Autoregulation of the Glial Gene reversed polarity in Drosophila melanogaster

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AUTOREGULATION OF THE GLIAL GENE \textit{REVERSED POLARITY} IN \textit{DROSOPHILA MELANOGASTER}

A Dissertation
presented in partial fulfillment of requirements
for the degree of Doctor of Philosophy
in the Department of Biology
The University of Mississippi

by

JAMIE L. WOOD

May 2015
ABSTRACT

During development, cells of the nervous system begin as unspecified precursors and proceed along one of two developmental paths to become either neurons or glia. I seek to understand more about the genes that control this process, focusing on the lesser understood of the cell types, glial cells. Using Drosophila melanogaster as a model system, previous work from my lab and others has established the role of the master regulatory transcription factor Gcm in directing neuronal precursor cells to assume a lateral glial fate. Gcm acts on many target genes, one of which is reversed polarity (repo). repo is necessary for proper glial cell differentiation; once activated, its expression is maintained throughout the life of the fly through currently unknown mechanisms. I propose that repo expression is maintained in an autoregulatory manner, whereby Repo protein acts as a transcription factor on its own regulatory DNA sequence. Three canonical Repo binding sites (RBSs) are located within the 4.3 kb repo cis-regulatory DNA (CRD). Using both S2 cell culture and in vivo expression systems, I have evidence that suggests Repo protein interacts strongly with one of these sites, designated RBS1, to induce the expression of reporter genes. Mutagenesis of RBS1 results in a significant decrease of reporter gene expression in both systems, while RBS2 and RBS3 appear to have no role in autoregulation of repo expression.
DEDICATION

This dissertation is dedicated with much love to the memory of my grandfathers, James F. “Frank” Kizziah, Jr. and Elmer B. Thompson, both of whom passed away as I pursued this degree.

“He was a million miles from a million dollars, but you could never spend his wealth.”

~ “Preacher”, OneRepublic
LIST OF ABBREVIATIONS AND SYMBOLS

β-gal  β-Galactosidase Protein
CNS  central nervous system
CRD  cis-regulatory DNA
gcm  glial cells missing Gene
Gcm  glial cells missing Protein
GMC  ganglion mother cell
kb  kilobases
lacZ  reporter gene that produces β-gal
NS  nervous system
PNS  peripheral nervous system
repo  reversed polarity Gene
Repo  reversed polarity Protein
RBS  Repo binding site
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS AND SYMBOLS</td>
<td>iv</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td><strong>CHAPTER</strong></td>
<td></td>
</tr>
<tr>
<td>I. BACKGROUND AND INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Overview of Nervous System</td>
<td>1</td>
</tr>
<tr>
<td>Gene Regulation</td>
<td>6</td>
</tr>
<tr>
<td>Genes of Drosophila Glial Cell Development</td>
<td>8</td>
</tr>
<tr>
<td>II. EXPERIMENTAL PROCEDURES</td>
<td></td>
</tr>
<tr>
<td>Construction of additional <em>repo-lacZ</em> and <em>luciferase</em> reporter genes</td>
<td>21</td>
</tr>
<tr>
<td>Mutation of Repo binding sites</td>
<td>21</td>
</tr>
<tr>
<td><em>Drosophila</em> S2 Cell Culture Luciferase Assays</td>
<td>22</td>
</tr>
<tr>
<td><em>Drosophila</em> S2 Cell Staining</td>
<td>22</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>23</td>
</tr>
<tr>
<td><em>Drosophila</em> genetics</td>
<td>23</td>
</tr>
<tr>
<td>III. RESULTS</td>
<td></td>
</tr>
<tr>
<td>Introduction</td>
<td>25</td>
</tr>
<tr>
<td>Results</td>
<td>26</td>
</tr>
</tbody>
</table>
Discussion........................................................................................................................................39

IV. CONCLUSIONS AND FUTURE DIRECTIONS............................................................................40

REFERENCES ....................................................................................................................................45

VITA................................................................................................................................................49
LIST OF FIGURES

