IDENTIFYING THE 5’END OF THE CAMTA1 GENES IN ZEBRAFISH

Morgan Fronk

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Honors Thesis

IDENTIFYING THE 5’END
OF THE CAMTA1 GENES IN ZEBRAFISH

by

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Submitted to Brigham Young University in partial fulfillment of graduation requirements for University Honors

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ABSTRACT

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OF THE CAMTA1 GENES IN ZEBRAFISH

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Congenital heart diseases (CHDs) are a significant cause of infant death and are frequently caused by mutations in transcription factors. Camta1 (calmodulin binding transcription activator 1) is a transcription factor that has been proposed as a modulator in embryonic heart development and a possible cause of CHDs. The only other known member of its family in vertebrates is involved in activating a hypertrophy gene program in adult heart failure. Unlike camta2, camta1 is expressed in the embryonic heart during heart looping. However, few studies have been done on camta1. In zebrafish, there are two camta1 ohnologs (homologs created through a duplication event), camta1a and camta1b. These have not yet been annotated fully so the 5’end is unknown, preventing further analysis and study. Here, we use 5’RLM-RACE (RNA Ligase Mediated Rapid Amplification of cDNA Ends) to determine the 5’end of camta1a and begin the process of understanding camta1 regulation through nucleotide BLAST. Future studies can build on this and elucidate the regulation of the camta1 genes in zebrafish by locating the 5’end of camta1b, determining the promoter regions of both genes, and beginning studies on its
downstream targets, which may reveal an important role for *camta1* in cardiac development.
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I. Background and Significance

Congenital heart disease (CHD) is the most common birth defect in the United States, affecting nearly 40,000 or 1% of live births annually (Hoffman and Kaplan, 2002). The etiology of CHDs is not fully understood, although recent research is establishing the significance of genetics in contributing to its incidence (Edwards and Gelb, 2016). Determining the genes involved in embryonic heart development can contribute to improved understanding of the gene mutations involved in CHDs and lead to better methods for diagnosis, treatment, and prevention. However, identifying these genes alone is not enough. It will also be necessary to understand the transcriptional regulation of these genes, which controls how these genes are expressed. Heart formation requires complex patterns of gene expression, requiring dynamic transcriptional regulation that is not well understood (Nemer, 2007). Elucidating these regulatory pathways will not only expand our knowledge of cardiac development but may also reveal links between these transcriptional regulators and CHDs.

Due to the complexity of gene expression required for cardiac development, many transcription factors are needed to regulate this process. Family-based heritability studies on CHDs have primarily identified transcription factors, indicating that many of the known causes of CHDs in humans are mutations in such factors (Reamon-Buettner and Borlak, 2010). Transcription factors identified in early heart morphogenesis include GATA4, Nkx2.5, Tbx5, Hand1, and Hand2. Mutations of these factors have been associated with many types of CHDs in humans, although their exact molecular mechanisms are not yet fully understood (Nemer, 2007). For example, Nkx2.5 mutations in humans are known to cause cardiomyopathy, septal defects, and conduction defects (Hanley et al., 2016; Xu et al., 2017a; Xu et al., 2017b), while the absence of the Nkx2.5
homologs, \textit{nrx2.5} and \textit{nrx2.7}, in zebrafish results in abnormal ventricular and atrial development (Targoff et al., 2009; Reamon-Buettner and Borlak, 2010). However, few of the downstream targets of Nkx2.5 have been identified (Targoff et al., 2009) and its role in the transcriptional regulatory network driving cardiac development is still being defined (Yan et al., 2017). As the role of transcription factors in heart development is elucidated, their causative role in CHDs will become clearer as well (Nemer, 2007).

