storage organelle, the sarcoplasmic reticulum, also contributes to the influx of calcium. Ion channels, called ryanodine receptors, allow calcium from the sarcoplasmic reticulum into the cytoplasm when the cell membrane is depolarized. The transverse tubules of muscle cells allow ryanodine receptors to directly contact dihydropyridine receptors (DHPRs) on the cell membrane. When the membrane is depolarized, the DHPRs open, resulting in a change in conformation. The conformational change can result in opening of ryanodine receptors if they are in contact with a DHPR. This is referred to as depolarization-induced calcium release. Ryanodine receptors can also be activated by binding of calcium. This is referred to as calcium-induced calcium release (Rossi and Sorrentino, 2002).

Ryanodine receptors are made up of four identical subunits. Each subunit has a molecular mass of approximately 500-600 kDa (Franck et al., 1998). Ryanodine receptors are found in various tissues, but are most abundant in striated muscle (Fill and Copello, 2002). They contain at least two functional domains: Regulatory sites are located on the amino-terminal cytoplasmic domain and transmembrane and pore forming regions are part of the carboxy-terminal domain (Rossi and Sorrentino, 2002). Binding sites for nucleotide, calmodulin, FKBP12, Ca^{2+} and phosphorylation sites are all present in the cytoplasmic domain (Rossi and Sorrentino, 2002).

1.2 RyR Isoforms

Three isoforms of ryanodine receptors have been identified in mammals with an overall amino acid identity of 66-70% (Rossi and Sorrentino, 2002). The majority of the divergence between the isoforms is confined to three major regions, which have been designated D1, D2 and D3. For the RyR1 isoform, the regions span amino acids 4254-4631, 1342-1403 and 1872-1923 respectively (Rossi and Sorrentino, 2002). The RyR isoform that is found primarily in skeletal muscle is referred to as RyR1, the isoform found mainly in cardiac muscle is designated RyR2, and the ubiquitously expressed isoform, RyR3, has been found in the brain, diaphragm and skeletal muscle (Morrissette et al., 2000). RyR3 comprises only 2-5% of the ryanodine receptors expressed in mammalian skeletal muscle (Sorrentino, 2003). RyR3 has also been suggested to have a role in development. The RyR3 isoform is expressed in all muscles in the embryo, and peaks 15 days after birth then declines until the expression in adult tissues is limited to mainly the diaphragm and a few other tissues (Sorrentino, 2003). In mammals, the three RyR isoforms are products of three different genes (Fill and Copello, 2002). The RyR isoforms first identified in non-mammals were designated α-RyR and β-RyR because the homology of these isoforms to mammals was uncertain (Franck et al., 1998). It has since been determined that these isoforms are homologous to RyR1 and RyR3 respectively. Two distinct isoforms have been shown to be discretely expressed in slow and fast twitch muscle of fish. These have been designated RyR1-slow and RyR1-fast, respectively (Franck et al., 1998). Slow and fast twitch muscle in fish are discretely compartmentalized which makes them a model organism to study expression of these isoforms.

Ryanodine receptors have shown a complex system of regulation because they are regulated by various endogenous molecules (Tripathy et al., 1995). Ryanodine receptors can be activated or inhibited by molecules such as Ca^{2+}, Mg^{2+}, ATP and calmodulin. The

three isoforms have shown different behaviours when studied under various concentrations of effector molecules. RyR1 activity is enhanced by calmodulin when the concentration of Ca^{2+} is in the nanomolar range, but inhibited by calmodulin when in higher, micromolar concentrations of Ca^{2+}. RyR2 does not show activation by calmodulin when Ca^{2+} is not bound, and it is inhibited when Ca^{2+} levels are in the micromolar range (Rossi and Sorrentino, 2002). The RyR2 isoform has been shown to be more sensitive to activation by micromolar concentrations of Ca^{2+}, and less sensitive to inhibition by millimolar concentrations of Ca^{2+} than RyR1. RyR3 has also shown to be less sensitive to both activation and inhibition by Ca^{2+}. RyR2 and RyR3 are both less sensitive to inhibition by Mg^{2+} than RyR1 (Morrissette et al, 2000).

Many factors have been shown to contribute to the physiological differences between fast and slow twitch muscle. Myosin in fast-twitch fibers has a higher maximum velocity of shortening and fast-twitch muscle also has a greater concentration of sarcoplasmic reticulum, and therefore a greater concentration of calcium pumps (Rome et al., 1996). Calcium pumps on the sarcoplasmic reticulum differ between fast- and slow-twitch muscle. Fast-twitch muscle has sarco/endoplasmic reticulum calcium ATPase-1 (SERCA1) and slow-twitch muscle has SERCA2. The concentration of parvalbumin, a protein that binds both Mg^{2+} and Ca^{2+}, is greater in fast-twitch muscle than slow-twitch muscle (Rome et al., 1998). This results in slow twitch muscle taking a longer time to achieve maximum contraction, and one-half relaxation (Morrissette et al., 2000).

1.3 Zebrafish as Model Organisms

Zebrafish (*Danio rerio*) have become a model organism for genetic and developmental studies. This can be attributed to their short generation time and their small genome size (Teh et al., 2005). The optical clarity of zebrafish embryos make them

ideal organisms for developmental studies. When referring to a stage of development in zebrafish embryos, a staging series is often used. The rate of development of zebrafish embryos is slightly different, even embryos from the same clutch develop at slightly different rates. A staging series ensures better reproducibility (Kimmel et al., 1995).

1.4 Zebrafish Development

1.4.1 Muscle Development

A map of cell fate at the organ and tissue level is available for as early as gastrulation (Kimmel et al., 1995). Somites first appear in zebrafish during the segmentation period. This period is defined as 10-24 hours post fertilization (hpf). Somites appear in the trunk and tail during this period, the anterior ones developing before the posterior ones (Kimmel et al., 1995). The first 18 somites develop at a rate of three per hour, and the remaining appear to develop at a rate of two per hour (Kimmel et al., 1995). The majority of the cells of each somite will develop into a myomere, or muscle segment, and the remaining will give rise to vertebral cartilage. The medial somatic epithelium appears to be the region (adaxial region) from which the first muscle fibers appear (Thisse et al., 1993). The pioneer cells may also be included in this region. These cells develop early in young trunk myotomes, where the horizontal myoseptum will form (Felsenfeld et al., 1991). The muscle pioneers are present at the apex of the myomere (Kimmel et al., 1995). The myotomes start to show swimming movements during the pharyngula period (24-48 hpf).

1.4.2 Brain Development

The brain starts to develop during the segmentation period. In the middle of segmentation the brain rudiment develops ten swellings, referred to as neuromeres. Two

of these will become the forebrain, one the midbrain and seven neuromeres make up the hindbrain (Kimmel et al., 1995).

1.4.3 Pharyngeal Arch and Swimbladder Development

Pharyngeal arches develop rapidly during the pharyngula period. There are a total of seven pharyngeal arches that develop from a primordial region. The swimbladder starts to develop around 42 hpf and inflates at four days post fertilization (Kimmel et al., 1995).