1. Identification of the 4.3 kb repo regulatory region via a lacZ reporter construct ..........15
2. The lack of Gcm interaction with the reporter construct eliminates reporter protein .......16
3. Ectopic β-gal reporter expression observed with ectopic Repo expression in repo-4.3-
lacZ embryos .................................................................................................................19
4. Four reporter constructs from the promoter bash of the repo cis-regulatory DNA (CRD) result in ectopic expression driven by actGal4/UASrepo .........................................................28
5. Ectopic expression of β-gal with specific repo-lacZ reporters in dissected stage 17 embryos ........................................................................................................................................29
6. Diagram of crossing strategy used to create heterozygous embryos to study effect of ectopic Repo expression on repo-lacZ reporters ..................................................................................30
7. The repo CRD contains three highly conserved canonical Repo binding sites (RBSs) ... 32
8. Expression of Repo in S2 cells ..........................................................................................35
9. Repo in S2 cells can interact with repo-luciferase constructs ........................................36
10. Mutation of the canonical RBS1 site significantly decreases luciferase expression in culture ........................................................................................................................................38
CHAPTER I

BACKGROUND AND INTRODUCTION

All complex animals, both invertebrates and vertebrates, have a nervous system, which allows them to process information from internal and external environments. This system is made of two different types of cells, neurons and glia. Neurons are the most well-understood of the two types as they have been extensively studied for a longer period of time as compared to glia. As development of the nervous system proceeds, an unspecified precursor cell will adopt one of these two cell fates; the path a precursor cell will take is dependent on the gene expression pattern of that cell. The purpose of this introduction is to provide an overview of the nervous system and the genes required for proper development, focusing on the model system for this study, *Drosophila melanogaster*, and specifically on the genes necessary for proper glial cell formation. This introduction will be divided into three major categories as follows: a broad overview of the nervous system, gene regulation, and genes necessary for *Drosophila* glial development.

Section 1: An Overview of the Nervous System

Most animals have a nervous system (NS) which contains two divisions, the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS is composed of the brain and spinal cord; the PNS contains all the remaining nerve tissue throughout the body. The nerve cells of the PNS are funneled to the CNS, which serves as the integration point for
information processing. The NS collects and processes all sensory information the animal receives from both internal and external environments, including chemical and physical stimuli and regulates cognitive functions. Two types of specialized cells form the NS, neurons and glia.

Not all animals possess this type of NS. Animals are placed into two broad categories based on their development as either diploblasts or triploblasts. Diploblasts develop only two germ layers during gastrulation, while triploblasts develop three. Diploblasts are the animals that will develop as radially symmetrical, the cnidarians and the ctenarians (Ghysen, 2003). These animals have a nerve net rather than a NS with two divisions. The nerve net is a type of sensory system made of neurons, and this system is spread throughout the animal with no distinct brain for information processing. Since diploblasts are the only animals with a nerve net, and since many triploblastic animals share common features in their NSs (anterior-posterior organization, orthogonality, the presence of mechanosensory organs (Ghysen, 2003)), this suggests common ancestry for the origin of NS in all triploblasts (Ghysen, 2003). For the remainder of this chapter, only the complex NS of triploblasts will be discussed.

Neurons are cells so well-understood that the basic structure and functions are included in all undergraduate introductory biology textbooks. Many different types of neurons are found throughout the NS of an animal, but all neurons share the same features. All neurons have dendrites, a cell body, and one axon. The dendrites receive information and carry it to the cell body, while the axon transmits information away from the cell body; information is processed as an electrical signal. The cell body contains the organelles of the neuron. Neurons are such attractive cells for study largely because of the physiological ability to generate electrical impulses, which result from changes in the overall charge on the interior and exterior of the neuron’s plasma membrane. When at rest, Na+/K+ pumps work to pump Na+ ions out of the cell
and $K^+$ ions into the cell, creating an overall positive charge outside of the membrane and a negative charge inside the membrane (resting potential). When the resting potential is disrupted, or when the neuron “fires”, $Na^+$ channels open creating a local area of depolarization on the membrane, which then becomes propagated by means of an action potential. This movement of the action potential down the axon is how information is transmitted within an individual neuron.

