Analysis of the Minimal Promoter from the Hatching Enzyme a Gene

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ANALYSIS OF THE MINIMAL PROMOTER FROM
THE HATCHING ENZYME 1a GENE

A THESIS SUBMITTED TO
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BY
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To my mom, Janina Kreczko
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ABSTRACT

Hatching, defined as a biochemical or biophysical mechanism that allows the embryo to leave its protective envelope, is found in most animals. In fish, reptiles and amphibians, mostly oviparous animals, this means the emergence of the embryo from an egg. In mammals, viviparous animals, hatching is performed by the blastocyst in order to shed the zona pellucida.

Fish, an oviparous animal, take advantage of a biochemical mechanism in order to hatch and emerge from their chorion, or egg envelope. The mechanism includes the use of hatching enzymes that are secreted in order to digest the envelope membrane. The genes controlling the expression of these enzymes are transcribed and translated early in development and are secreted from the animal itself to perform their function. The gene, which controls expression of the protein, is in turn regulated by an upstream region called the promoter. It is the main goal of this project to clone and characterize the minimal promoter of the hatching enzyme gene within the *Danio rerio*, zebrafish genome. This fragment will contain all the necessary regulatory elements to bind transcription factors and drive gene expression. The identification and analysis of the minimal promoter of the hatching enzyme gene may also produce a molecular tool that consists of an extremely short and strong promoter to be used in conjunction with a variety of genetic screens as a reporter.
CHAPTER I

INTRODUCTION

During development most animals have to leave their protective envelope, using a biophysical or biochemical mechanism called hatching. In mammals, viviparous animals, hatching is performed by the blastocyst in order to shed the zona pellucida (Yasumasu, Iuchi, & Yamagami, 1988). In fish, reptiles and amphibians, mostly oviparous animals, this means the emergence of the embryo from an egg. This phenomenon, which transforms an animal from intracapsular life to a free-living larva, can occur via a biophysical, biochemical or a combination of both means. Biophysical mechanisms use physical forces, for example pecking, mastication, a blow from the tail or an increase in embryonic pressure, in order to break the egg envelope. This type of hatching is seen in chickens, lizards and ducks, to name a few. A biochemical mechanism utilizes enzymatic activity to soften the egg envelope and make it more susceptible to breakage (Yamagami, Hamazaki, Yasumasu, Masuda, & Iuchi, 1992). Fish take advantage of both methods in order to hatch and emerge from their chorion or egg envelope. As a result they express hatching enzymes that are secreted in order to soften and digest one layer of the envelope membrane followed by embryonic muscular movements to rupture the outer layer of the membrane.
**Egg Envelope**

The fish chorion is a thick non cellular and multilayered coat completely surrounding the fish oocyte (Figure 1) (Cotelli, Andronico, Brivio, & Lamia, 1988).

![Chorion](image)

**Figure 1.** Image of pre-hatching zebrafish embryo surrounded by its chorion (Schrandt, 2009).

It is a key player in the process of fertilization, particularly when it comes to species specificity and preventing polyspermy. It also protects and isolates the egg and embryo from outside environmental conditions. The chorion is composed of proteins and glycoproteins arranged into two layers, (1) a thin outer vitelline layer, directly adjacent to the outer surface, and (2) an extremely thick chorionic layer, bordering the embryo. The outer layer is constructed of three heterogeneous glycoproteins, zona pellucida 1 (ZP-1), zona pellucida 2 (ZP-2) and zona pellucida 3 (ZP-3). These three glycoproteins are synthesized in the female liver and moved to the ovary via the blood stream. ZP-1 plays a
role in sperm binding while ZP-2 and ZP-3 act as sperm receptors. The zona pellucida domain, found in all three glycoproteins, is an essential characteristic common in all vertebrate egg envelopes (Hamazaki, Nagahama, Iuchi, & Yamagami, 1989).

An unfertilized egg is soft and fragile; however upon fertilization the glycoprotein components are polymerized via a transglutaminase to form $N^\epsilon$-(γ-L-glutamyl)-L-lysine isopeptide cross links between the glycoprotein subunits of the envelope. These cross links create a hard and turgid structure, both in mechanical potency and opposition to environment, that provides the fragile embryo with protection from both chemical and physical stresses (Figure 2) (Mari, Yasumasu, Shimizu, Sano, Iuchi, & Nishida, 2010) (Yamagami, Hamazaki, Yasumasu, Masuda, & Iuchi, 1992).

The reproductive method of most oviparous animals starts off by the production of an ovum that is very delicate and susceptible to damage. Following fertilization, to avoid physical adversity and allow the embryo to mature, the egg develops protective layers which result in a sturdy and impenetrable envelope. Ironically, there comes a time when the developed embryo is required to fracture the chorion and emerge.

In order to digest and soften the chorion, the embryo secretes a hatching enzyme, produced by the hatching gland. Teleostean hatching enzymes have been well studied, specifically medaka (Oryzias latipe), and zebrafish (Danio rerio), and amazingly it has been discovered that molecular structures of hatching enzymes from fish to birds are conserved (Yasumasu, Mao, Sultana, Sakaguchi, & Yoshizaki, 2005). These enzymes are zinc metalloproteases belonging to the astacin family zinc endopeptidases. All the family
members, forming an orthologous group, are composed of approximately 200 amino acids with characterized consensus sequences (Quesada, Sanchez, Alvarez, & Lopez-Otin, 2004). The zinc metalloproteases have a consensus sequence of HExxHxxGFxxHExxRxDR, the zinc motif, which binds zinc ions, and SxMHY, the methionine turn and two intramolecular disulfide bridges formed by four conserved cystein residues. The teleostean hatching enzymes have a unique twenty amino acid segment in the N-terminus containing two extra cysteine residues (Kawaguchi, Yasumasu, Hiroi, Naruse, Inoue, & Iuchi, 2006).

The enzymatic properties and gene structure of hatching enzyme have been most studied in medaka. The medaka hatching enzyme is proposed to be a system assembled of two proteases, a high choriolytic enzyme and a low choriolytic enzyme, HCE and LCE respectively. The two function cooperatively in order to solubilize the egg envelope. HCE swells the inner layer of the chorion by digestion and simultaneously releasing small water soluble peptides to increase the envelope’s volume (Kawaguchi, et al., 2005). The LCE solubilizes the inner layer and allows for hatching (Yasumasu, Iuchi, & Yamagami, 1988). The hatching of the medaka embryo is dependent on the cooperation of both proteases to fully disintegrate and digest the inner layer while the outer layer is broken by the embryo’s movement (Yasumasu, et al., 2010). While HCE and LCE are 55% similar to one another it has been decided that all hatching enzyme cDNA’s are homologues to the HCE protease (Kawaguchi, et al., 2005).
Figure 2. Predicted changes within the Zebrafish chorion. After fertilization the crosslinks are formed, creating a hard and turgid structure, HE1a swells the chorion which causes the crosslinks to dissolve and makes the chorion susceptible to physical pressure (Yasumasu, et al., 2010).
All three paralogues are clustered on chromosome 22 and transcribe in the same direction. The relative position of the three genes is 3.5 and 3.2 kilo basepairs (kbp) between \( HE1a \) and \( HE1b \) and \( HE1b \) to \( HE2 \), correspondingly (Figure 3) (Kawaguchi, Yasumasa, Hiroi, Naruse, Suzuki, & Iuchi, 2007). The amino acid sequence of \( HE1a \) and \( HE1b \) are 99% identical to each other and 60.8% identical to \( HE2 \) (Sano, Inohaya, Kawaguchi, Yoshizaki, Iuchi, & Yasumasa, 2008). It is interesting to note that the zebrafish hatching enzyme gene cluster is internal to an aldehyde oxidase 3 (\( aox \) 3) intron.

Figure 3. Map of ZHE gene cluster on chromosome 22. Arrows represent the direction of transcription while the dashed lines denote coding exons (Ensembl, 2012).

Based on molecular phylogenetic analysis, it is theorized that an ancestral hatching enzyme system consisted of a single enzyme and due to duplication and diversification many species use a two enzyme system (Figure 4). When comparing medaka to zebrafish, the latter does contain two enzymes \( HE1 \) and \( HE2 \), however over time it appears \( HE2 \) lost its proteolytic abilities and gained detrimental mutations in its
regulatory region, resulting in low expression (Mari, Yasumasu, Shimizu, Sano, Iuchi, & Nishida, 2010).

Zebrafish hatching enzyme 1a and 1b are 21 kilo Dalton (kDa) zinc metalloproteases that are homologous to the high choriolytic hatching enzyme in medaka (Kawaguchi, Yasumasu, Hiroi, Naruse, Inoue, & Iuchi, 2006). In 1998, Yasumasu et.al. performed Northern blot and RT-PCR analysis to observe gene and RNA expression. Northern blot analysis showed a strong HE1a signal at 11.5 hours post fertilization and no HE2 signal. RT-PCR detected HE1a transcript levels after 19 cycles of PCR and HE2 after 28 cycles. The above data plus a negative result by whole mount in situ hybridization suggests that HE1 is the hatching enzyme responsible for chorion degradation (Sano, Inohaya, Kawaguchi, Yoshizaki, Iuchi, & Yasumasu, 2008). HE1 is sufficient to digest the chorionic layer and allow for the embryo to rupture the vitelline layer.