1.5 Calcium Signalling During Development

Calcium has an important role in the regulation of development (Creton, 2004; Creton et al., 1998; Jaffe, 1999; Webb and Miller, 2003). Patterns of free calcium have been described in zebrafish embryos throughout the developmental process (Creton et al., 1998). The initial calcium signalling events have been correlated with specific morphological events, however as development continues and becomes more complex, the calcium signals appear to become more complicated as well (Webb and Miller, 2003). The re-initiation of development in arrested eggs appears to be activated by calcium (Webb and Miller, 2003). At this stage calcium signalling takes the form of intracellular waves. Calcium signalling has also been shown to play an important role in the segregation process during the zygote period (Leung et al., 1998). The fast calcium waves that occur during these periods have been shown to be generated by calcium induced calcium release from the endoplasmic reticulum (Webb and Miller, 2003). Calcium waves are still occurring during the cleavage period, however by the blastula period, individual waves can no longer be resolved (Webb and Miller, 2003). Intercellular calcium signalling is first observed during the blastula period. In this period, localized calcium spikes are seen in the cells that eventually develop into the enveloping

layer, and calcium may play a role in the formation of the yolk syncytial layer (Webb and Miller, 2003). The gastrula period exhibits intercellular calcium waves (Gilland et al., 1999). Webb and Miller (2003) suggest that these waves may provide positional information to cells, or may coordinate cell responses. Three areas of calcium signalling during the segmentation period are observed: One in the tail bud and posterior region, a second one in the trunk and a third in the anterior region (Webb and Miller, 2003). The calcium signalling in the trunk may have a role in somite formation (Webb and Miller, 2003). Because calcium appears to have such an important role in development, understanding the expression and distribution of RyR isoforms is essential.

The objective of this study was to identify the RyR isoforms in zebrafish and characterize them by sequencing, phylogenetic analysis and gene expression studies in developing zebrafish embryos. I have identified two isoforms of RyR in zebrafish, thought to be RyR1 and RyR3.

2. Materials and Methods

2.1 RNA Extraction and First Strand cDNA Synthesis

Specific zebrafish tissues were dissected from frozen zebrafish using a dissecting microscope. Slow twitch skeletal (red), fast twitch skeletal (white), and cardiac muscle were carefully dissected and immediately placed in TRIzol reagent (Invitrogen Life Technologies) to inhibit DNase or RNase activity. TRIzol reagent was used to extract RNA from the three types of muscle tissues as well as whole zebrafish (Chomczynski, 1987). The tissues were homogenized with a motorized teflon pestle and glass mortar. TRIzol reagent uses a phenol chloroform separation technique, which allows RNA to be isolated from other cellular components. Total RNA was ethanol precipitated, dried, resuspended in RNase free water, and quantified by ultraviolet spectrophotometry.

20 μg of total RNA was used to synthesize first strand cDNA. A 20 μl solution of 1μg/μl of RNA and 0.005μg/μl of Oligo dT_{15} (Invitrogen Life Technologies) was heated to 95°C for five minutes, and immediately chilled on ice. Once the solution was chilled, 12μl of 5x Oligo dT_{15} buffer (Invitrogen Life Technologies), 2μl of 10mM dNTPs (Invitrogen Life Technologies), 24μl H_2O, 1 μl RNAsin (Invitrogen Life Technologies), and 1 μl of reverse transcriptase (Invitrogen Life Technologies) were added and mixed. The solution was incubated at room temperature for 15 minutes, followed by 30 minute incubation at 37°C. The reaction was stopped by adding 40 μl of 10 x TE (pH 8.0). The first strand cDNA was precipitated by adding 10 μl of 3M sodium acetate and 250 μl of 95% ethanol. The cDNA was precipitated at -20°C overnight and pelleted by centrifugation at 14,000rpm. A Speedvac centrifuge was used to dry the pellet. The pellet was dissolved in distilled, RNAse and DNAse free water.

2.2 Primer Design

Both degenerate and zebrafish-specific RyR primers were designed for polymerase chain reaction (PCR). Degenerate primers were designed based on alignments of conserved regions of the RyR message from frog, rabbit and fish (see appendix 7.1). The CODEHOP program was used to design degenerate primers (Rose et al., 1998). This program designs primers with a 3' degenerate end, across four codons of highly conserved amino acids and a 5'-non-degenerate end approximately 20 – 30 base pairs long. The primers designed using this program were, ZFF1 (5'-CGAGGGAGGAGAGGGAGARGANGARRT-3'), ZFR1 (5'-CTGCTTGGAAGCAGGGTGNATNGTCCA-3'), ZFF2 (5'-GGCTTTCAACTTCTTCCGAAARTTYTAYAA-3'), and ZFR2 (5'-CAGCATCGCTCCTGGTACATYTTCCA-3'), where R was either A or G, Y was either C or T, and N was A, G, C or T.

Zebrafish-specific primer pairs, ZF53F1 (5'-AGAGTGCGCTGGACTTTGAT-3') and ZF53R1 (5'-TCTCCATGTCCTCCTTCACC-3'), were designed using the Primer 3 program (Rozen and Skaletsky, 2000) based on a sequence obtained from the zebrafish genome Ensembl database (Wellcome-Sanger Trust).

2.3 Reverse Transcriptase Polymerization Chain Reaction (RT-PCR)

45 µl of mastermix and 5 µl of template were used for each reaction of RT-PCR. The mastermix had a concentration of 1x PCR buffer (Invitrogen Life Technologies), 1.5 mM $MgCl_2$ (Invitrogen Life Technologies), 0.2 mM dNTPs, 1.0 µM of each forward and reverse primer and 2.5 U of Taq DNA polymerase (SuperScript™ II Reverse Transcriptase, Invitrogen Life Technologies). The initial denaturation step was 95˚C for nine minutes. The subsequent denaturation step was 95˚C for one minute, followed by an annealing step at 60˚C for one minute and thirty seconds and an extension step at

72°C for one minute. These three steps were cycled 35 times and a final extension step at 72°C for seven minutes completed the reaction.

The products of the RT-PCR reaction were fractionated on 1% low melt agarose gels and purified by either gel purification (Wizard® PCR Preps DNA Purification System, Promega) or with QIAquick Nucleotide Removal Kit (QIAGEN) (Figure 1).

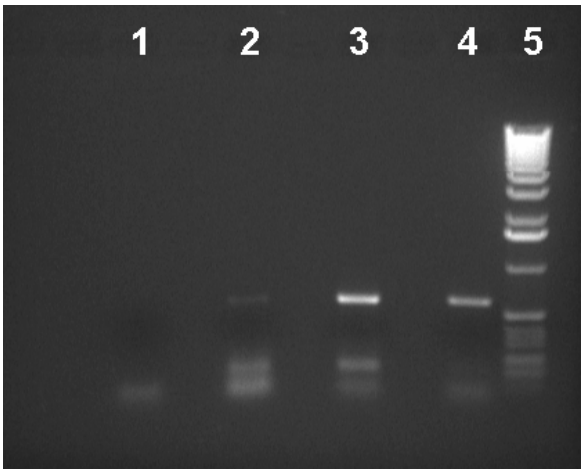

Figure 1: RT-PCR products using ZF F1/R1 primers with whole zebrafish cDNA (lane 4), zebrafish red muscle cDNA (lane 3) and zebrafish white muscle cDNA (lane 2). A control reaction was run in lane 1

2.4 Cloning of PCR products

The purified RT-PCR products were ligated with pGEM®-T Easy vectors (Promega Biotech), transformed into competent *Escherichia coli* (*E. coli*) cells and plated on LB media plates containing 0.05 mg/ml ampicillin (Invitrogen Life Technologies). The plates were incubated at 37°C for 24 hours.

2.5 PCR Detection of Recombinants and Plasmid Purification

Seven colonies were randomly picked from each plate and PCR was performed using M13 forward (5'-GTTTTCCCAGTCACGAC-3') and M13 reverse (5'-

CAGGAAACAGCTATGAC-3') primer pairs. Sites for the M13 forward and reverse primers flank the insert, to allow amplification of the partial RyR sequences. The PCR program consisted of four steps, of which the second, third and fourth step were cycled 35 times. The initial denaturation step was 95˚C for five minutes. The second step was 95˚C for one minute, followed by an annealing step at 55˚C for thirty seconds and an extension step at 72˚C for one minute. The reactions were run on a 1% agarose gel, and the colonies containing inserts were identified by viewing the gel (Figure 2). Bacterial cultures were grown overnight, with colonies showing the expected band size, in LB media containing 0.05mg/ml ampicillin.