To transmit information between neurons, or between the neuron and another target, (e.g., a muscle cell), neurotransmitters are released from vesicles across the synapse. This is the miniscule space that separates the axon of the transmitting neuron from the receiving cell. The neurotransmitters will bind to specific receptors on the target cell and elicit the correct response.

Neurons can be classified in different ways, including anatomical (Masland, 2004), molecular (Kodama, et.al, 2012), electrophysiology (Markram, 2004), and functional (Sharpee, 2014).

Glia are the second type of cell found in the NS; while these cells are much less well-known and well-studied than their neuronal cousins, glia are far more numerous in the NS than neurons. Despite the gap in our understanding between neurons and glia, we know that glia play many critical roles in the NS, both during development and the mature NS. These include, but are not limited to, regulation of neuronal stem cell proliferation, axon pathfinding, axon ensheathment, synapse formation and maintenance, regulation of the blood-brain barrier, and immunological functions (reviewed in Stork, et.al, 2012). Four major types of glia are found in vertebrates: astrocytes, oligodendrocytes, Schwann cells, and microglia (Corty and Freeman, 2013; reviewed in Barres, 2008).

Two classes of astrocytes, fibrillary and protoplasmic, are found throughout the brain, and astrocytes of both classes ensheathe synapses to regulate ion and neurotransmitter levels (Barres, 2008). Oligodendrocytes and Schwann cells are the myelinating cells of the NS; both of
these cells extend their own membranes around the axons of neurons. Oligodendrocytes wrap axons in the CNS while Schwann cells do the same in the PNS. Myelination allows the speed of electrical impulses traveling along the axon to increase, and by extension the information traveling from cell to cell. (Barres, 2008) Myelination is only seen in vertebrates. Microglia reside within the CNS and function as immune cells; they are unusual in origin as they are derived from a lineage of myeloid cells that move into the developing brain before the blood-brain barrier is erected. These cells are also mobile within the brain; they can seek out sites of injury and function in repair. Because of their role in immunity, microglia are rich targets for research in brain health and disease (Barres, 2008).

*Drosophila melanogaster* is an excellent model for use in studying the nervous system for several reasons. Fruit flies share the two major divisions of mammalian nervous systems, the CNS and PNS, neurons and glia are found in the NS, and many genes needed to form the NS in *Drosophila* share the same function in vertebrates. In addition, *Drosophila* have only four chromosomes, making genetics experiments much simpler. Unlike vertebrates, whose CNS forms on the dorsal side, the insect nervous system begins developing on the ventral side of the embryo. Initial formation of the nervous system begins around stage 5 in the *Drosophila* embryo when a portion of the ectoderm becomes modified into the neuroectoderm (Campos-Ortega and Hartenstein, 1997). Different classes of progenitor cells give rise to neurons and/or glia, depending on their location in the NS. Within the CNS, neuroblasts derive from the neuroectoderm in a stereotypical pattern, are designated based on their position and gene expression patterns, and are a mixed population of progenitor cells that give rise to neurons and/or glia (Goodman and Doe, 1993; Campos-Ortega and Hartenstein, 1997). Included in this population are the glioblasts, neuroglioblasts and neural progenitors (Campos-Ortega and
Hartenstein, 1997; reviewed in Jones, 2001). Glioblasts give rise to the longitudinal glia and derive specifically from the lateral neuroblasts; these cells undergo symmetrical division to produce two cells unlike the asymmetrical division of other neuroblasts (Goodman and Doe, 1993). Neuroglioblasts are further subdivided as Type 1 or Type 2, and each type leads to specific subsets of daughter cells. After one division, Type 1 neuroglioblasts produce two cells, one of which behaves as a glioblast and the other that behaves as a neuroblast. The glioblast gives rise to glia only, while the neuroblast gives rise to neurons only (reviewed in Jones, 2001). Type 2 neuroglioblasts behave more as a traditional stem cell showing asymmetrical divisions. The neuroglioblast divides several times, producing a ganglion mother cell (GMC) with each division; the GMC then divides to produce either neurons or glia depending on gene expression patterns (reviewed in Jones, 2001). In the PNS, sensory organ precursors are the progenitor cells that give rise to neurons and glia (reviewed in Jones, 2001). Approximately 30 neuroblasts are produced within each abdominal hemisegment, leading to about 350 neurons and 30 glial cells.