The \textit{camta1} (calmodulin binding transcription activator) gene has been proposed as a possible candidate for regulation of cardiac development and involvement in CHDs (Schwartz and Schneider, 2006). Little research has been done on this gene, but the only other member of its family in vertebrates, \textit{camta2}, is known to activate a hypertrophy gene program in adult heart failure in mice (Song et al., 2006) through the interaction of its CG-1 domain with the homeodomain of Nkx2.5 to activate transcription of hypertrophic genes (Schwartz and Schneider, 2006). The amino acid sequences of Camta1 and Camta2 share many homologous regions, including the CG-1 domain (figure 1) (Long et al., 2014). \textit{Camta2} is expressed only in adults while \textit{camta1} is strongly expressed in the embryonic mouse heart (Song et al., 2006). RNA-seq time course experiments by Dr. Jonathon Hill have determined that \textit{camta1} is transiently expressed during heart morphogenesis, peaking at approximately 48 hours post fertilization (hpf) (figure 2) (Hill et al., 2017), which is when heart looping occurs in zebrafish (Bakkers,
The brief temporal expression of *camta1* in the embryonic heart suggests that it has a very specific duty during that time period, rather than a more general one. Computational analysis data show that *camta1* has a high number of interactions with other molecules, suggesting its role as an important regulator (figure 3) (Hill et al., 2017).

Preliminary CRISPR knockouts of *camta1* performed by the Hill lab in zebrafish show heart defects (figure 4) (courtesy of Z. Taylor). Embryos injected with CRISPR sgRNAs targeting *camta1a* show a dilated atrium, shrunken ventricle, and lack of proper heart looping. This phenotype is significant because it is similar to the Nkx2.5 phenotype.
Figure 3. Graph of known and predicted gene regulatory interactions for cluster A1. Size of the node indicates the number of interactions it has with other genes. Camta1 (circled in red) has the largest node and thus the largest number of interactions, marking it as a likely regulator (Hill et al. 2017).

Figure 4. A. 48 hpf embryo with a healthy heart. The ventricle and atrium are roughly equal in size and heart looping can be seen. B. 48 hpf embryo with camta1a knocked out using CRISPR/Cas9 technology. The heart has an enlarged atrium, shrunken ventricle, and a looping defect (courtesy of Z. Taylor).
(Targoff et al., 2008), and Nkx2.5 is a known partner of Camta2 (Song et al., 2006). These data indicate that camta1 plays an important role in embryonic heart development, making it a promising candidate for further research.

While humans have only one camta1 gene, there are two camta1 ohnologs (camta1a and camta1b) in zebrafish due to a whole-genome duplication (WGD) event that occurred in an ancestral species approximately 350 million years ago. These genes show identical temporal expression patterns (figure 5), suggesting that each retains an important role.

Figure 5. Temporal expression patterns of camta1a and camta1b. Normalized read counts are shown from 30 to 72 hpf. Note the similar pattern, peaking at approximately 48 hpf (Hill et al. 2017).

Again, we see that peak expression occurs around 48 hpf, simultaneous with heart looping in zebrafish (Bakkers, 2011). Regions conserved on the promoter regions of both camta1a and camta1b can thus be assumed to have essential functions and can be compared to human camta1 in future studies (Kleinjan et al., 2008).

Most Camta1 research has been conducted in the plant Arabidopsis thaliana, which contains a large family of orthologs closely related to Camta1 in vertebrates. One study recently revealed that A. thaliana uses Camta proteins to regulate specific gene responses
to different calcium levels. Calcium signaling is sensed by a Ca$^{2+}$-calmodulin-CAMTA complex to code for specific gene expression responses in response to stress (Liu et al., 2015). While this interaction has only been studied in plants so far, the calmodulin-binding IQ domain is conserved in vertebrates. The potential to respond to changing calcium levels has significant implications for Camtas in heart development. Calcium signaling has been shown to play an important role in cardiac development through transcriptional regulation (Li et al., 2002; Linask et al., 2001), specifically during cardiac looping (Porter et al., 2003). Other calmodulin-dependent enzymes have already been implicated in cardiac hypertrophy and regulation of growth and function in response to calcium in cardiomyocytes (Frey et al., 2000; Passier et al., 2000). We propose that Camtas behave similarly to other calmodulin-dependent enzymes and their counterparts in A. thaliana.