Plasmids were purified from bacterial cultures using the Spinbind kit (FMC), which uses a microcentrifuge system to isolate plasmid DNA. Plasmid minipreps were sent for off site sequencing at the University of Toronto Sick Kids Hospital. Sequences were edited and compared by BLAST submissions to the GenBank database. Homology to RyR sequences was inferred by sequence similarity.

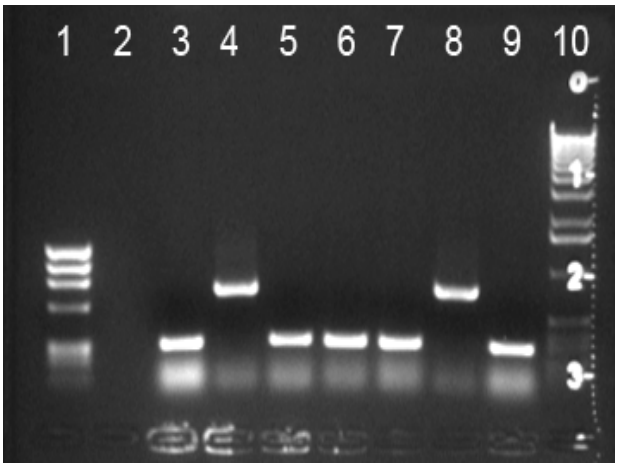

Figure 2: Agarose gel showing the RT-PCR products from *E. coli* colonies using M13 forward and M13 reverse primer pairs. Lanes 4 and 8 show the expected size of a vector containing an insert of the amplified region of a RyR gene (ZF6 and ZF2 respectively)

2.6 *In situ* Hybridization to Embryos

Whole mount *in situ* hybridization was performed by Bernard Thisse and Christine Thisse at the University of Strasbourg, France (Thisse et al., 2004). Embryos were fixed at particular stages with paraformaldehyde. This was followed by protein digestion and post fixation. Digoxegenin labelled RNA probes were prepared using ZF2, ZF6 and ZF53-7 cDNA according to Boehringer instructions (Cat. #1175025). Plasmids were linearized using restriction enzymes, and the cDNA was used to create anti-sense RNA probes labelled with digoxigenin. Zebrafish embryos were incubated with the DIG-labelled RNA probes overnight at 55˚C. The excess probes were removed and the embryos were stained for at least 30 minutes. Pictures of the embryos were taken and adjusted for contrast and brightness using Photoshop software.

2.7 Phylogenetic Analysis

The ZF2 and contig sequences were aligned with other RyR isoforms using Clustalx (Thompson, 1997). Bootstrapping was performed on the alignment using the Seqboot program of PHYLIP, with 500 replicates. This statistical technique randomly samples columns of the alignment with replacement in order to determine the variation between sequences. The alignment was sampled 500 times and a parsimony analysis was performed on the output file using the Protpars program of PHYLIP. The PHYLIP program Consense determined the arrangement of the tree that best fit the sequences.

3. Results

3.1 Sequencing

A random selection of cloned PCR products from two sets of RyR primer pairs yielded four sequences that show sequence identity to published RyR sequences. The degenerate primers, ZFF2/ZFR2, amplified the sequences designated ZF2, ZF6 and ZF8, and the primers, ZF53F1/ZF53R1, amplified the sequence designated ZF53-7. Alignment of the four sequences showed that two distinct sequences were identified (see appendix 7.2); the ZF2 sequence appeared to be different from the other three sequences (Figure 3). The nucleotide sequences of ZF6, ZF8 and ZF53-7 were translated, in silico, into protein sequences and aligned. From the alignment, the sequences appeared to be derived from the same gene. The ZF6 and ZF8 nucleotide sequences were 99% similar, with only three nucleotides of 496 differing. The protein sequences of ZF6 and ZF8, however, were identical (Figure 3). The ZF53-7 sequence extends further in the 5' direction than the ZF6 and ZF8 sequences. 99 amino acids of the three deduced protein sequences overlap. The alignment of these 99 amino acids was 98% similar. Each sequence was compared to the Genbank database using tBLASTn, which uses a protein sequence to search against a translated nucleotide database (Table 1). Only one isoform came up in the top three matches for each sequence. The ZF2 protein sequence resulted in matches with RyR1 isoforms, and the ZF6, ZF8 and ZF53-7 protein sequences all resulted in matches with RyR3 isoforms. A sequence contig was constructed of the ZF6, ZF8, and ZF53-7 sequences (see appendix 7.3). Pairwise comparison of the ZF6/ZF8/ZF53-7 contig with published RyR isoform sequences was performed to determine sequence similarities. The greatest similarities were seen when compared to the RyR3 sequences (Table 2).

Figure 3: Alignment of deduced ZF6, ZF8, ZF2 and ZF53-7 zebrafish amino acid sequences. Identical amino acids are shaded black, functionally conserved amino acids are shaded in grey.

Table 1: Top three matches using a tBLASTn search of Genbank database for ZF2, ZF6, ZF8 and ZF53-7 amino acid sequences. Accession numbers of matching sequences are shown. E values are shown in parenthesis.

Sequence	Match 1 (E value)	Match 2 (E value)	Match 3 (E value)
ZF2	Pig RyR1, NM_001001534 (1e-78)	Pig RyR1, M91452 (1e-78)	Pig RyR1, M91451 (1e-78)
ZF6	Human RyR3, NM_001036 (2e-82)	Mouse RyR3, AK132464 (3e-80)	Rabbit RyR3, X68650 (3e-80)
ZF8	Human RyR3, NM_001036 (3e-80)	Mouse RyR3, AK132464 (3e-80)	Rabbit RyR3, X68650 (3e-80)
ZF53-7	Mouse RyR3, AK132464 (1e-82)	Bullfrog RyR3, D21071 (1e-82)	Rat RyR3, AF130881 (1e-82)

Table 2: Percent sequence similarity of contig amino acid sequence with RyR1 sequences from Human (NP_000531) and Mouse(NP_033135), RyR3 sequences from Human (NM_001036) and Rabbit (X68650) and RyR2 sequences from Human (NP_001026) and Mouse (NP_076357)

Sequence	Human RyR1	Mouse RyR1	Human RyR3	Rabbit RyR3	Mouse RyR2	Human RyR2
Contig	90%	89%	**92%**	**92%**	86%	86%
Human RyR1		99%	91%	91%	92%	92%
Mouse RyR1			91%	91%	92%	92%
Human RyR3				99%	90%	90%
Rabbit RyR3					89%	89%
Mouse RyR2						100%

The ZF2 sequence was distinct from the other three protein sequences (Figure 3). The amino acid sequence similarity between the ZF6/ZF8/ZF53-7 contig sequence and ZF2 is 91%, and the nucleotide sequence similarity was 81%. The ZF2 amino acid sequence was aligned with two sequences of each isoform to determine sequence variation between isoforms (Table 3). The sequence similarity of 97% between ZF2 and the RyR1 sequences from human and mouse was evident in the alignment (Figure 4).