In the mature Drosophila embryo, the CNS is organized as two major tracks of axons that parallel either side of the ventral midline. These two tracks are connected by neurons that cross the midline. Eight to ten glial cells wrap the neurons of the major PNS nerve tracks in the abdomen (reviewed in Jones, 2001). Most glial cells, collectively called “lateral glia”, are derived from neuroectoderm; the only exceptions are the midline glia, which arise from mesectoderm. Glial cells in Drosophila are grouped into three major categories based on position and morphology: surface glia, cortex glia and neuropile glia (Ito, et. al, 1995; reviewed in Jones, 2001). Surface glia completely ensheath the axons of the CNS and peripheral neurons. Cortex glia are a special subset of glial cells called cell body glia, and these are found in the
cortex of the ventral nerve cord. Finally, neuropile glia ensheath nerve roots, the neuropile and commissures.

**Section 2: Gene Regulation**

The overarching question of developmental biology is this: how do plants and animals transition from clusters of identical cells to organisms composed of a variety of different cell types? One facet of the developmental process is gene regulation, the expression of the correct gene at the correct time and place as the organism develops, and/or the prevention of gene expression at the correct time and place. In this regard, the coding sequence of a gene is not the crucial element, but rather the specific sequences of DNA that recruit factors that allow the gene to be expressed or repressed. These sequences are generally called *cis*-regulatory elements and have several components, including promoters, enhancers, and silencers.

Promoter sequences are required for basal levels of transcription and are the most well-understood of all regulatory sequences; general transcription factors (TFs) and RNA polymerase II interact with promoter sequences to begin gene transcription. Enhancer sequences are traditionally defined as DNA sequences that are necessary for the maximum level of transcription for a given gene, act in concert with other TFs to recruit RNA polymerase II, and are located either upstream or downstream from the gene they regulate (Ong and Corces, 2011). Whether or not a gene is transcribed is largely determined by the availability of the enhancer sequences by the TFs and other proteins, meaning that enhancers play a large role in gene regulation. Enhancers are also crucial for tissue-specific gene expression and pattern formation in a developing embryo (Spitz and Furlong, 2012). Silencers are DNA sequences that reduce the amount of transcription of the gene they regulate; instead of binding TFs, these sequences bind repressor proteins that will inhibit transcription.
A classic example of cis-regulatory DNA (CRD) from *Drosophila* genetics is the regulatory DNA of the *even-skipped (eve)* gene. This pair-rule gene defines the odd-numbered parasegments in the developing embryo and is expressed in alternating stripes. The expression in these stripes is dependent on the amount of activator and repressor proteins present to interact with the *cis*-regulatory region of *eve*. Bicoid, Hunchback, Kruppel, and Giant work in concert to control the expression of *eve* in these alternating stripes. For stripe 2, Bicoid and Hunchback are required as activators, while Kruppel and Giant act as repressors. When the concentrations of Kruppel and Giant are low, repression is absent and *eve* is expressed if, simultaneously, the concentrations of Bicoid and Hunchback are high. Initial studies of the CRD for stripe 2 confirmed Giant as a repressor by mutating Giant binding sites in an *eve*-lacZ reporter line; this mutated line showed an increase in the number of stained cells, indicating when repression is absent the CRE is active in more cells (Small, et.al, 1992).