Taken together, this evidence suggests a model where camta1 translocates to the nucleus and binds Nkx2.5 under specific circumstances to play an important role in the activation of certain heart development genes. According to our model, stress causes calcium signaling. This calcium signaling could be caused by voltage-gated calcium channels, but it is more likely caused by developmental stressors like the build-up of blood pressure in areas where certain structures of the heart, like the ventricle, need to form. This stress-induced calcium signaling leads to the activation of calmodulin and subsequent formation of Ca$^{2+}$-calmodulin-Camta complexes. These complexes then translocate to the nucleus (figure 6B). We hypothesize that this complex then interacts with Nkx2.5 in the nucleus to activate transcription of genes necessary for ventricular specification or maintenance(figure 6C). The full picture of how this plays out cannot be
fully understood, however, without understanding what is occurring upstream of camta1 in its regulatory regions (figure 6A).

If camta1 is indeed an important regulator in heart development, it is essential to understand how it is regulated itself. We hypothesize that the important regulatory regions will be conserved between both ohnologs, as they have identical temporal expression patterns. However, it is currently difficult to determine the regulation of camta1 because its 5’ end is not fully annotated in zebrafish, so its promoter region and transcription start site (TSS) are unknown, preventing bioinformatic comparison of the regulation of both ohnologs. Defining the 5’ end and locating the TSS would enable identification of the promoter, which would allow for further characterization and annotation of camta1 (Kim et al., 2012). Recent bioinformatic analysis performed in the Hill lab has indicated that the incomplete annotation may be due to exon one of both camta1a and camta1b being located in the wrong place in the genomic annotation (figure 6).
7, *camta1a* not pictured), but this cannot be confirmed until the zebrafish 5’UTR is determined. Once the TSS has been identified, its genomic location can be determined and homology analysis can be performed by aligning the putative promoter regions of *camta1a* and *camtalb* to identify conserved regulatory elements in both ohnologs (Kleinjan et al., 2008).

To determine the *camta1a* and *camtalb* TSS, we used 5’RACE (Rapid Amplification of cDNA Ends) using a primer set designed to both *camta1a* and *camtalb*. Amplification of the 5’ end of *camta1a* was successful and confirmed that the annotation does have an error in the genome assembly preventing proper annotation of the gene. Surprisingly, the alignment of the identified 5’ end of zebrafish corresponded to exon 3 of mouse Camta1, indicating that there is either an alternative TSS for *camta1a* in the heart, or that the 5’ end is not conserved between species. These results will be used to guide future experiments to study the transcriptional regulation of *camtal* in the developing zebrafish heart.

Figure 7. *camtalb* alignment compared to current annotation of zebrafish chromosome 11. This was determined by blasting a mouse exon one to the zebrafish genome but cannot be confirmed until the zebrafish 5’UTR is determined. (courtesy of A. Martin).
II. Materials and Methods

Primer design

Inner and outer primers were designed for both *camta1a* and *camta1b* using Geneious primer design software and following the specifications given in the 5’RLM-RACE protocol (Invitrogen). OligoAnalyzer 3.1 (Integrated DNA Technologies) was used to check for self-dimers, heterodimers, hairpins, and GC content (Table 1). 5’RLM-RACE uses a two-step PCR to increase specificity and thus requires two specific inner and outer primers for each gene (figure 8). There is one primer for the initial PCR and another for the second, embedded PCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td><em>camta1a</em> inner</td>
<td>CGTGGAGCATTTTGGTAGACAT</td>
</tr>
<tr>
<td><em>camta1a</em> outer</td>
<td>TTCCACCTGTGTCTCTCTTTG</td>
</tr>
<tr>
<td><em>camta1b</em> inner</td>
<td>GCCTTAGATTGATTGGGAGGGT</td>
</tr>
<tr>
<td><em>camta1b</em> outer</td>
<td>TTAGCAGGTGTCTCGTGAGATGG</td>
</tr>
</tbody>
</table>

Table 1. Primers used for 5’RLM-RACE

Heart isolation

Zebrafish embryos (*Danio rerio*) with hearts expressing GFP were collected and incubated for 48 hpf before being dechorionated. Hearts were then removed using a
protocol modified from Burns and MacRae, 2006. Briefly, GFP-positive embryos were fragmented with a 19-gauge needle in L-15 Leibowitz cell culture media enriched with 5% FBS. A pipet was used to separate the hearts underneath an Olympus SZX16 stereomicroscope based on green fluorescence. Extracted hearts were then placed in TRI Reagent (Zymo), homogenized with an insulin needle, and stored at -80 °C until RNA extraction.