Figure 4. Theorized evolutionary pathway of hatching enzyme resulting in a two enzyme system used by certain species (Kawaguchi, Yasumasu, Hiroi, Naruse, Suzuki, & Iuchi, 2007).
Gene Regulation

Based on in situ hybridization expression patterns, he1a is first activated during the segmentation period (10-24hpf) and inactivated at hatching (48-72hpf). Expression is also restricted to the hatching gland region, located on the surface of the yolk, with a very specific necklace like pattern (Thisse, et al., 2001). Thus, the *HE1a* gene has a strict embryonic expression pattern that is spatially and temporally regulated.

Transcription is regulated by proteins that bind to specific non-coding DNA sequences and control RNA polymerase activity. The gene transcription control region, the promoter contains specific sequences that are recognized by proteins, known as transcription factors. Core promoter elements, indispensable to the regulation of many genes, are the TATA box and a transcription start site. The promoter also contains specific transcription factor binding sites or enhancers which are essential for the expression of a particular gene. Enhancers, although part of the promoter can be located very far from the gene they are regulating.

Conserved non-coding sequences, those that are similar across species, are of interest due to their potential for gene regulation. Changes in gene regulation are believed to account for a majority of the differences between species. Therefore, study of promoter regions of orthologous or paralogous genes can uncover differences in the presence and or position of transcription factor binding sites.

The zebrafish hatching enzyme has been selected for this research due to its many interesting characteristics and the lack of a complete understanding of the mechanism
controlling its early and very specific regulation. The zebrafish shows a loss of the two hatching enzyme system and a return to the single enzyme system, while maintaining similar hatching mechanisms with those of other species. As will be shown in this study the regulatory regions of these enzymes also show a level of conservation. Furthermore, the complete understanding behind the mechanism of this regulatory element will not only provide insight into the hatching enzyme expression, but make possible the use of this promoter alongside a reporter gene as a marker for early development and transposable element integration.

**Gateway Cloning**

Phage lambda has two main life cycles, (1) the lysogenic lifecycle and (2) the lytic lifecycle. The lysogenic lifecycle results in the integration of the phage into the bacterial genome. The second lifecycle, the lytic lifecycle results in the production of new λ virus particles. Bacteriophage lambda is a virus that infects *E.coli* cells by integrating itself into the bacterial genome using special attachment sites, *att* sites. The bacterial site, *attB*, and the phage site, *attP* recombine to result in a bacterial genome containing phage DNA and a new set of hybrid flanking attachment sites. Recombination is catalyzed by the expression of special enzymes that can bring the two special attachment sites together, cut them and then religate the DNA (Lederberg & Lederberg, 1953).

The Gateway system is made up of two reactions, a BP reaction and an LR reaction, taken from the lysogenic and lytic pathways respectively. The BP and LR
reactions are executed using a special enzyme mix, BP Clonase II and LR Clonase II respectively, and the addition of vectors, either provided or designed, that include the complementary att sites. The initial step is the isolation of a gene and the final product is the generation of an expression clone that allows for the expression of that gene. The BP reaction calls for the PCR of your DNA of interest to result in a fragment flanked by a specific sequence in order to produce attachment sites, att sites. The final product of this reaction combines the PCR product, an Invitrogen provided vector and a BP clonase enzyme mix that contains lysogenic enzymes to generate an entry clone, whereas the LR reaction combines the entry clone, a destination vector plus an LR clonase mix to generate your expression clone.

All expression clones for this project were generated using the Invitrogen MultiSite Gateway ® Pro technology. This technology allows for the cloning of multiple DNA sequences into an expression vector using site specific recombination that is based on the lifecycle of bacteriophage lambda (λ). Recombination, in biology, refers to the breaking of a DNA strand and attaching it to another strand. The MultiSite Gateway Pro technology has built upon nature’s design and provides a quick and efficient method to repeatedly transfer DNA fragments. The system uses two sets of specific nucleotide sequences, att sites, to ensure directionality and the conservation of the reading frame, plus a set of enzyme mixes to drive the recombination reactions. There are two main steps in the two fragment recombination system. Step one is the insertion of a DNA fragment into an Invitrogen supplied plasmid and step two is the generation of a clone
ready for gene expression (Figure 5).

**BP Reaction**

The BP clonase II enzyme mix is used to drive the reaction and mimics the lysogenic pathway. In a 2 fragment recombination BP reaction, a portion of the PCR product that includes the gene of interest transferred into a vector, pDONR 221 P1-P5, via recombination (Figure 6).

---

**Figure 5.** Schematic of the Two Fragment Recombination MultiSite Gateway Pro Cloning Strategy showing points of recombination, used in this study (Invitrogen, 2006).
Figure 6. Schematic of the pDONR™ 221 P1-P5r entry vector (Invitrogen, 2006).

The attP sites on the donor plasmid recombine with the attB sites on the PCR product and the reaction will transfer the ccdB cassette out, along with the chloramphenicol resistance gene and transfer in the PCR product. If there are vectors that do not recombine with the PCR product the resulting plasmid will still contain the ccdB cassette which prevents wild type *E.coli* growth. The kanamycin resistance gene present on the plasmid permits for selection of bacteria that only contain the pDONR plasmid backbone.

**LR Reaction**

The LR clonase enzyme mix mimics the recombination in the lytic pathway. In an LR reaction, two entry clones, containing the DNA elements of interest are being recombined and transferred into a destination vector. The destination vector has to contain an attR1 and attR2 site along with a ccdB cassette. In this study the destination
vector is the pDEST miniTol2 rfa_verB. This vector is based on a naturally occurring transposable element that is found in vertebrate genomes and has been engineered to contain the Gateway recombination cassette to facilitate cloning (Urasaki, Morvan, & Kawakami, 2006). The use of a transposable element allows for the integration of the construct into the organism’s genome. This makes the probability of expression much higher and allow for the construct to be integrated into the genome at high efficiency and the insert passed down to offspring.

**Zebrafish**

In the 1970s George Striesinger, from University of Oregon chose *Danio rerio*, the zebrafish, as his vertebrate model organism. He wanted a system that is simpler than a mouse system and easy to manipulate its genetics. *Danio rerio* is a freshwater fish that can be found in the rivers of northern India, northern Pakistan, Nepal and Bhutan, as well as any pet shop around the world. It is a small 2.5-3.8 cm, striped fish that is easy to maintain and has many characteristics that make an ideal model organism (Figure 7).

The zebrafish is a vertebrate organism and is very similar, in terms of embryogenesis, to higher vertebrates. However, the zebrafish egg develops outside the female allowing access to the developing embryo. Mating pairs are easy to set up as there are visible differences between males and females and one mating can result in hundreds of eggs. This organism can breed early, approximately 90 days from conception, and they breed often, daily if pushed. The fertilized embryos are covered by a transparent chorion. Hence development can be observed from the fertilization stage on. Embryonic
development lasts about three days before hatching into free living larvae. These characteristics allow for easy, quick manipulation and expression of various genetic markers which can be used in development and functional studies.

Figure 7. Zebrafish fertilized zebrafish eggs two hours post fertilization and an adult zebrafish (Oregon State University, 2011).
CHAPTER II
MATERIALS AND METHODS

**Oligonucleotides**

The design of the oligonucleotides to act as primers for the generation of the constructs had to include the sequences essential to the Gateway cloning technique. All primers had to include their corresponding *att* sites to ensure recombination. The forward primers required the *attB1* site to be located on the 5’end of the regulatory region sequence. All five forward primers consist of a common 5’ 29 bp sequence, which contains the *att* site, plus a specific 3’ end sequence. The backward primer had the *attB5r* site attached to its 3’ end and an internal BamHI site (underlined below). The 5’ common sequence is GGG GAC AAG TTT GTA CAA AAA AGC AGG CT while the backward primer is GGG GAC AAC TTT TGT ATA CAA AGT TGT GGA TCC TTG CCT CAG TGT GTA GAG ATG TTC AGG AT. Specific primer sequences are listed in Table 1.

Table 1. Sequences of primers used.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HE1a -497 primer</td>
<td>GTT Tag TAgAATGCATCGCTAAACTTGAGAA</td>
</tr>
<tr>
<td>HE1a -370 primer</td>
<td>TAT ATG TAT GAAGTGCA ACC AACTCCAGGCA TAGCTAG</td>
</tr>
<tr>
<td>HE1a -270 primer</td>
<td>TAT ATG TAAAAAAAC AAAAAATAAAAAGTCTATTCACT</td>
</tr>
<tr>
<td>HE1a -213 primer</td>
<td>TAT ATG TAA GAG GCC ACT TCC CCT GT ACTGT AAA AC</td>
</tr>
<tr>
<td>HE1a -148 primer</td>
<td>TAT ATG TAACACATTAGT CC CG A AAA ACTAAGACCC</td>
</tr>
<tr>
<td>HE1a Backward primer</td>
<td>GGG GAC AACTTTTTGT TACA AAG TTG GTG AGT C T C AGT GAG ATG TTT CAG GAT</td>
</tr>
</tbody>
</table>
Nomenclature

All constructs were named using the abbreviated promoter name and the number of nucleotides upstream from the translational start. All clone names and their descriptions are listed in Table 2.