Development of new technology, such as whole genome sequencing, has led to studies that show the importance of chromatin in gene regulation. New types of studies, such as genome-wide association studies, reveal the different chromatin conformations found at various types of DNA regulatory sequences. Enhancers in particular are characterized by nucleosome instability and specific histone modifications that are conducive to transcription (reviewed in Ong and Corces, 2011). Within *Drosophila*, a major class of proteins that modify chromatin are the Polycomb group (PcG) proteins. PcG proteins were identified in *Drosophila* early in the 2000s as repressors of Hox genes; PcG proteins restrict Hox gene expression to the correct region within the developing embryo (Sawarkar and Paro, 2010). Five different protein complexes in *Drosophila* form the PcG; two of them are known as repressor complexes, PRC1 and PRC2. Each of these large repressor complexes has several different components, none of which
directly bind DNA (Bantignies and Cavalli, 2011). To carry out repression, these complexes act on histone proteins in two different ways. PRC2 will trimethylate histone H3 at lysine-27, while PRC1 ubiquitinates lysine 110 of histone H2A (Bantignies and Cavalli, 2011). The trimethylated H3 histone allows PRC1 to bind to the chromatin using the Polycomb subunit; once the PRC1 complex has bound and ubiquitinated the H2A histone, RNA polymerase II is impaired from initiating transcription (Bantignies and Cavalli, 2011).

Since neither of these complexes bind directly to DNA, other protein factors that do bind DNA are required to recruit Polycomb (Sawarkar and Paro, 2010). The specific regions of DNA that bind these proteins are called Polycomb response elements (PREs) (Bantignies and Cavalli, 2011). Another of the PcG complexes, Pho-repressive complex (PhoRC), is suggested to play a role in the recruitment of PRC1 and PRC2 to these response elements. Unfortunately, *Drosophila* PREs have no sequence similarities and a consensus sequence has not been identified (Bantignies and Cavalli, 2011).

The Trithorax group (TrxG) protein complexes act as antagonists to PcG, removing repression and allowing gene expression. Less is known about this group of proteins than PcG. In *Drosophila*, several complexes of proteins contain TrxG proteins, similar to the PcG setup (Schuettengruber, et.al, 2007). Another trimethylation mark may also be necessary for binding of some of these TrxG components, this time at histone H3 lysine residue 4, and possibly for the transcriptional activation of some genes (Schuettengruber, et.al, 2007).

**Section 3: Genes of *Drosophila* Glial Cell Development**

Since neurons and glia arise from common progenitor cells, genetic switches must exist to direct these cells to take one of these developmental paths. Two seminal papers, published in *Cell* (1995), along with a third paper published in *Development* (1996), identified glial cells
missing (gcm) as a binary switch that, when expressed in neuronal progenitors, directs those cells to become glial cells (Jones, et.al, 1995; Hosoya, et.al, 1995; Vincent, et.al, 1996). gcm was identified in each paper using a different method; Jones, et.al (1995) used an EMS screen, Hosoya, et.al (1995) used a P-element insertion screen, and Vincent et.al (1996) used the rA87 line, which expresses β-galactosidase in glial cells, to perform mutagenesis of the gene next to the lacZ insertion. All groups observed that upon mutation of the gene, the number of glial cells was diminished. Jones, et.al and Hosoya, et.al (1995) cloned gcm and used in situ hybridization to demonstrate the expression pattern of the gene in wildtype embryos. gcm expression is first detected at stage 11 in the NS of developing embryos and is no longer detected by stage 15. All three papers confirm when gcm is mutated, glial cells are transformed into neurons (Jones, et.al, 1995; Hosoya, et.al, 1995; Vincent, et.al, 1996); however, Jones, et.al (1995) conducted the most detailed analysis of the cell morphology with electron microscopy of the developing CNS and PNS to show the lack of glial cells in the gcm mutant embryos. gcm’s role was further characterized through ectopic expression in fly embryos using the UAS/GAL4 system. Here, Jones, et.al and Hosoya, et.al (1995) demonstrated that overexpressing gcm in developing neuroblasts resulted in all of those cells being converted into glial cells. Taken together, these studies identified gcm as a binary switch acting in GMCs; if gcm is expressed in the progenitor cell, it develops as a glial cell, while lack of expression results in neuronal differentiation.