Table 3: Percent sequence similarity of the amino acid sequence of ZF2 with RyR1 sequences from Human (NP_000531) and Mouse(NP_033135), RyR3 sequences from Human (NP_001027) and Chicken (NP_996757) and RyR2 sequences from Human (NP_001026) and Mouse (NP_076357)

Sequence	Human RyR1	Mouse RyR1	Human RyR3	Chicken RyR3	Mouse RyR2	Human RyR2
ZF2	**97%**	**97%**	93%	92%	90%	90%
Human RyR1		100%	95%	93%	93%	93%
Mouse RyR1			95%	93%	93%	93%
Human RyR3				97%	94%	94%
Chicken RyR3					93%	93%
Mouse RyR2						100%

```
ZF2              1 ---AFNFFRKFYNMSEDEDEPDMKCDDMMTCYLFHMYVGVRAGGGIGDEIEDPAGDEYEL
Mouse RyR1    4850 TVVAFNFFRKFYNKSEDEDEPDMKCDDMMTCYLFHMYVGVRAGGGIGDEIEDPAGDEYEL
Human RyR1    4853 TVVAFNFFRKFYNKSEDEDEPDMKCDDMMTCYLFHMYVGVRAGGGIGDEIEDPAGDEYEL

ZF2             58 YRVVFDITFFFFVIVILLAIIQGLIIDAFGELRDQQEQVREDMETKCFICGIGSDYFDST
Mouse RyR1    4910 YRVVFDITFFFFVIVILLAIIQGLIIDAFGELRDQQEQVKEDMETKCFICGIGSDYFDTT
Human RyR1    4913 YRVVFDITFFFFVIVILLAIIQGLIIDAFGELRDQQEQVKEDMETKCFICGIGSDYFDTT

ZF2            118 PHGFETHTMEEHNLANYMFFLMYLINKDETEHTGQESYVWKMYQSDA-------------
Mouse RyR1    4970 PHGFETHTLEEHNLANYMFFLMYLINKDETEHTGQESYVWKMYQERCWDFFPAGDCFRKQ
Human RyR1    4973 PHGFETHTLEEHNLANYMFFLMYLINKDETEHTGQESYVWKMYQERCWDFFPAGDCFRKQ

ZF2              ------
Mouse RyR1    5030 YEDQLS
Human RyR1    5033 YEDQLS
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Figure 4: Alignment of ZF2 amino acid sequence with RyR1 sequences from Mouse (NP_033135) and Human (NP_000531). Consensus between the sequences is shown in black, functionally conserved amino acids are shown in grey.

3.2 Phylogenetic Analysis

A multiple sequence alignment of the derived amino acid sequence for the ZF6/ZF8/ZF53-7 contig sequence, the ZF2 clone and selected published RyR sequence was used to determine the phylogenetic position of the zebrafish sequences. The corresponding amino acid sequence derived from the *Drosophila* RyR gene was designated as an outgroup sequence (Figure 5). The phylogenetic analysis was performed using the Protpars program of PHYLIP (Felsenstein, 1992) based on multiple bootstrapped datasets (Seqboot). A consensus tree was determined using the Consense program of PHYLIP. The ZF6/ZF8/ZF53-7 contig sequence clustered with the frog RyR3 sequence with a bootstrap value of 75, whereas the ZF2 sequence clustered with the RyR1 sequences with a bootstrap value of 80 (Figure 6).

```
FrogRyR1          1 AFNFFRKFYNKSEDEDEPDMKCDDMMTCYLFHMYVGVRAGGGIGDEIEDPAGDEYELYRV
RabbitRyR1        1 AFNFFRKFYNKSEDEDEPDMKCDDMMTCYLFHMYVGVRAGGGIGDEIEDPAGDEYELYRV
FishRyR1slow      1 AFNFFRKFYNKSEDEDEPDMKCDDMMTCYLFHMYVGVRAGGGIGDEIEDPAGDEYELYRV
ZF2               1 AFNFFRKFYNMSEDEDEPDMKCDDMMTCYLFHMYVGVRAGGGIGDEIEDPAGDEYELYRV
FrogRyR3          1 AFNFFRKFYNKSEDEDDPDMKCDDMMTCYLFHMYVGVRAGGGIGDEIEDPAGDPYEMYRI
ZFContig          1 AFNFFRKFYNKSEDEDAPDMKCDDMMTCYLFHMYVGVRAGGGIGDEIEDPAGDPYELYRI
RabbitRyR2        1 AFNFFRKFYNKSEDGDTPDMKCDDMLTCYMFHMYVGVRAGGGIGDEIEDPAGDEYEIYRI
Drosophila        1 AFNFFRKFYIQEEDE-EVDKKCHDMLTCFVFHLYKGVRAGGGIGDEIGDPDGDDYEVYRI

FrogRyR1         61 VFDITFFFFVIVILLAIIQGLIIDAFGELRDQQEQVKEDMETKCFICGIGSDYFDTTPHG
RabbitRyR1       61 VFDITFFFFVIVILLAIIQGLIIDAFGELRDQQEQVKEDMETKCFICGIGSDYFDTTPHG
FishRyR1slow     61 VFDITFFFFVIVILLAIIQGLIIDAFGELRDQQEQVKEDMETKCFICGIGSDYFDTTPHG
ZF2              61 VFDITFFFFVIVILLAIIQGLIIDAFGELRDQQEQVREDMETKCFICGIGSDYFDSTPHG
FrogRyR3         61 VFDITFFFFVIVILLAIIQGLIIDAFGELRDQQEQVREDMETKCFICGIGNDYFDTTPHG
ZFContig         61 LFDITFFFFVIVILLAIIQGLIIDAFGELRDQQEQVKEDMETKCFICGIGNDYFDRTPHG
RabbitRyR2       61 IFDITFFFFVIVILLAIIQGLIIDAFGELRDQQEQVKEDMETKCFICGIGNDYFDTVPHG
Drosophila       60 IFDITFFFFVITILLAIIQGLIIDAFGELRDQLESVKDNMESNCFICGMGKDFFDIVPHG