Table 2. Table of clone names and their description.

<table>
<thead>
<tr>
<th>Clone Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pENTR L1/He1a148/L5</td>
<td>Entry clone constructed using promoter fragment truncated 148 upstream of the translational start.</td>
</tr>
<tr>
<td>pENTR L1/He1a213/L5</td>
<td>Entry clone constructed using promoter fragment truncated 213 upstream of the translational start.</td>
</tr>
<tr>
<td>pENTR L1/He1a270/L5</td>
<td>Entry clone constructed using promoter fragment truncated 270 upstream of the translational start.</td>
</tr>
<tr>
<td>pENTR L1/He1a370/L5</td>
<td>Entry clone constructed using promoter fragment truncated 370 upstream of the translational start.</td>
</tr>
<tr>
<td>pDESTminiTol2He1a148:MCFP</td>
<td>Destination clone constructed using the entry clone containing the truncated 148 nucleotide promoter fragment and the cyan fluorescent protein</td>
</tr>
<tr>
<td>pDESTminiTol2He1a213:MCFP</td>
<td>Destination clone constructed using the entry clone containing the truncated 213 nucleotide promoter fragment and the cyan fluorescent protein</td>
</tr>
<tr>
<td>pDESTminiTol2He1a270:MCFP</td>
<td>Destination clone constructed using the entry clone containing the truncated 270 nucleotide promoter fragment and the cyan fluorescent protein</td>
</tr>
<tr>
<td>pDESTminiTol2He1a370:MCFP</td>
<td>Destination clone constructed using the entry clone containing the truncated 370 nucleotide promoter fragment and the cyan fluorescent protein</td>
</tr>
</tbody>
</table>

**HE1a Promoter PCR**

The initial plasmid, pENTRHE1a-497, was constructed using isolated zebrafish genomic DNA as the PCR template. An initial 25µl reaction was carried out in 0.2ml
PCR tubes with the following amounts of reagents per tube:

- 19.8µl  diH$_2$O
- 2.5µl  Polymerase Buffer
- 0.5µl  dNTPs
- 0.5µl  Forward primer
- 0.5µl  Reverse Primer
- 1.0µl  PfuUltra High Fidelity DNA polymerase

The tubes were placed in a BioRadCyclePCR thermo-cycler and allowed to proceed at the following conditions:

1. Denaturation  95°C for 5 minutes
2. Denaturation  95°C for 30 seconds
3. Annealing  62°C for 30 seconds
4. Extension:  72°C for 4 minutes
5. Repeat steps 2-4 30 times
6. Extension:  72°C for 10 minutes
7. Preservation  4°C for $\infty$

After the amplification, 5µl of the PCR product was run out on a 1.0% agarose/TAE gel in order to verify the product. A positive PCR result was sufficient to proceed with setting up preparative 50µl reactions in order to isolate product for cloning. These reactions were carried out using the same thermo-cycler conditions. PCR products were then PEG purified (as described in Liset.al) to extract DNA from the reaction mixture and resuspend in 25µl of ddH$_2$O.

**BP Reaction**

The BP protocol calls for equal molar amounts of the pDONR 221 P1-P5r vector and the PCR product. In this procedure both were adjusted to a concentration of 150ng to be used in the BP reaction. In a 1.5ml microcentrifuge tube the following reagents were added and mixed as follows:
150ng pDONR 221 P1-P5r
150ng PCR product
2µl BP Clonase II
enough diH2O to bring the reaction to 8µl

(It is important to note that the Invitrogen protocol calls for 1X TE Buffer, however based on our own experiments diH2O appears to provide the same results or better.) The mixture was incubated for 1 hr at 25°C, in the thermo-cycler to ensure a constant temperature. After 1 hr, 1.0µl of Proteinase K was added to each reaction and incubated for 10 min at 37°C. Proteinase K is a serine protease and is used to inactivate nucleases that might damage the plasmid.

BP reactions were then transformed into chemically competent DH5α E.coli cells using New England BioLabs High Efficiency Transformation Protocol. The transformed cells were spread on LB/kanamyacin plates and placed in a 37°C incubator overnight. The next day, 12 single colonies were picked and grown in 50mL centrifuge tubes containing 5mL LB media and 5µl (1000X) kanamyacin for 24 hours at 37°C, shaking.

**Plasmid Isolation**

To isolate the entry clones from the E.coli broth, the cells were mini-prepped using a FermentasGeneJet ® Plasmid MiniPrep Kit. The plasmid, once isolated, was digested with restriction enzyme, ApaLI for the -497, -370, and -270 clones and PagI for the -148 clone, in order to verify the presence of a plasmid with the correct size and orientation. The restriction enzymes were chosen based on their presence in the plasmid
and the location of their cut site. Multiple clones of each construct were digested (Figure 8).

Figure 8. Restriction Digest of BP entry clones. (A) pENTR L1/HE1a270 (B) pENTR L1/HE1a148 (C) pENTR L1/HE1a213 (D) pENTR L1/HE1a370.

The pENTR L1/He1a148/L5 (Figure 8B) construct restriction digest produced two bands; the lower band migrated to right below 1kb and the upper band between 1.5kb and 2kb. The expected plasmid size was 927bp and 1.8kbp, respectively. The remaining three constructs first produced one band at around 1.7kb and the second band between 1kb and 1.5kb (Figure 8). These are consistent with the expected migrations; the upper band being constant for the three clones at 1.790kb and the lower bands at 1.042kb, 1.1kb and 1.2kb respective to the -213, -270, and -370bp Entry clones. In addition to the restriction digest, all clones were sequenced to confirm the correct orientation.
Following the initial confirmation, via restriction analysis, an aliquot of the mini-prepped DNA was sent out for DNA sequencing (Genewiz Inc.). The sequence results were compared to maps of the expected plasmids using DNASTAR Lasergene 7 SeqMan software, confirming the presence and correct orientation of the HE1a promoters. A representative map of pENTR L1/He1a370/R5 is shown in Figure 9, all other break points are visible on the pENTR L1/He1a370/R5 map.

![Figure 9. Map of pENTR L1/He1a370/R5.](image)

The resulting plasmids pENTR HE1a -470, pENTR HE1a -370, pENTR HE1a -270, pENTR HE1a -213, and pENTR HE1a -148 containing the promoter fragments were obtained.
The successful BP reactions led to the generation of entry vectors. The expression vector requires the recombination of two entry clones and one destination vector. For this project, the second entry clone, pENTR L5/CFP/L2 contained the membrane bound cyan fluorescent protein (MCFP) and the destination vector was pDESTminiTol2 rfa verb (Figure 10).

The pENTR L5/MCFP/L2 entry clone, provided by Dr. Eric Schroeter, was grown, isolated and confirmed via restriction digest before use. Digestion with MluI produced two bands, one around 1kb and the second right below 3kb, these are consistent with the expected 923bp and 2.7kb bands. To confirm the integrity of the miniTol2 destination vector, provided by Dr. Schroeter, the plasmid was digested with PstI to produce a correct band of about 5kb.

Each of the entry clones containing different sized HE1a promoter fragments, and pENTR L5/MCFP/L2, containing a membrane bound cyan fluorescent protein were used in the LR reactions with pDESTminiTol2 rfa verb as a target. The destination vector contains the required att sites as well as an ampicillin and chloramphenicol resistance gene. Recombination between the two entry clones and the expression vector results in the generation of a plasmid, pDEST miniTol2 He1a #: MCFP, containing the HE1a promoter, MCFP and a selectable marker (where # indicates the number of nucleotides upstream of AUG).

**LR Reaction**

The LR reaction requires 50 femtamoles (fmoles) of each entry clone. Based on
their size, the required amount of entry clones used were 19.3ng, 19.1ng, 18.7ng, and 18.3ng of the pENTER HE1a -470, pENTER HE1a -370, pENTER HE1a -270, pENTER HE1a -213, and pENTER HE1a -148 clones, respectively. In a 1.5mL microcentrifuge tube the following reagents were combined:

- 50fmoles pENTR L5/MCFP/L2
- 50fmoles pENTR L1/HE1a/L5
- 50fmoles pDEST miniTol2 rfa verb
- Enough diH2O to bring the reaction to 8µl
- 2µl of LR Clonase II

The mixture was incubated for 16 hrs at 25°C, in the thermo-cycler to ensure a constant temperature. After 16 hrs, 1.0µl of Proteinase K was added to each reaction and incubated for 10 min at 37°C. The LR reaction was then transformed into DH5α E.coli cells using ampicillin plates.