The initial differentiation of lateral glial cells is not due solely to gcm. Kammerer and Giangrande (2001) and Alfonso and Jones (2002) characterized a second gcm gene, gcm2, after the initial identification of this homolog by Akiyama, et.al (1996) in a genetic screen for the gcm-motif. gcm2 has a minor role in glial cell development, but it is essential for macrophage differentiation. gcm2 is located approximately 30 kb 5’ of gcm, just upstream of thioredoxin and
contains the same unique gcm-motif as other members of this family of proteins (Akiyama, et.al, 1996; Kammerer and Giangrande 2001; Alfonso and Jones, 2002). The expression pattern of gcm2, as shown through in situ hybridization, mirrors that of gcm, but gcm2 is expressed at lower levels in the cells (Kammerer and Giangrande 2001; Alfonso and Jones, 2002). When gcm2 is ectopically expressed in developing neuroblasts using the UAS/GAL4 system, these cells are converted into glia, as is the case with gcm ectopic expression (Kammerer and Giangrande 2001; Alfonso and Jones, 2002). This result indicates that gcm2 is sufficient for gliogenesis; however, Alfonso and Jones (2002) showed that gcm2 is necessary for gliogenesis by creating a deletion for gcm2 in Drosophila that was homozygous viable. When these flies were crossed to other flies that carried deletions for both gcm and gcm2, resulting embryos that contained only one copy of gcm and no copies of gcm2 showed consistent defects in glial cells, such as decreased numbers of longitudinal glia (Alfonso and Jones, 2002).

Jones, et.al and Hosoya, et.al (1995) were able to confirm, based on the DNA sequence, that gcm codes for a novel protein that had no known homology to other proteins. The authors confirmed localization of the protein to the nucleus (Jones, et.al, 1995) and the presence of a nuclear localization signal (NLS) (Hosoya, et.al, 1995). The most significant characterization of the Gcm protein was published by Akiyama, et.al (1996) and Schreiber, et.al (1997) which showed gcm produces a novel transcription factor with a highly conserved N-terminal DNA binding domain. Both groups determined the recognition sequence through binding assays using randomized oligonucleotides (Akiyama, et.al, 1996; Schreiber, et.al, 1997); however, the differences in techniques may account for the slight variation seen in the consensus binding sequence. Akiyama, et.al (1996) found the Gcm recognition sequence to be 5’-(A/G)CCCGCAT-3’, while Schreiber, et.al (1997) found 5’-ACCCG(T/C)AT-3’ as the recognition sequence.
Schreiber, et.al (1997) note that this consensus sequence is similar to the binding sequence necessary for POU-domain proteins; however, these proteins cannot recognize the Gcm sequence, and Gcm cannot bind to the POU recognition sequence. Therefore, the Gcm domain, or Gcm box, is unique. Schreiber, et.al published a second paper in 1998 which provided a more detailed analysis of the Gcm box, including which specific residues in the consensus sequence are critical for Gcm interaction. Using the sequence 5’-ATGCGGGT-3’ and a sequential mutational analysis, residues 2 and 3 (T and G) and residues 6 and 7 (G and G) were shown to be the most critical, and these results were the same in both Drosophila Gcm and mouse GCM (mGCM), indicating a strong level of conservation of function (Schreiber, et.al, 1998). The authors also determined that Gcm/mGCM interacts with the DNA as a monomer, and that seven cysteine amino acids are necessary for the interaction with the DNA (Schreiber, et.al, 1998).