FrogRyR1        121 FETHTLEEHNLANYMFFLMYLINKDDTEHTGQESYVWKMYQERC
RabbitRyR1      121 FETHTLEEHNLANYMFFLMYLINKDETEHTGQESYVWKMYQERC
FishRyR1slow    121 FETHTLDEHNLANYMFFLMYLINKDETEHTGQESYVWKMYQERC
ZF2             121 FETHTMEEHNLANYMFFLMYLINKDETEHTGQESYVWKMYQSDA
FrogRyR3        121 FETHTLQEHNLANYLFFLMYIINKDETEHTGQESYVWKMYQERC
ZFContig        121 FETHTLQEHNLANYLFFLMYLINKDETEHTGQESYVWKMYQERC
RabbitRyR2      121 FETHTLQEHNLANYLFFLMYLINKDETEHTGQESYVWKMYQERC
Drosophila      120 FDTHVQKEHNLANYMFFLMHLINKPDTEYTGQETYVWNMYQQRS
```

Figure 5: Alignment of ZF2 and ZF6/ZF8/ZF53-7 contig amino acid sequences with published RyR sequences, used for phylogenetic analysis. Consensus between the sequences is shown in black, functionally conserved amino acids are show in grey.

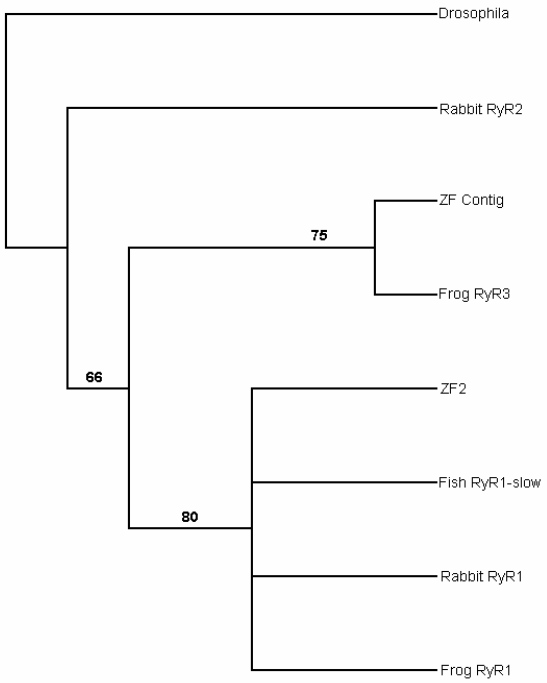

Figure 6: Protein parsimony tree based on multiple sequence alignment of cloned RyR sequences and selected published RyR sequences including Rabbit RyR2 (U50465), Frog RyR3 (D21071), Fish RyR1-slow (U97329), Rabbit RyR1 (X15209), and Frog RyR1 (D21070). The deduced amino acid sequence from the Drosophila RyR gene (NM_057646) was the designated outgroup. The programs, Seqboot, Protpars and Consense of the PHYLIP program package were used to create the tree. Bootstrap values are given as a percentage of 500 replicates.

3.3 *In situ* Hybridization to Zebrafish Embryos

The ZF2, ZF6 and ZF53-7 clones were used to synthesize antisense RNA probes for *in situ* hybridization to zebrafish embryos. The ZF6 and ZF53-7 derived probes revealed the same pattern of expression. Expression was not seen in these embryos until the middle of somitogenesis, where there was weak expression in the somites. At 24 hours post-fertilization (hpf) expression was seen in the myotomes (Figure 7A). Expression in these tissues continued throughout the observations until five days post fertilization (Figure 7B, C, D). At 5 days post fertilization, the expression was only at the myotomes boundaries (Figure 7D). At 48 hpf, expression was evident in the midbrain and hindbrain as well as one of the branchial arches (Figure 7C, E). Expression of the ZF2 sequence was not observed until 24 hpf (Figure 8A, B). From 24 hpf to 48 hpf, expression in the myocytes was visible (Figure 8C, D, E, F). There was no expression at the level of the myoseptum or in the newly formed myotomes (posterior). At 5 days post fertilization there was labelling in the swimbladder (Figure 8G, H, I).

Differences were seen between the two sequences in the labelling patterns of the myotomes. The ZF2 sequence had more homogeneous labelling in the tissues, whereas the ZF53-7 sequence showed more accumulation at the tips of the myofibrils (Figure 9).

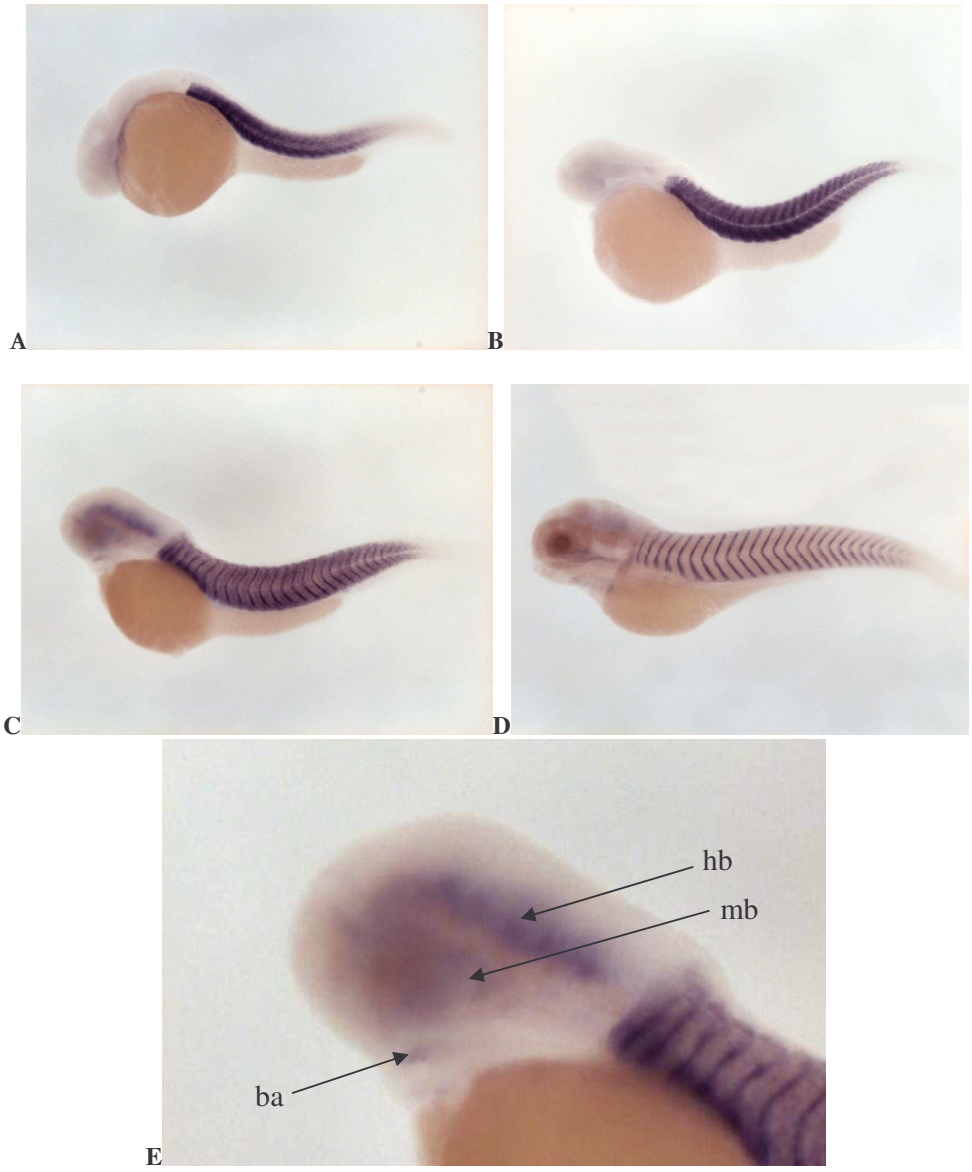

Figure 7: Expression of the ZF53-7 sequence in zebrafish embryos. Lateral view of embryo is shown at 24 hours post fertilization (hpf) (A), 36 hpf (B), 48 hpf (C) and five days post fertilization. Expression is visible in the myotomes from 24 hpf to five days post fertilization. Expression in the hindbrain (hb), midbrain (mb) and branchial arch (ba) is evident at 48 hpf (E).

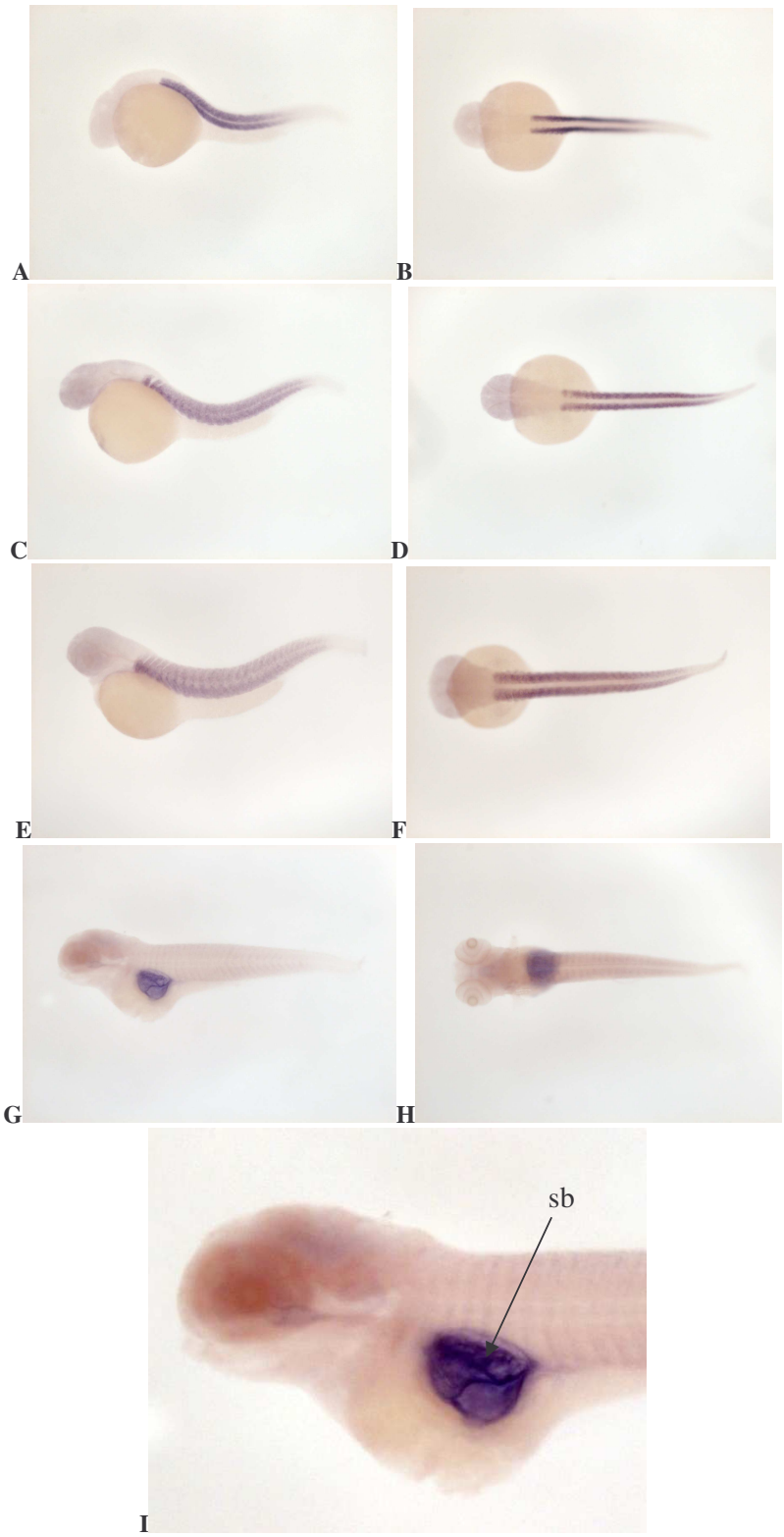

Figure 8: Expression of the ZF2 sequence in zebrafish embryos. Lateral and dorsal view of embryos are shown at 24 hours post fertilization (hpf) (A, B), 36 hpf (C, D), 48 hpf (E, F), and five days post fertilization (G, H). Expression of the sequence in the myotomes is evident from immediately post fertilization to 48 hpf. Expression in the swimbladder (sb) is apparent at five days post fertilization (I).

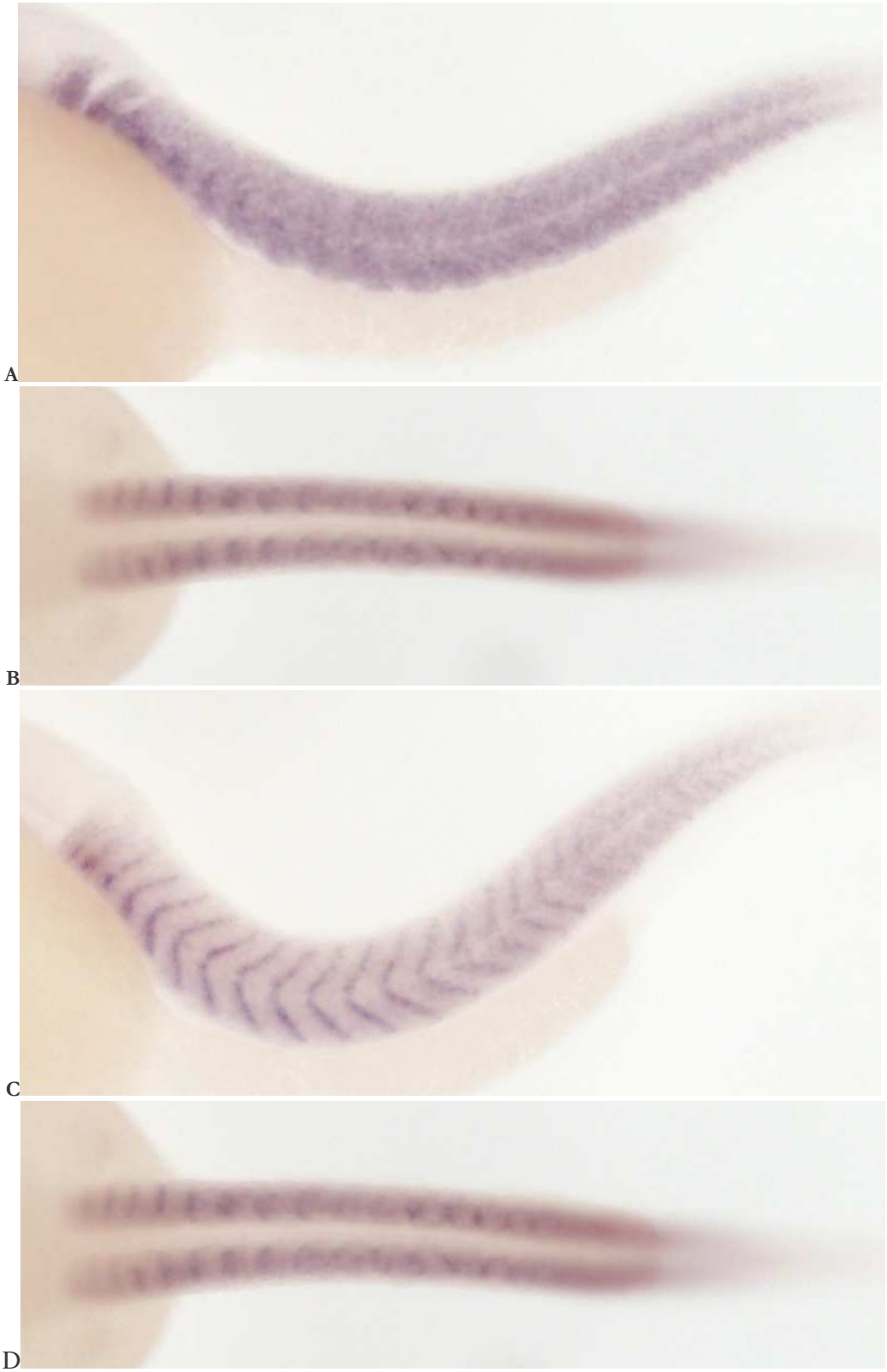

Figure 9: Expression of the ZF2 sequence (A, B) and the ZF53-7 sequence (C, D) in the myotomes at 36 hours post fertilization (hpf).

4. Discussion

4.1 Sequencing

The results of this study identify two distinct isoforms of ryanodine receptors in zebrafish. tBLASTn searches of Genbank were done to first identify which isoform each sequence most resembled. Alignment of the sequences to published sequences from Genbank suggests that the ZF2 sequence is the RyR1 isoform and the ZF6, ZF8 and ZF53-7 sequences are the RyR3 isoform. The partial clones that were identified correspond to the carboxy terminus of the gene. This region is not in one of the three divergent regions seen in RyR genes (Rossi and Sorrentino, 2002), therefore, some sequence similarity should be expected between all isoforms. This was seen when the sequences were aligned to other published RyR sequences. The sequence similarity between the isoforms in this region allows for the degenerate primers to potentially amplify all of the isoforms. The ZF6/ZF8/ZF53-7 contig sequence had highest sequence similarity with the RyR3 sequences of other organisms, however a relatively high sequence similarity was seen between the other isoforms as well. This may have been due to variances in the ZF6, ZF8 and ZF53-7 sequences when constructing the contig. The ZF2 sequence was more easily classified as a RyR1 isoform because of the differences when compared to the other isoforms. There is no published RyR1-fast sequence for this region of the gene published in Genbank. Therefore, from the sequence alone, a conclusion can not be made about which RyR1 isoform was cloned. The results of the tBLASTn search in Genbank allowed identification of the RyR3 sequence, but the RyR1 sequence could only be classified as RyR1, not as the slow or fast isoform.

4.2 Phylogenetic Analysis

The phylogenetic analysis further confirmed which isoform each sequence resembled. Because only one RyR gene has been identified in *Drosophila* (Ebbinghaus-Kintscher et al., 2006), the Drosophila sequence served as an outgroup sequence, allowing the tree to be rooted. The bootstrap values for each isoform were significant, at 75 and 80 percent. The evolutionary relationship between the ZF2 clone and the other RyR1 sequences was significant, however no relationships within this cluster were significant. The phylogenetic analysis did allow identification of the evolutionary relationships between the two zebrafish isoforms and other published RyR isoforms, clustering the zebrafish RyR1 isoform with the published RyR1 isoforms and the zebrafish RyR3 isoform with a published RyR3 isoform.