An LR product, pDEST miniTol2He1a#:MCFP, was generated for each promoter truncation construct. Prior to being used for zebrafish egg injection, each plasmid was analyzed via restriction digest and sequencing (Figure 11). The pDEST miniTol2He1a370:MCFP destination vector (Figure 11D), when digested with Mph1103I produced two bands, one between 1.5 and 2.0kb and the second a little over 3kb. The expected bands were 1.690 and 3.292kb. The 270 (Figure 11C) vector digested with ApaL1, produced one lower band between 1 and 1.5kb and an upper band right above 2kb. The expected digest showed three bands at 1243, 1405, and 2234.
Figure 10. Maps of pENTR L5/CFP/L2 and pDESTminiTol2 rfa verb.
The difference was attributed to the two smaller bands being unresolved from each other. pDESTminiTol2He1a148:MCFP (Figure 11A) and pDESTminiTol2He1a213:MCFP (Figure 11B) were digested with Bsp120I and each was expected to produce two bands, one at 4146 and the second at either 614 or 678 respectively. Both digests produced an upper band between 4 and 5kb and a lower band above 500bp. Following the initial confirmation, via restriction analysis, an aliquot of the mini-prepped DNA was sent out for DNA sequencing to Genewiz Inc. All clones yielded a 100% contig when compared to the prepared maps.

Figure 11. Restriction Digest of LR destination clones. (A) pDESTminiTol2He1a148:MCFP (B) pDESTminiTol2He1a213:MCFP (C) pDESTminiTol2He1a270:MCFP (D) pDESTminiTol2He1a1370:MCFP.

**Zebrafish Embryo Injection**

Each construct was injected into one cell stage zebrafish embryos using a custom built pneumatic injection apparatus (Figure 12) (E. Schroeter personal communication). Constructs were injected into both wild type zebrafish and Tg (pax6-DF4:gap43-CFP) q1.
transgenic fish (hereafter referred to as Q01).

**Assay of Promoter Constructs in *Daniorerio* Embryos**

Each injected embryo was screened and scored for the presence or absence of cyan fluorescence in the hatching gland at 24 hours post fertilization. All screening was done using a Olympus SZX16 fluorescent microscope and pictures were taken using a Nikon D5000 camera controlled from a computer using Camera Control Pros 2 software. In addition, some embryos were imaged on an Olympus Fluoview FV1000 confocal microscope.

Figure 12. Diagram of injection apparatus.
CHAPTER III

RESULTS

To determine the minimal promoter of the hatching enzyme 1a gene, promoter truncation plasmids were generated and assayed for expression. Promoter lengths were designed based on the conservation of non-coding regions between Teleost species. Expression plasmids were constructed using Gateway® cloning technology. Each plasmid contains a 5’ fragment of the upstream regulatory region of He1a to drive expression with a cyan fluorescent protein (CFP) reporter gene downstream for detection. These constructs were injected into one cell stage fertilized zebrafish eggs. The eggs were then allowed to develop for 24 hours, after this period of time the one day old embryos were screened for fluorescence using a fluorescent microscope.

The initial rationale for cloning the promoter fragments was based on an alignment of the upstream regions using four teleostean hatching enzymes. Utilizing NCBI, Ensemble and the University of California Santa Cruz Genome Browser, six hatching enzymes genes were identified: two from *Oryzias latipes* (medaka), two from *Takifugu rubripes* (fugu), one from *Tetraodon nigroviridis* (tetraodon) and one from *Danio rerio* (zebrafish). The alignment was scored at 100% and 75% consensus identity.

The first set of primers were designed to prime forward at 24bp upstream of the first 100% consensus and prime backwards from the ATG start site. Based on those
primer binding locations, this construct was expected to contain the-497 nucleotides upstream of the HE1a gene.

The first construct pDESTminiTol2He1a-497:McFP was successfully cloned and scored for positive expression before four more truncation constructs were designed (Figure 13). The fully functional pDESTminiTol2He1a-497:McFP clone sequence was then added to the upstream region alignment. The alignment was re-scored, again at 100% and 75% consensus identity, however now with eight sequences (Figure 14).

Starting at the ATG of the genes coding sequence truncation points were picked to be in places where there were obvious breaks in homology. The shortest truncation construct was designed to encompass 148bps upstream of the AUG translational start site. This construct contains a region of 48 nucleotides with a 100% consensus between the species (Figure 14). The exact truncation point was based on a four nucleotide gap in homology found at that location, when compared to the three other teleosts, but still containing the TATA box. The next construct, HE1a-213, starts 213bp upstream of the AUG and isolates a location of the HE1a-497 clone prior to a gap in the consensus. HE1a-270 and HE1a-370 were chosen to provide break points between these two consensus islands to narrow down the minimal promoter. The backward primer for all constructs was identical to the initial HE1a-497 backward primer which ends the fragment one nucleotidie upstream of the AUG (Figure 14).
Injections

Initially the pDESTminiTol2He1a497:MCFP construct was injected into one cell stage zebrafish embryos, to assay whether this fragment contains a functional promoter.

Figure 13. Schematic of linearized expression plasmids.
Figure 14. Alignment of hatching enzyme promoter (approx. 520bp upstream of coding region), (1) start of clone, (2) first cut off site (~ 370bp upstream), (3) second cut off site (~ 270bp upstream), (4) third cut off site (~ 213bp upstream), (5) fourth cut off.
The plasmid was injected into wild type zebrafish embryos. The expected expression pattern consisted of a necklace-like pattern located on the anterior yolk identical to the pattern of RNA expression shown by \textit{in situ} hybridization (Figure 15).

![Figure 15. Expected HE1a expression necklace-like pattern (Thisse, et al., 2001).](image)

The expression of the mRNA is detectable by 12hpf (Sano, Inohaya, Kawaguchi, Yoshizaki, Iuchi, & Yasumasu, 2008). Fluorescence of CFP expressed from the injected plasmid was easily detected by 24hpf.

Four additional \textit{hatching enzyme 1a} promoter truncation constructs were designed to be injected into zebrafish one cell stage embryos to determine the gene’s minimal promoter. The promoter lengths were based on a four species hatching enzyme regulatory region alignment. The four constructs lengths were 370, 270, 213, and 148bps upstream of the ATG start site. The zones of truncation were determined based on regions of 100% identity throughout all species as described earlier.
The pDESTminiTol2He1a370:MCFP, pDESTminiTol2He1a270:MCFP, and pDESTminiTol2He1a213:MCFP, plasmids showed positive expression patterns in both wild type and Q01 zebrafish. Q01 is a transgenic zebrafish line that expresses in both the nervous system and muscle but does not express in the hatching gland. This line was used to facilitate locating zebrafish in the dish, once located the fish was then screened for CFP expression in the hatching gland (Figure 16A-D). The shortest plasmid pDESTminiTol2He1a148:MCFP was negative for expression (Figure 16A) despite injecting and screening nearly 500 embryos.

To determine if there were significant differences in expression between the promoter lengths expression was scored using a low (+), medium (++) , high (+++) criteria. Due to the ability to distinguish individual cells the embryos were separated based on the number of cells expressing. A low level of expression showed one or very few positive cells (Figure 16C), medium level showed an intermediate number of cells expressing (Figure 16B), and a high level showed a full necklace like pattern (Figure 16D).

The pDESTminiTol2He1a370:MCFP plasmid was injected into 607 embryos and showed expression in 302 embryos with 22.9% showing low expression, 19.8% showing intermediate expression and 5.8% showing high expression, pDESTminiTol2He1a270:MCFP was injected into 602 embryos and was positive in 378 with 24.1% showing low expression, 23.5% showing intermediate expression and
14.3% showing high expression. The pDESTminiTol2He1a213:MCFP construct expressed in 276 out of 552 embryos with 23.9% showing low expression, 17.4% showing intermediate expression and 8.7% showing high expression, and pDESTminiTol2He1a148:MCFP was injected into 495 embryos and showed no expression (Figure 17). The following table shows specific expression levels (Table 3).

Table 3. Promoter Expression Levels given as % embryos expressing.

<table>
<thead>
<tr>
<th>Promoter Length</th>
<th>0</th>
<th>+</th>
<th>++</th>
<th>+++</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>-370</td>
<td>51.5</td>
<td>22.9</td>
<td>19.8</td>
<td>5.8</td>
<td>586</td>
</tr>
<tr>
<td>-270</td>
<td>38.1</td>
<td>24.1</td>
<td>23.5</td>
<td>14.3</td>
<td>609</td>
</tr>
<tr>
<td>-213</td>
<td>50.0</td>
<td>23.9</td>
<td>17.4</td>
<td>8.7</td>
<td>552</td>
</tr>
<tr>
<td>-148</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>495</td>
</tr>
</tbody>
</table>

Expression of the fluorescent reporter in the full hatching gland was obtained with constructs containing the 213, 270, 370, and 497bp of sequence upstream of the translational initiation site. This shows that a sequence of only 213 base pairs is sufficient to show high and specific promoter activity in the embryo during transient expression. It is interesting to note that the -270 construct appears to yield a higher percentage of high level expression, almost double the -370 construct. However due to this is most
likely due to variability in plasmid injections rather than the existence of a positive regulatory role in this region.