*RNA extraction*

RNA was extracted from the isolated hearts with the Direct-zol RNA MicroPrep kit (Zymo Research), following the included protocol. Due to the difficulty of extracting only heart RNA and maintaining purity, RNA concentrations were low (40-50 ng/μL), yielding less than 1 μg of RNA for the 5’RACE, enough for only a small reaction. The 5’RACE protocol recommended using 10 μg of RNA for a standard reaction, so we also extracted RNA from whole zebrafish embryos using TRI Reagent (Zymo) and the Direct-zol RNA MicroPrep kit (Zymo Research) to obtain the needed amounts of material. Concentrations were higher (ranging from 250-350 ng/μL), but only provided 4 μg.

5’RLM-RACE

Extracted RNA from hearts or whole embryos was used for 5’RLM-RACE with the FirstChoice RLM-RACE kit (Invitrogen), following the included protocol. We performed the small reaction, using up to 1 μg of RNA, several times and the standard reaction, using approximately 4 μg of whole embryo RNA, once. A positive control from
the kit was performed alongside these reactions, along with two non-template controls in the PCR steps.

**TOPO TA Cloning and Sequencing**

The PCR product from the completed 5’RLM-RACE was cloned into bacteria using the Zero Blunt TOPO PCR Cloning kit (Invitrogen). Bacteria was then spread on LB plates containing Kanamycin and incubated at 37°C for 24 hours. Isolated colonies were then chosen and transferred to 3 ml LB containing Kanamycin and incubated for an additional 24 hours at 37°C. To prepare the bacteria for sequencing, DNA was extracted using the GenElute Plasmid Miniprep kit (Sigma-Aldrich), following the included protocol. The Big Dye sequencing reaction was then performed using standard methods and read on an Applied Biosystems 3730xl DNA Analyzer at the BYU DNA Sequencing Center.

**Data Analysis**

The 5’RACE insert was determined by using SnapGene to align sequencing results against the PCR Blunt II TOPO vector. This insert was then aligned against the zebrafish genome using NCBI Blast and analyzed and compared to mouse *camta1* using the Ensembl genome browser.
III. Results

In order to locate the TSS on the *camta1* genes in zebrafish, we conducted 5’RACE (Rapid Amplification of cDNA Ends). 5’RACE, also known as anchored PCR, is used to amplify the 5’ ends of RNA strands when only a partial sequence is available. We chose to use RNA Ligase Mediated RACE (RLM-RACE) because it improves on classic RACE by preventing fragmented RNA from being amplified (figure 9). We performed both standard (10 ug of starting whole embryo RNA) and small reactions (<1 ug starting heart RNA) of the RACE, along with a positive control and non-template controls. Samples were run on an agarose gel for visualization (figure 10). The standard reaction (lanes 3-6) did not show any amplification, likely due to insufficient starting
material (4 ug vs. 10 ug). However, the small reactions for *camta1a* (lanes 7 and 8) yielded clear bands at approximately 80 bp. Neither the standard nor small *camta1b* reactions (lanes 5, 6, 9, and 10) yielded visible bands. The positive controls (lanes 11 and 12) also did not amplify anything. This may be due to using rSAP (shrimp alkaline phosphatase) instead of CIP (cow intestine phosphatase) on the first step of preparing the positive control (see figure 9), as the kit ran out of CIP. Although we believed rSAP should have the same effects as CIP, it may have affected the reaction by failing to remove the 5’ phosphate group on fragmented mRNA or DNA, which might have been amplified later. The non-template controls (lanes 13 and 14) showed nothing, as expected.