4.3 Gene Expression in Zebrafish Embryos

Calcium is an important second messenger during development (Creton, 2004; Creton et al., 1998; Jaffe, 1999; Webb and Miller, 2003). Describing the expression and regulation of RyR isoforms during zebrafish development is essential in order to further understand the role that calcium plays during development.

Ryanodine receptor isoforms have been shown to be expressed in a tissue specific manner in vertebrates. Two RyR1 isoforms have been found to be discretely expressed in either fast or slow twitch muscle, the RyR2 isoform has been found in cardiac muscle and the RyR3 isoform has been shown to be ubiquitously expressed.

The RyR1 clone and the RyR3 clone showed different expression patterns in zebrafish embryos. Both of the clones were expressed in the muscles through similar stages of development, although a different pattern was observed even in these tissues. The RyR1 sequence had more homogeneous labelling in the tissues, whereas the RyR3 sequence showed more accumulation at the tips of the myofibrils. Somites can give rise

to the axial skeleton (sclerotome) or the muscles of the trunk (myotome) (Stickney et al., 2000). The majority of the somite in fish gives rise to myotome. The sclerotome goes on to form the ribs and vertebrae and is identified in zebrafish in the ventral part of the somite (Lewis and Bremer, 1927). The adaxial region of the somite appears to be where the first muscle fibers are formed (Thisse et al., 1993). This region is a part of the medial somitic epithelium, adjacent to the notochord (Kimmel et al., 1995).

At five days post fertilization, the RyR1 clone showed labelling in the swimbladder. A study done by Franck, et al. (1998) identified a single RyR isoform expressed in the swimbladder of toadfish that was not expressed in slow twitch muscle. The isoform was designated RyR1-fast. The toadfish swimbladder is thought to be one of the fastest vertebrate muscles known (Rome et al., 1996). Expression of the RyR1 clone is not seen in the swimbladder until 5 days pf, however, observation of the embryos preceding 5 days pf was 72 hours earlier. Expression in the swimbladder may have been seen within this period if hybridization was performed on zebrafish within this 72 hour span. According to Kimmel et al. (1995) the swimbladder of zebrafish first appears around 42 hpf, during the high-pec stage, but does not inflate until approximately 72 hpf. At this stage the fish also begins to swim about actively. A study done by Zeddies and Fay (2005) determined that the swimbladder in zebrafish are not functional until 5 days pf, when the fish start to respond to acoustic stimuli. The expression of the RyR1 clone corresponds to this pattern of swimbladder development. A functional swimbladder contracts and expands which involves the use of ryanodine receptors. Therefore it is expected that a RyR gene would be expressed around the same time that the swimbladder starts to function.

The ZF6 and ZF53-7 sequences which correspond to the RyR3 isoform show expression in the brain at 48 hpf. The RyR3 isoform was originally identified in the brain of rabbit (Hakamata et al., 1992). In developing mouse brain, RyR3 was shown to be

expressed in the brain during the embryonic stage (Mori et al., 2000). The clones appear to be the RyR3 isoform due to the expression seen in the brain, however the RyR1 and RyR2 isoforms are expressed in the brain of mice throughout development as well (Mori et al., 2000). Expression of the zebrafish RyR3 clone in specific regions of the brain can not be determined using these photographs. The hindbrain and the midbrain appear to be expressing the RyR3 sequence, but the specific regions of these parts of the brain can not be determined. According to Mori et al. (2000), in embryonic mouse models the RyR1 isoform was most abundant in the rostral cortical plate, the RyR3 isoform was prominent in the caudal cortical plate and the hippocampus, and low levels of the RyR2 isoform were expressed in the diencephalons and brainstem during the embryonic stage. However, according to a study done by Rosemblit (1999), the RyR2 isoform is first expressed in the developing heart in mice, which was not seen with the expression of the ZF6 or ZF53-7 clones. We can conclude that the ZF6 and ZF53-7 clones do not appear to be RyR2, but appear to be the RyR3 isoform.

4.4 Future Studies

Further research needs to be done on the RyR isoforms in zebrafish. Two more isoforms need to be identified and characterized. The RyR1 isoform that has been identified can be further characterized to confirm that it is the RyR1-fast isoform. *In situ* hybridization studies can be done with all of the isoforms to determine the expression patterns in adult zebrafish. This method will help to differentiate the RyR1-fast and RyR1-slow isoforms, since there are no sequences published from this region of known fast isoforms. As well, once specific primers are designed for each isoform, the location of each gene in the zebrafish genome can be determined using radiation hybrid mapping. This will further support the identification of four distinct isoforms.

5. Conclusions

1. Two distinct ryanodine receptor isoforms have been identified in zebrafish using sequence, phylogenetic and gene expression analyses.

2. The two zebrafish isoforms cluster with published RyR1 and RyR3 isoforms in a phylogenetic analysis with bootstrap values of 80 and 75 respectively.

3. The two zebrafish isoforms show different patterns of expression in the myotomes, brain and swimbladder of zebrafish embryos.

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7. Appendices

7.1 Alignment for Degenerate Primer Design

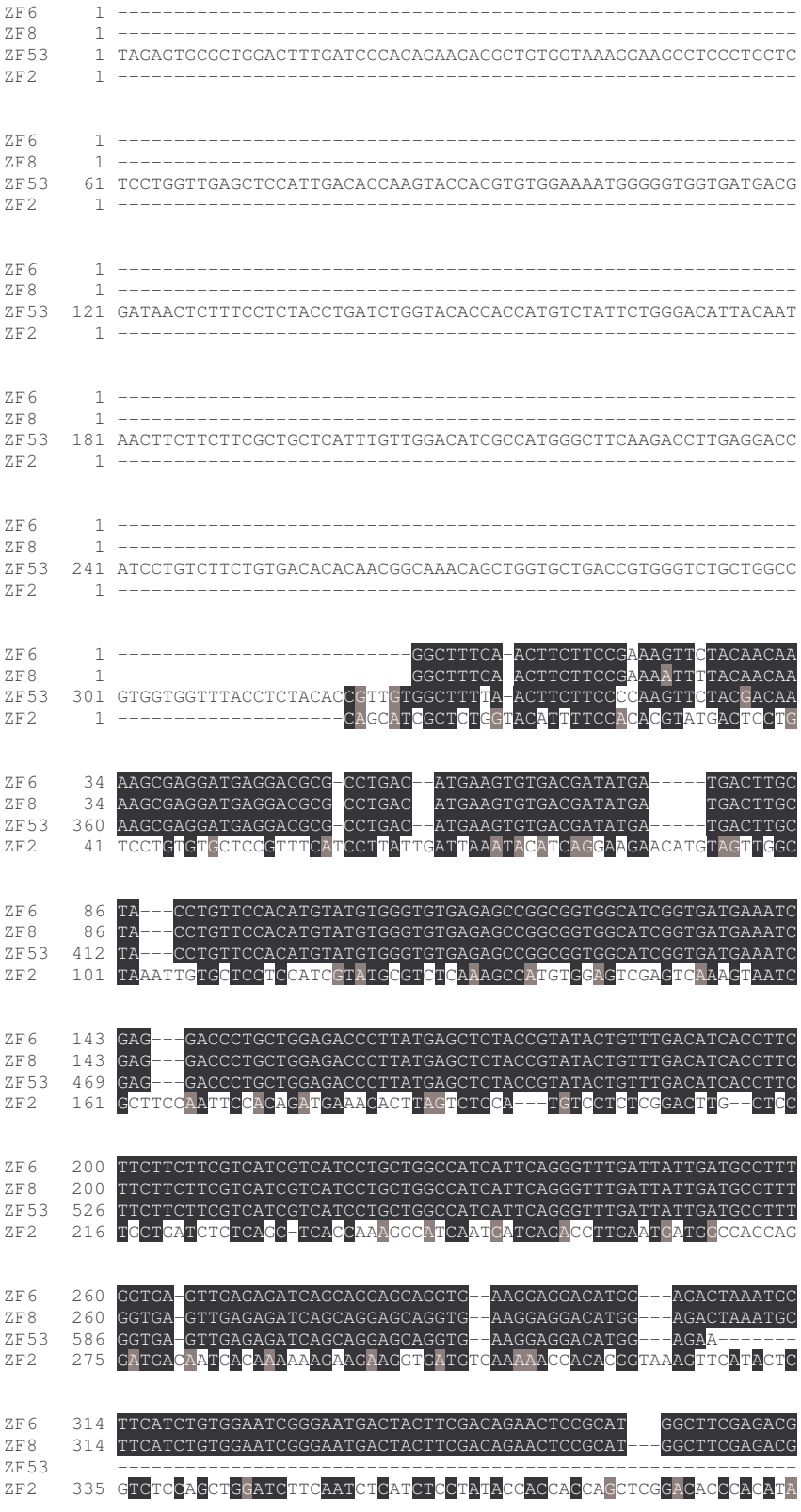
```
FrogRyR1          ILSSVTHNGKQLMMTVGLLAVVVYLYTVVAFNFFRKFYNKSEDEDEPDMKCDDMMTCYLF
FishRyR1slow      ILSSVTHNGKQLMMTVGLLAVVVYLYTVVAFNFFRKFYNKSEDEDEPDMKCDDMMTCYLF
RabRyR1           ILSSVTHNGKQLVMTVGLLAVVVYLYTVVAFNFFRKFYNKSEDEDEPDMKCDDMMTCYLF
FrogRyR3          ILSSVTHNGKQLVLTVGLLAVVVYLYTVVAFNFFRKFYNKSEDEDDPDMKCDDMMTCYLF
RabRyR2           ILSSVTHNGKQLVLTVGLLAVVVYLYTVVAFNFFRKFYNKSEDGDTPDMKCDDMLTCYMF
                  ************::***************************** * ********:***:*