Figure 16. Expression patterns of injected expression vectors into one cell stage embryos; (A) pDESTminiTol2He1a148:MCFP showing no expression (B) pDESTminiTol2He1a213:MCFP showing positive expression (C) pDESTminiTol2He1a270:MCFP showing positive expression (D).
Computational Analysis of Potential Regulatory Regions

The 213bp region upstream of the HE1a gene is sufficient to drive normal gene expression; however the 148bp region yields no expression. As a result, I sought to identify any regulatory regions that might be located in the area between these two break points and essential for normal expression. Any potential regulatory regions should show two properties; high sequence conservation relative to an orthologous species and contain a potential transcription factor binding sites (TFBS).

Evolutionarily conserved regions were identified by looking at percent identity in the 213bp region using multiple alignments of four species, each containing two paralogues of the hatching enzyme gene: fugu (Takifugu), stickleback (Gasterosteus), medaka (Oryziaslatipes), and zebrafish (Danio rerio) (Figure 18). Within the -213 region, 26% of the residues show complete homology and 75% identity was seen at 28% of the nucleotides.

In order to identify potential TFBS, the 65bp region between the 148 and 213 cut off sites, was reanalyzed for percent identity and the presence of TFBS. More than half, 52%, of the nucleotides showed at least 75% identity among the 8 genes with 12% showing complete identity. It is important to note that a majority of the regions that a not 100% matching are due to differences only between the two zebrafish paralogues, He1a and He1b.
Figure 17. Promoter Activities in all injected embryos for pDESTminiTol2He1a370:MCFP, pDESTminiTol2He1a270:MCFP, pDESTminiTol2He1a213:MCFP, and pDESTminiTol2He1a148:MCFP. Expression levels are as follows: not expressing (o), low expression (+), intermediate expression (++) and high expression (++). Activities are given as percentage of embryos expressing based on total numbers reported as in Table 3.

Within the 65bp region that separates the non-functional and fully functional minimal promoter one highly conserved motif was identified. This motif consists of five bases, CTCCT, three bases show 100% conservation in the species alignment and two bases show 75% identity (Figure 19). The two nucleotides that are not conserved are within the medaka genome and are flipped with TC to CT. This therefore suggests that this short sequence is highly important.

Using Genomatix© MatInspector, a TFBS identifier, the HE1a promoter region was searched against a large library of TFBS. The -148 non-functional fragment
contained a majority of the conserved regions including a TATA box and a CCAAt box, both yielding 100% identity (Figure 18). Conserved sequences within this fragment were identified as general transcription factor binding sites and while highly important, not unique to the HE1a gene and not likely to be providing differential expression in the hatching gland.

The 65 base pair region immediately upstream of the non-functional promoter identified four potential TFBS; a NF-κβ, a homeodomain, TWIST and KLF binding sites. The KLF, Krüppel-like factor, coaligns to the CTCCT motif with 100% identity (Eaton, Funnell, Sue, Nicholas, Pearson, & Crossley, 2008). It is interesting to note that while the three paralogues show a high level of conservation, the KLF binding site is missing from the non-functional not expressing He2 paralogue.
Figure 18. Clustal W alignment of Teleost HE promoter regions from fugu, stickleback, medaka, and zebrafish with the various truncation cut sites emphasized with a box. First box represents the 270 break point, the second box represents the 213 break point while the third box represents the 148 break point. Nucleotides highlighted in black have complete identity, those in dark grey are missing one nucleotide and light grey are matching at six of the eight species.
Figure 19. Clustal W alignment of Teleost HE promoter region between the 213 and 148 break points. The red box highlights the CTCCT motif that is conserved in all four species and all 8 genes with the exception of one CT to TC flip in the medaka genome.
Figure 20. Clustal W alignment of zebrafish HE1a (line 1), HE1b (line 2), and HE2 (line 3). The KLF motif is highlighted with the red box.
CHAPTER IV
DISCUSSION

In this study, the *hatching enzyme 1a* minimal promoter the regulatory region immediately upstream of the gene was identified and aligned with the regulatory regions of five other hatching enzymes. Based on a comparative study of the alignment, five truncated fragments were designed. All fragments contain the 5’ initiation sequence of the coding region plus various consecutive non-coding regions. These fragments were recombined into entry clones, via Invitrogen MultiSite Gateway® Pro cloning technology, and positioned upstream of a CFP reporter gene. These final expression vectors were then injected into one cell stage zebrafish eggs and scored for expression.

Based on data from the expression assay a minimal promoter was identified. This fragment was then analyzed for the presence of both general and specific transcription factors. This analysis looked at not only the hatching enzyme paralogues of zebrafish but also its orthologues in other species to determine essential regulatory sites.

This study has successfully identified a small minimal promoter of the zebrafish hatching enzyme 1a gene driving expression in the hatching gland. Only a 213bp region upstream of the transcriptional start site is sufficient to drive high levels with no ectopic expression. The data also suggest that the essential regulatory element is a KLF transcription binding site. First the supporting data for these conclusions will be discussed, followed by the implications of this work.
Truncated promoter constructs were placed upstream a fluorescent protein and injected into one cell stage zebrafish embryos. The expression was assayed using a fluorescent microscope and compared to normal He1a expression. The generation of various construct lengths allowed for a more accurate assessment of essential promoter components. The initial construct consisted of a 497bp region upstream the ATG. Unknown at the time, this construct invades into another gene’s protein coding region that was later added to the annotated gene assembly (Ensembl, 2012). Acyl-coA oxidase, AOX, flanks the entire He1a, Helb and He2 coding region. The hatching enzyme exons are located in a large intron of the AOX 3 gene. In fact, both the -497 and the -370 constructs include a chunk of an AOX 3 exon. Knowing this it is not a big surprise that is the regions defined by these deletions are dispensable to function. The smallest truncation construct that yielded normal expression patterns was only 213bp long. This is a remarkably small region which is capable of producing high levels of specific expression. On the other hand the -148 produced no expression, suggesting that an element critical for He1a gene expression is located in the 65bp region between these two break points.

Multiple sequence alignments showed conserved regions in the zebrafish He1a promoter relative to its orthologs in other teleost species. These alignments also contained potential general transcription factor binding sites, for example a TATA box located 81 nucleotides upstream of the AUG and a CAAT box a little further upstream. This is amazing homology for a noncoding sequence between 8 genes and 4 species.

Of interest were any conserved motifs between the 148 and 213 fragments since
they would be considered essential. In the 65 nucleotide sequence there is one sequence that shows conservations across all 8 hatching enzyme genes and four species. A five nucleotide motif was identified 163bps upstream of the translational start. This motif had great potential for being an essential regulatory element for the following reasons; (1) it is located in a region that if deleted from the rest of the promoter it deactivates Helae expression, (2) is located in the hatching enzyme regulatory regions of three other fish species and seven other hatching enzyme promoter regions and (3) is missing from the inactive He2 gene.

A search of various TFBS databases, including TRANSFAC and MatInspector produced one result for this sequence. The nucleotide motif CTCCT acts as a binding site for Klf, Krüppel-like factor (Eaton, Funnell, Sue, Nicholas, Pearson, & Crossley, 2008). The Klf family is a zinc finger transcription factor family. These factors contain a highly conserved DNA binding domain composed of three zinc fingers. The zinc fingers bind GC-rich or CACC box elements. The family consists of both activators and repressors of transcription. Interestingly Klf4 is also one factor that has been shown to be essential for hatching gland gene expression. In a study done by Gardiner et. al. (2007) injected Klf4 morpholinos do not develop a hatching gland and fail to hatch. These morpholino experiments also showed significant down regulation of He1a, showing that Klf4 is required for Helae synthesis (Gardiner, Gongora, Grimmond, & Perkins, 2007), (Figure 21).

It is interesting that He2 is missing the CTCCT motif in its regulatory region
(Figure 17). If in fact the Klf4 binding site is essential for normal gene expression it stands to reason that the missing Klf4 binding site within He2 might be responsible for the non-transcription of He2. The He2 loci can then be considered a pseudogene that has lost its ability to transcribe by deletion of the Klf4 transcription factor binding site.

![Quantitative real time-PCR validation of gene expression](image)

It is also important to point out that Klf4 in situ hybridization does show an identical expression pattern to He1a in the hatching gland in the same early stages and isolates Klf4 throughout the lateral plate mesoderm (Figure 20).

In order to confirm the importance of CTCCT motif the next step in this analysis would be to either rescue expression by reintroducing the hypothesized critical transcription factor binding site in He2 or delete the transcription factor binding site exclusively within an active He1a promoter. The hypothesized critical transcription site could be rescued
from the -148 by generating a longer promoter fragment. By using 165 base pairs of the translational start site will just include the Klf4 TFBS. It would also be important to show that Klf4 does in fact bind to the CTCCT binding site via biochemical assays.