![Figure 10. Gel run to confirm 5’RACE results. Lane 1: 1 kb ladder 2: 100 bp ladder 3-4: Standard A 5-6: Standard B 7-8: Small A 9-10: Small B 11-12: Positive control 13: NTC with A primers 14: NTC with B primers](image-url)
The small *camta1a* samples were sequenced and the sequencing results were used to determine the insert (figure 11), which was then aligned to the zebrafish genome. This yielded a 100% match with a 61-base pair (bp) segment including bases 30,659,109-30,659,169 on the forward strand of chromosome 23 in the GRCz11 Primary Assembly. Interestingly, the current annotation of chromosome 23 (where *camat1a* is found) in zebrafish places the insert on the reverse strand, running at 30,431,333-30,061,364 (see figure 12). When compared to the mouse genome, our insert aligned to Camta1 exon 3 (data not shown).

![Figure 11. Small A sequencing results compared against the PCR II Blunt TOPO vector to determine the insert for BLASTing. The asterisk (*) marks sample F2, which was used to BLAST against the zebrafish genome.](image1)

![Figure 12. Comparison of the positioning of our insert in the current annotation and chromosomal alignment of chromosome 23.](image2)
IV. Discussion and Conclusions

Here, we determined the location of the 5’end of camtala through 5’RLM-RACE and nucleotide BLAST. This is an important step for continuation of camtal studies in zebrafish in the future. Analysis of the 5’end of camtala has already been very revealing, suggesting several important steps for future studies.

The chromosomal alignment of our insert with the zebrafish genome supports our prediction that the current annotation of camtal in zebrafish is incorrect (see figures 7 and 10). Significantly, our insert falls near a contig boundary, where annotation mistakes are more likely to occur. Once the 5’end of camtalb is located, it will be interesting to see if it, too, is aligned differently than it appears in the current annotation.

Our insert also shows up in exon 3 of camtal in the mouse genome, rather than at the 5’end as expected. There are three main possibilities for this occurrence:

1. *The insert we discovered is not the actual 5’end* – This is unlikely as 5’RACE (and especially 5’RLM-RACE) is generally highly accurate. The RLM-RACE protocol incorporates a dephosphorylation step to prevent ligation of the 5’ primer to fragmented RNA or DNA. In addition, the BLAST alignment extends across an exon boundary, suggesting that our results are not from fragmented genomic DNA.

2. *Evolutionary divergence between mice and zebrafish* – Figure 1 shows that the first functional domain of Camtal begins at amino acid 63, and the determined 5’ end is less than 100 bp from the annotated mouse ATG. Thus, this insert could have been deleted in the zebrafish without affecting the functionality of the protein. Evolutionary differences between mice and zebrafish might have resulted in a deletion only in camtala or in both
camta1a and b in zebrafish. This distinction cannot be made until the 5’end of camta1b is located and aligned to the mouse genome, though. If evolutionary divergence is the cause of this result, our plan to use homology analysis to locate the promoter in the future may not work.

3. Alternative TSS – There is a possibility that has an alternative TSS in the heart. This hypothesis could be tested by performing 5’RACE with RNA from different tissues. Interestingly, the standard reaction performed using whole embryo RNA showed a faint band of a different length than the small reaction, which used only heart RNA (compare lane 3 to lanes 7 and 8 of figure 8).

We propose that future studies of camta1 focus on locating the 5’end of camta1b through 5’RLM-RACE and continue analysis of the 5’ends of both genes to determine their correct annotation and to identify their promoter regions. The alternative splicing hypothesis can also be proven through an additional 5’RACE comparing RNA from hearts, brains, and whole embryos. As camta1 studies continue in zebrafish, the 5’ends of both genes will soon be fully elucidated, allowing for further studies to better understand regulation of these genes and determine their proper annotation. As understanding of camta1 increases, its putative role in heart development will also be revealed, leading to breakthroughs in the treatment and prevention of CHDs.
References