FrogRyR1          HMYVGVRAGGGIGDEIEDPAGDEYELYRVVFDITFFFFVIVILLAIIQGLIIDAFGELRD
FishRyR1slow      HMYVGVRAGGGIGDEIEDPAGDEYELYRVVFDITFFFFVIVILLAIIQGLIIDAFGELRD
RabRyR1           HMYVGVRAGGGIGDEIEDPAGDEYELYRVVFDITFFFFVIVILLAIIQGLIIDAFGELRD
FrogRyR3          HMYVGVRAGGGIGDEIEDPAGDPYEMYRIVFDITFFFFVIVILLAIIQGLIIDAFGELRD
RabRyR2           HMYVGVRAGGGIGDEIEDPAGDEYEIYRIIFDITFFFFVIVILLAIIQGLIIDAFGELRD
                  ********************* **:**::****************************

FrogRyR1          QQEQVKEDMETKCFICGIGSDYFDTTPHGFETHTLEEHNLANYMFFLMYLINKDDTEHTG
FishRyR1slow      QQEQVKEDMETKCFICGIGSDYFDTTPHGFETHTLDEHNLANYMFFLMYLINKDETEHTG
RabRyR1           QQEQVKEDMETKCFICGIGSDYFDTTPHGFETHTLEEHNLANYMFFLMYLINKDETEHTG
FrogRyR3          QQEQVREDMETKCFICGIGNDYFDTTPHGFETHTLQEHNLANYLFFLMYIINKDETEHTG
RabRyR2           QQEQVKEDMETKCFICGIGNDYFDTVPHGFETHTLQEHNLANYLFFLMYLINKDETEHTG
                  *****:*************.*****.*********:*******:*****:****:*****