Figure 22. Klf4 expression pattern (Thisse & Thisse, Fast Release Clones: A High Throughput Expression Analysis, 2004).

The identification of the minimal promoter not only tells us more about the hatching mechanism of this model organism but provides us with a tool that can be used for screening transgenics. This usually involves the incorporation of a small reporter into a transposable element vectors that will be used for making transgenics. After waiting an appropriate time after injection, embryos can be screened for expression of the report to determine if transgenesis is successful. The same can be used to determine if transposable elements have passed onto the progeny of these injected fish as well as the maintenance these fish lines.

Currently the only promoter of like size and high levels of expression is the
cardiac myosin light chain 2 promoter (cmlc2). This 250bp fragment drives expression exclusively in heart muscle and has been used in the widely distributed Tol2Kit transposon vector kit (Huang, Tu, Hsiao, Hsieh, & Tsai, 2003). This promoter however has some definite drawbacks. Expression is not detectable until 2-3 days post fertilization making fast assessment of transgenesis take longer than necessary. In addition, this promoter expresses in the heart, a tissue that has a high level of research interest. Heart expression reduces the utility of the Tol2kit constructs for this area of research by not permitting the evaluation of GFP or RFP expression from other promoters.

Use of the He1a promoter as a replacement for the cmlc2 promoter has several advantages. It is smaller, at only 213 base pairs, is detectable very quickly, as soon as 11 hours post fertilization and is found in a structure that has no homology to mammals. Expression is confined to the pre-hatching period of development and is completely gone 3 days post fertilization, leaving the embryo easily accessible to various areas of research.
CHAPTER V

IN VIVO IMAGING OF ZEBRAFISH RETINAL BIPOLAR CELL DENDRITIC DYNAMICS

When looking at the human eye we can immediately observe four key features, the pupil, iris, cornea, and sclera. The first two features play crucial roles when it comes to light sensing, the pupil allows light to enter the eye while the iris controls the amount of light entering. The next two features have more physical roles, the cornea is a transparent surface that covers both the pupil and the iris, and the sclera is a supporting wall for the entire eyeball. When the eye is more closely analyzed it is possible to distinguish three layers, the external, intermediate and internal layers formed by the sclera and cornea, iris, and retina respectively (Mann, 1964). The light sensing part of the eye is the retina. Visible light is interpreted by the visual system to develop a depiction of the surroundings. Light first enters through the cornea and is refracted; it is then adjusted by the iris and enters the pupil, goes through the lens and is inverted and projected on the retina. When light enters the retina the signal is converted to a nerve impulse and is sent to the brain via the optic nerve where it gets interpreted.

The retina is located in the back of the eye and is adjacent to the optic nerve, which runs to the brain, and blood vessels that vascularize the eye. The retina is part o
the nervous system that derives from the neural tube; it is about 5mm thick in humans and contains five cell types organized into five cell layers (Rodieck, 1973) (Figure 23). The layers starting with the outer most are, retinal pigment epithelia (RPE), outer limiting membrane (OLM), outer nuclear layer (ONL), outer plexiform layer (OPL) inner nuclear layer (INL), inner plexiform layer (IPL) and the ganglion cell layer (GCL). This organization maintains all cell bodies in the nuclear layers whereas synapses are located in the plexiform layers. The ONL contains the cell bodies of photoreceptors (PR), whose axons reach into the OPL. Within the OPL, photoreceptor axons and bipolar cell (BPC) dendrites form the first synapses and allow for the flow of electrical impulses. It is also within the OPL that bipolar cells connect with horizontal cells (HC) in order to modify and stabilize the signal input from photoreceptors. Horizontal cell bodies, bipolar cell bodies, and amacrine cell bodies are located in INL. Bipolar cell axons extend to the IPL and synapse with retinal ganglion cells and amacrine cells, where the signal is regulated before it continues down to the retinal ganglion cell body, located in the GCL. Ganglion cell axons form the optic nerve which transmits the signal to the visual cortex for processing (Kolb H. , 1991) (Figure 24).

**Retinal Development**

The retina is derived from the neural tube and its development can be separated into two steps, (1) invagination of optic vesicles and (2) cell migration. During the first step optic vesicles fold in on themselves and form a cup shaped structure composed of two layers. Layer one, the outer wall, will develop the pigment epithelium while layer two, the inner wall, will result in the formation of a single-layered pseudo stratified. Step two involves
the movement of cells in order to generate the correct cell types and to localize in the correct layers. The cells of the retina are susceptible to interkinetic migration, a back and forth movement of the nuclei in which certain phases of the cell cycle occur in specific locations. While nuclei are moving the cells extend their cytoplasmic processes from the pigment epithelium to the internal limiting membrane. Once mitosis occurs the daughter cells extend their processes toward the opposite surface and wait until the nucleus migrates so that they can break their original connection. It is then that the daughter cell can either re-enter the cell cycle or become postmitotic neuroblasts (Lamb, Collin, Pugh, & Pugh Jr., 2007). These neuroblasts will then differentiate into one of five retinal cell types, each having a specific period of generation. The first cells to differentiate are ganglion cells, the second wave are amacrine, horizontal and cone photoreceptors and the last cells to be generated are bipolar and Muller glial cells. Rod photoreceptors develop throughout the entire period of development.

The polarization of ganglion cells directs their axons towards the optic nerve while the dendrites extend toward the rear of the eye, this forms the first synaptic layer, the IPL. In the IPL axons start extending before the cell body is localized in its layer with its dendrites reaching out after localization. Conversely in the OPL, dendritic and axonal processes are seen to extend and orient before the final completion of the The first synapses to form in this layer are those between photoreceptors and horizontal cells while the last are bipolar cells and photoreceptors (Mann, 1964).
Figure 23. Section of an adult human eye showing the path light takes when entering the eye plus the cell layers of the retina (Kolb H., Simple Anatomy of the Retina, 2011).

Figure 24. A simple diagram of the vertebrate retina (Kolb H., Simple Anatomy of the Retina, 2011).
Photoreceptors

The first layer in the retina is the outer nuclear layer (ONL) and it contains the cell bodies of photoreceptor cells. Photoreceptor cells act as biological sensors that detect light within the retina and they are the pre-synaptic partners of bipolar cells. Photoreceptors are a specialized type of neuron which can convert electromagnetic radiation or light into an electrical impulse.

Photoreceptors consist of two types; rods and cones, each type having their own characteristic morphology but also sharing some common features. Each photoreceptor is made up of four main parts, axon terminal, cell body, inner segment, and outer segment. The axon terminal releases glutamate, a neurotransmitter, to bipolar cells dendrites. Next is the cell body containing the nucleus and all the organelles found in a normal cell. Following is the inner segment which is full of mitochondria whose sole purpose is to provide ATP to the sodium (Na+) Potassium (P+) pump, ribosomes and membranes that assemble opsins. Lastly is the outer segment, which is the light absorbing segment and is essentially a modified cilia that houses disks full of opsins and Na+ channels (Figure 25).

There are differences between the two photoreceptor cell types. Rods, which are extremely sensitive to the levels of photons present and are used in low levels of light, are slim and rod shaped. The inner and outer segments fill the area between cones and stretch to the pigment epithelial cells. Cone outer segments, on the other hand, are used with bright light because they require high levels of photons for stimulation. Cones are conical and have a thicker body shape. The cell bodies are located right below the outer limiting pigment epithelial (Kolb 1970).
Within the outer segment, membrane bound discs contain visual pigments which make it possible for the retina to respond to light. This pigment consists of a protein called opsin and vitamin A derived chromophore. Opsins are membrane bound G protein-coupled receptors that are 35-55 kDa in size. They are light sensitive and bound to the retinal within the membrane.

Zebrafish have one type of visual pigment within the rod photoreceptor called rhodopsin, and four types of cone visual pigments. Rhodopsins are used in night vision whereas cone opsin is utilized in color vision. The four types of cones and their subdivision is based on the wavelength at which the highest light absorption is observed (λ max) absorption maxima. The four types of opsins are long λ sensitive (LW), middle λ
sensitive (MW), short λ sensitive (SW), and ultraviolet sensitive (UW). Long λ sensitive opsin has its λ max in the red region of the electromagnetic spectrum (about 650nm), the middle λ sensitive opsin is sensitive to the green region (510 nm), the short λ sensitive opsin reacts to the blue region (about 475 nm) and the UV opsin absorbs between (10-400nm) (Burnes & Lamb, 2003).

**Bipolar Cells**

In the retina, the bipolar cell is found in the inner nuclear layer and transfers its signal from its dendrites in the outer plexiform layer, to ganglion cells, in the ganglion cell layer, via its axons. There are two types of bipolar cells ON-center and OFF-center. To understand how these two differ it is essential to understand the basics of how a signal is transmitted within the retina.

An ON bipolar cell response, elicited in the presence of light, has a depolarized membrane, which results in an excitatory pathway. A depolarized membrane induces an increase in the neurotransmitter, glutamate being released to the ganglion cell. An OFF bipolar cell response, stimulated by the loss of light, results in the hyperpolarization of the membrane. The hyperpolarized membrane decreases the concentration of glutamate released to the ganglion cells at the synapse.

Each photoreceptor makes contact with both ON- and OFF- bipolar cells, the ON bipolar cell then synapses with an ON-ganglion cell while the OFF bipolar cell synapses with an OFF ganglion cell. Photoreceptors release glutamate to bipolar cells. In the dark (absence of light) there is a continuous flow of glutamate whereas in the presence of light the release of glutamate is decreased. Glutamate acts as a ligand to metabotropic
receptors (mGluRg) and AMPA/KA receptors found on ON and OFF bipolar cells respectively. The continuous release of glutamates keeps the AMPA/KA channels open and the OFF bipolar cell depolarized while mGluR6 channels are kept closed and the ON bipolar cells hyperpolarized. The presence of light decreases glutamate release from the photoreceptors. The decrease in glutamate results in the closure of the AMPA/KA channels and the hyperpolarization of OFF bipolar cells and the opening of mGluR6 and depolarization of ON bipolar cells. Hyperpolarized OFF bipolar cells decrease the amount of neurotransmitter sent to ganglion cells. While depolarized ON bipolar cells increase the amount of glutamate sent to ganglion cells (Figure 26).

It is also important to note that bipolar cells come in a great variety of morphologies and physiologies (Ayoub & Copenhagen, 1991). Besides being classified as ON or OFF, bipolar cells can also be divided by their stratification in the IPL and invagination relative to the photoreceptors. A bipolar cell is a midget cell when it is monostratified and comes in contact with only one cone photoreceptor, while a diffuse bipolar cell is multistratified and contacts many photoreceptors. A bipolar cell can also be flat or invaginating. A flat dendritic tip lies on the surface of the photoreceptor presynaptic terminal while an invaginating dendritic tip penetrates the terminal and approaches the synaptic ribbon (Wu, Gao, & Maple, 2000).

The development of dendritic arborization can be categorized into three stages, all contributing to the final size and complexity of a neurons structure. Stage one is the early phase of initial outgrowth and elongation. During this period there is minimal branching and the dendrites that have already developed are tipped with growth cones.
Figure 26. Schematic of ON and OFF bipolar cells. In the dark (left) there is a continuous release of glutamate to the bipolar cells. Glutamate ensures that the AMPA/KA receptors, found on OFF bipolar cells, stay open while mGluR6 receptors, on ON bipolar cells, remain closed. The trigger of light decreases the amount of glutamate being released, which closes the AMPA/KA OFF bipolar cell receptors and the cell becomes hyperpolarized while the ON bipolar cells open their mGluR6 receptors and become depolarized.

There are no synapses forming, however processes are being added and lost. Stage two is synaptogenesis, a more extensive and complex stage which marks the development of filopodial protrusions on the shafts of dendrites and the beginning formation of synapses. Stage three is known as maturity, a time when the dendrites reach their final size and structure. In some cases the filopodia are replaced by spines and the final synapses are made. Filopodia and growth cones are extremely dynamic and tend to extend and retract extensively, however the arbor overall stays balanced in regards to these numbers.
Prior *in vivo* work done on other cell types in the retina, specifically ganglion cell dendrites, demonstrate a pruning mechanism of initial outgrowth and subsequent elimination of dendrites during development as well as the possibility of a directed elimination of processes (Kolb H., *Organization of the outer plexiform layer of the primate retina: electron microscopy of Golgi-impregnated cells*, 1970). It was not until 2007 that Mummet. *et al.*, from his work using *in vivo* time lapse imaging with ganglion cell dendrites, suggested that it is both mechanisms at work.

For this study we propose that there are two models that might predict how dendrites of the bipolar cells develop and form connections with their presynaptic partners. The first model, directed outgrowth, theorizes that dendrites are targeted and will make connections with a single or few attempts. In this type of growth dendrites show specificity as to where they extend their processes in order to make connections with photoreceptors. The second model, growth and remodeling, uses a trial and error method. The dendrites will make contacts with inappropriate partners, retract, and try again until the correct presynaptic partner is found.

This study will be testing these two models by observing the development of individual bipolar cells and their interaction with photoreceptor cells in the zebrafish the retina. By employing both molecular and microscopy techniques it is possible to visualize both bipolar cells and photoreceptors *in vivo*. Using a combination of fluorescent proteins and endogenous retina promoters we can specifically look at certain cell types via confocal microscopy and monitor their interactions.
Figure 27. Diagram showing the possible strategies that bipolar cells and photoreceptors make their connections. The directed outgrowth model states that the dendrites are targeted and those cells will find their presynaptic partners quickly whereas the growth and remodeling model theorizes the formation of the synapses is done via trial and error.

**Experimental Design**

To study the strategy used by ON bipolar cells to achieve their final dendritic architecture it was important to design an experiment that yielded a fish that expressed fluorescent proteins in photoreceptor cells and bipolar cells in a manner that allowed for simultaneous visualization. This experiment is composed of two parts, a molecular and observational component.

In a previous study, a transgenic fish was generated in which bipolar cells expressed a membrane bound yellow fluorescent protein (mYFP) (Schroeter et. al. 2006). The specific expression of the fluorescent protein is controlled by a cell specific promoter, in this case the *nyx* gene promoter. The *nyx*, nyctalopin, gene encodes a leucine rich proteoglycan that is shown to be mutated in human Congenital Stationary Night Blindness (Bech-Hansen et. al. 2000). When the promoter is linked with mYFP there is expression of the fluorescence in a subset of ON bipolar cell (hence forth referred to as
the Q16 fish line) (Schroeter et. al. 2006). As a complement to this fish, the next step was to use a similar methodology to visualize photoreceptor cells.

Using a similar experimental design, SWS1, a gene that is expressed solely in blue sensitive cones and has a promoter region which retain its functionality once isolated, was used to visualize photoreceptors. The promoter was fused with a fluorescent protein, of a different color, mCFP, and its stability was verified by expression in a zebrafish embryo. The stable expression plasmid was injected into the embryos of the nyx:mYFP line and visualized for simultaneous expression of both cell types and using confocal microscopy the dynamic behaviors of the cells could be observed and studied.

Material and Methods

All cloning was done using Invitrogen Gateway Technology (as described in Chapter II). In order to ensure a successful cloning reaction it was necessary to begin with SWS1 promoter primers that contain the correct attachment sites flanking the primer sequence. SWS1 is located on Danio reiro chromosome 4, from base pair (bp) position 14,068,192 to 14,070,845 and can also be found in the bacterial artificial chromosome library in BAC 266 A5 (ensemble). Four primers were designed to isolate the SWS1 promoter region located 5.5kb upstream of the gene. One set of primers consisted of only the promoter forward and backward sequences used to identify and amplify the region, whereas the second set contained att sites flanking the promoter forward and backward sequences, attB1 and attB5r respectively.

Two test PCR amplification reactions were set up using BAC DNA as a template, one containing regular primers and the other containing Gateway Primers. Each reaction
contained 15µl of reagents and was carried out in 0.2ml PCR tubes with the following amounts of reagents per tube:

Gateway Primers

- 11.6 µl dH2O
- 1.5 µl 10X Accuprime PCR Buffer I
- 0.3 µl SWS1 B1 forward primer
- 0.3 µl SWS1 B5r reverse primer
- 1.0 µl BAC 266 A5 template (concentration)
- 0.3 µl Accuprime TaqDNA Polymerase High Fidelity (50µl)

Regular Primers

- 11.6 µl dH2O
- 1.5 µl API Buffer (get official name)
- 0.3 µl SWS1 forward primer (concentration)
- 0.3 µl SWS1 reverse primer (concentration)
- 1.0 µl BAC 266 A5 template (concentration)
- 0.3 µl Accuprime DNA polymerase (concentration)

The tubes were then placed in the PCR machine and the reaction was as follows:

1. Denaturation: 94 °C for 1 minute
2. Annealing: 55° C for 45 seconds
3. Extension: 68° C for 6 minutes
4. Repeat steps 1-3 35times
5. Extension: 68° C for 6 minutes
6. Preservation: 4° C for ∞

After the amplification, 5µl of the PCR product was run out on a 1.0% agarose/TAE gel in order to verify the product. A positive PCR result was sufficient to proceed with setting up preparative 50µl reactions in order to isolate product for cloning. These reactions were carried out using the same thermo-cycler conditions. PCR products were then PEG purified (as described in Liset.al) to extract DNA from the reaction mixture and resuspend in 25µl of ddH2O.
BP Reaction.

The BP reaction (described in Chapter II) required the following reagents to run to completion.