FrogRyR1          QESYVWKMYQERCWDFFPAGDCFRKTYEDQLG
FishRyR1slow      QESYVWKMYQERCWDFFPAGDCFRKQYEDQLS
RabRyR1           QESYVWKMYQERCWDFFPAGDCFRKQYEDQLS
FrogRyR3          QESYVWKMYQERCWDFFPAGDCFRKQYEDQLG
RabRyR2           QESYVWKMYQERCWEFFPAGDCFRKQYEDQLN
                  **************:********** *****.
```

7.2 Alignment of ZF6, ZF8, ZF53 and ZF2 Nucleotide Sequences



7.3 Alignment of ZF8, ZF6, ZF53-7 and Contig Sequence

```
ZF8       1 ------------------------------------------------------------
ZF6       1 ------------------------------------------------------------
CONTIG    1 SALDFDPTEEAVVKEASLLSWLSSIDTKYHVWKMGVVMTDNSFLYLIWYTTMSILGHYNN
ZF53-7    1 SALDFDPTEEAVVKEASLLSWLSSIDTKYHVWKMGVVMTDNSFLYLIWYTTMSILGHYNN

ZF8       1 ----------------------------------------------AFNFFRKFYNKS
ZF6       1 ----------------------------------------------AFNFFRKFYNKS
CONTIG   61 FFFAAHLLDIAMGFKTLRTILSSVTHNGKQLVLTVGLLAVVVYLYTVVAFNFFXKFYXKS
ZF53-7   61 FFFAAHLLDIAMGFKTLRTILSSVTHNGKQLVLTVGLLAVVVYLYTVVAFNFFPKFYDKS

ZF8      13 EDEDAPDMKCDDMMTCYLFHMYVGVRAGGGIGDEIEDPAGDPYELYRILFDITFFFFVIV
ZF6      13 EDEDAPDMKCDDMMTCYLFHMYVGVRAGGGIGDEIEDPAGDPYELYRILFDITFFFFVIV
CONTIG  121 EDEDAPDMKCDDMMTCYLFHMYVGVRAGGGIGDEIEDPAGDPYELYRILFDITFFFFVIV
ZF53-7  121 EDEDAPDMKCDDMMTCYLFHMYVGVRAGGGIGDEIEDPAGDPYELYRILFDITFFFFVIV

ZF8      73 ILLAIIQGLIIDAFGELRDQQEQVKEDMETKCFICGIGNDYFDRTPHGFETHTLQEHNLA
ZF6      73 ILLAIIQGLIIDAFGELRDQQEQVKEDMETKCFICGIGNDYFDRTPHGFETHTLQEHNLA
CONTIG  181 ILLAIIQGLIIDAFGELRDQQEQVKEDMETKCFICGIGNDYFDRTPHGFETHTLQEHNLA
ZF53-7  181 ILLAIIQGLIIDAFGELRDQQEQVKEDME--------------------------------

ZF8     133 NYLFFLMYLINKDETEHTGQESYVWKMYQERC
ZF6     133 NYLFFLMYLINKDETEHTGQESYVWKMYQERC
CONTIG  241 NYLFFLMYLINKDETEHTGQESYVWKMYQERC
ZF53-7      --------------------------------
```