1.0 µl pDONR 221 P1-P5r
.34 µl SWS1 PCR product
6.66 µl dH₂O *
2.0 µl BP Clonase II **

All steps performed were identical to those described in the HE BP Reaction in Chapter II. The resulting plasmid, pENTR L1/SWS1/R5, consisted of the SWS1 promoter and a P1-P5r vector, as seen in Figure 29.

LR Reaction.

The LR reaction (described in Chapter II) required the following reagents to run to completion.

50fmoles pENTR L5/MCFP/L2
50fmoles pENTR L1/SWS1/L5
50fmoles pDEST miniTol2 rfa verb
enough diH₂O to bring the reaction to 8µl
2µl of LR Clonase II

All steps performed were identical to those described in the HE BP Reaction in Chapter II. The resulting plasmid, pDESTminiTol2 SWS1:MCFP (Figure 30), consisted of two entry clones, pENTR L1/SWS1/R5 and pENTR L5/CFP/L2, and the miniTol 2 destination vector, the latter seen in Figure 10.

Zebrafish Injection and Microscopy.

All fish were injected as described in Chapter II. For the injection of the SWS1 construct three fish lines were utilized, WT, roy orbison (roy), and Q01. Roy fish have a
decreased number of iridophores, a pigment, which normally make visualization of the retina difficult. The Q01 fish are a transgenic line that has all cells labeled with a membrane targeted cyan fluorescent protein (“TG[pax6-DF4:MCFP]°°”.

Figure 28. Schematic of the SWS1 promoter.
Figure 29. Map of pENTR L1/SWS1/R5.

Figure 30. Map of pDEST miniTol2 SWS1:MCFP construct.
Each injected embryo was initially screened and scored for the presence or absence of cyan fluorescence in the retina at 3 days post fertilization. All screening was done using a Olympus SZX16 fluorescent microscope and pictures were taken using a Nikon D5000 camera controlled from a computer using Nikon Camera Control Pro 2 software. Embryos were then exposed to .003% phenylthiourea (PTU) at 12hpf. PTU prevents the formation of melanin in the retinal pigment epithelium increasing transparency for microscopy. Larvae ready to be imaged were immobilized using anesthetic tricain and then embedded in 1% LMT agarose. Embedded fish were then covered with fish water and imaged using an Olympus Fluoview 1000 confocal microscope.

Results

To study the strategy used to make initial synapses with synaptic partners it is necessary to visualize both players, in this case photoreceptors and bipolar cells in vivo. To be able to determine the ON bipolar cell dendritic arborization strategy it is necessary to visualize bipolar cell dendrites. This led to the construction of SWS1:mCFP clone. Generation of the construct, pDESTminiTol2 SWS1:mCFP, was successful and allowed for visualization of cone photoreceptors three days post fertilization.

It is possible to visualize both photoreceptors and bipolar cells by injecting the SWS1 construct into the Q16 fish. It was seen that the signal is not at high levels and even though it is possible to visualize both cells the photoreceptors start to fade quickly and require a higher laser intensity to be used. Higher laser intensity in turn causes the bipolar cells to bleach and detection is lost. Due to the above mentioned issues it was necessary to suspend the use of the SWS1 construct.
Even though it was not possible to visualize the photoreceptors it is still possible to look at the dendritic arborization of bipolar cells by imaging the Q16 fish line. In order to determine the timing of experimentation, the initial imaging was done 6 days post fertilization. It was expected that the arbors would be fully formed by that time and in fact that was the case (figure 31). A 6 day old Q16 fish shows the fully formed dendritic arbor of bipolar cells. We see the cell body along with the dendrites and spinules projecting off of the dendrites themselves. In this example an ectopic projection that would be going into the photoreceptor layer. It was verified that this projection does extend and then retracts back into the bipolar cell layer (image not provided).

The next step was to visualize the bipolar cells at four days post fertilization. A image showing a portion of the retina (figure 33) captured the entire bipolar cell including dendrites, cell body and axons. Here we can see that everything has stratified to their corresponding layers. When looking at a close up of the dendritic arbor of an individual bipolar cell we see a fully formed arbor along with the presence of spinules (Figure 32)

The window for arbor development was narrowed down to 0-4 days. At this point the imaging was done with shorter time intervals to try to capture the dynamics of the bipolar cell dendrites. Images were taken starting at 3 days (noon of the third day, the first point at which CFP expression starts) and continued for a number of hours. Time lapse images spanning a five hour interval (images taken every hour) show very little dynamics (Figure 34). Figure 36 shows the presences of a dendritic arbor along with full formed dendrites and spinules. We see a slight movement in some of the spinules.
Figure 31. Confocal visualization of bipolar dendritic arbors of a 6 day old zebrafish. This image shows the cell bodies (A), dendritic arbor (B) and spinule (C) projecting from the dendrites. An ectopic projection is also visible in this image (D).
Figure 32. Confocal image of a portion of the zebrafish retina at four days post fertilization. The entire bipolar cell layer, including dendrites, cell bodies and axons are visible from this image.
Figure 33. Close up of an individual bipolar cell at four days post fertilization. We see a fully formed arbor including spicules extending into the synaptic layer of the retina.

A six hour time lapse of an individual bipolar cell (initial image taken at noon on day 3) shows very little movement of its dendrites or spicules (Figure 35 and 36).

The transgenic fish line Q16 allows for visualization of bipolar cells three days post fertilization. Based on the above images the dendritic arbors are already near maturity at the time that the fluorescent protein starts expressing.

Discussion

Previous studies using the zebrafish retina have showed a great deal of growth and retraction of processes in amacrine cells, ganglion cells and ON bipolar cell axons
These processes are believed to find their synaptic partners via the exploration of their environment followed by the elimination of projections, a technique referred to as pruning. Mummet. Al. in 2007 showed that at least within ganglion cells, there seems to be a targeting mechanism that allows the projections to appropriately locate their developing strata followed by the overgrowth and elimination of projections to isolate a specific synaptic partner.

Initial studies done by Schroeter (unpublished work, personal communication). suggest a method of “overgrowth and remodeling” utilized by ON bipolar cell dendrites during the organization and stratification of the OPL, as opposed to “directed outgrowth”. The directed outgrowth model illustrates that the dendrites of bipolar cells will grow from the cell body and make contact with their presynaptic partners with a high level of specificity. On the other hand, the process of overgrowth and remodeling suggests that the bipolar cell dendrites will extend past the intended target then retract and continue to make connections with various partners until the right one is found.

This study looked at a time frame between 3 and 4 days. During this time frame the retina is still immature and is forming its very specific and organized layers. During this time period the bipolar cell dendrites seem to have established their architecture. The dendrites show no or very little dynamics with hours between any sorts of change in morphology. This is contrary to that what occurs on the other side of the same cell.
Figure 34. Confocal image of a 3 day bipolar cells. We see a fully formed arbor that shows very little movement in the five hours of imaging.

Figure 35. Confocal images of a bipolar cell found in a 3 day old zebrafish. The time lapse spans a six hour period.
Bipolar cell axons show dendritic architectural changes every fifteen minutes (Schroeter, Wong, & Gregg, 2006). Based on these observations there are two possibilities. One possibility is that the dendrites experience more of a directed outgrowth method and the presynaptic partners are found quickly and those connections are established fast. Another possibility is that the bipolar cell dendrites do have a comparably dynamic morphology but it is very early on in the development of the retina, at the time not visualizable in the Q16 fish. This rapid change and structuring of the dendritic architecture can potentially take place before the gene comes on.

**Future Experiments**

In order to make any valid conclusions on the dynamics of the ON bipolar cell dendrites it would be necessary to eliminate all the various possibilities. First image the cells at a more frequent interval and see if you can catch those spicules forming and the dendrites extending. Secondly, if possible, use another promoter to drive fluorescent expression in the bipolar cells. This gene would have to come on very early to see the entire time frame that the retina develops. Currently no such promoter exists.

It would also be interesting to see what the photoreceptor axons are doing at these times. In order to visualize the photoreceptors a more stable gene has to be chosen as the driving force of the photoreceptor visualization.
Figure 36. Confocal image of a bipolar cell found in a 3 day old zebrafish. The image shows the difference in individual cells after a six hour time gap.
REFERENCE LIST


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VITA

Anna Matejczyk was born in Bialystok, Poland and immigrated to the United States at the age of seven. Since then she has been living in the Chicago-land area. Anna began her undergraduate career at Loyola University Chicago, Illinois. She graduated from Loyola University in 2008 with a B.S. in Biology with an emphasis in Molecular Biology and a minor in Chemistry. Anna joined the Department of Biology as a research assistant to Dr. Eric Schroeter in early 2009. Later that year she entered the Graduate Program to pursue her Master of Science degree. She was awarded a two-year stipend and tuition scholarship by the Department of Biology Graduate School for 2009-2011. During her time as a graduate student she had the opportunity to share her knowledge and passion for biology as an instructor to Biology 111 and 112 laboratories. Anna is currently employed at Northwestern University where she continues to further enhance her new found enthusiasm for teaching by assisting in the formulation of a new biology curriculum as well as instructing Biology Lab at the Northwestern School of Continuing Studies.