While Gcm is necessary for the initial differentiation of glia in Drosophila, its expression is transient. As a transcription factor, Gcm activates the expression of other genes to further differentiate glial cells. As development proceeds, one of these is reversed polarity (*repo*). *repo* codes for another transcription factor produced exclusively (within the nervous system) in all lateral glial cells and is regulated directly by Gcm; it was identified in a series of closely published papers, each with different methods of isolation. Xiong et.al (1994) initially characterized the *repo* allele in a P-element mutagenesis screen for effects on the adult Drosophila visual system; this allele was named reversed polarity because the phenotype produced is one of a reversed reading on an electroretinogram. Using reporter lines for the mutant *repo* allele, the authors determined that expression was present in glial cells of all life stages of Drosophila; *in situ* hybridization with *repo* in the embryos showed that expression was confined to glial cells (Xiong, et.al, 1994). Xiong, et.al (1994) also showed that Repo protein
contains a homeodomain that is most similar to the paired-like group. In a paper published in October of the same year, Campbell et.al (1994) isolated a cDNA they called rk2 from a library screen of homeobox genes in the Drosophila eye imaginal disc; they determined from sequence data that the protein contained a homeodomain belonging to the paired class. To complete their analysis of this newly identified protein, the authors generated an antibody that matched in situ mRNA expression patterns (Campbell, et.al, 1994). The staining patterns observed in embryo and larval tissues faithfully replicate the staining patterns observed with the repo gene from Xiong, et.al (1994), an indication that rk2 is the same gene as repo (Campbell, et.al, 1994). Campbell, et.al (1994) also generated two null mutations for rk2 and examined embryological glial and neuronal development with a series of markers. Mutant rk2 embryos at stage 14 show normal glial development, but by stage 16, the glial cells have become disorganized; in addition, Rk2 is necessary for Prospero expression in longitudinal glia in stage 16 embryos (Campbell, et.al, 1994). However, the CNS tracts appear to be unaffected in the rk2 mutants (Campbell, et.al, 1994). These results suggest rk2 has a role in the later stages of glial cell development and is not required for initial glial cell determination. Early the next year, Halter et.al (1995) published results identifying repo through a cDNA library screen of genes regulating the developmental gene fushi tarazu (ftz). Using the regulatory DNA of ftz in a library screen, Campbell, et.al (1995) identified a protein that bound to the sequence, protecting the DNA from DNase digestion. The results of a competition binding assay revealed the sequence with the best competition for binding to the protein was 5’CAATTA3’ (Campbell, et.al, 1994), an indication that this is a homeodomain protein. The authors identified the protein as Repo based on the previously published work of Xiong, et.al (1994). Campbell, et.al (1995) also generated an antibody to Repo and showed that nuclear localization of Repo begins in glial cells of stage 11
embryos and continues thereafter, again replicating results of Xiong, et.al (1994) and Halter, et.al (1994). Campbell, et.al (1995) also used their Repo antibody to stain embryos of *Schistocerca gregaria* (locust) and found the antibody could recognize Repo in glial cells of these embryos, indicating evolutionary conservation of Repo. Analysis of *repo* mutant alleles shows defects in glial development in terminal stages (Campbell, et.al, 1995); these results are consistent with Xiong, et.al (1994) and should match since the same *repo* mutant alleles were analyzed.

Yuasa, et.al (2003) published a significant characterization of Repo protein, confirming the binding site and showing interactions with other transcription factors. To confirm 5’CAATTA3’ as the Repo binding site (RBS), two plasmids were created for use in S2 cell culture experiments. The reporter plasmid had two 5’CAATTA3’ motifs fused to the luciferase reporter gene and was co-expressed with a second plasmid that had repo cDNA fused to an actin promoter (Yuasa, et.al, 2003). The production of Repo protein in the S2 cells was able to generate high levels of luciferase activity; however, when 5’CAATTA3’ was mutated to 5’CAGTTA3’, the levels of luciferase dropped significantly (Yuasa, et.al, 2003). The 5’CAATTA3’ motif was confirmed as the RBS *in vivo* using a specific *lacZ* reporter gene containing a portion of the *ftz* regulatory DNA (Yuasa, et.al, 2003). The regulatory sequence contained two copies of 5’CAATTA3’, and β-galactosidase expression was seen in glial cells of the PNS and specific cells of the CNS in a wildtype background. When this reporter construct was expressed in a *repo* mutant background, no reporter staining was observed, indicating that Repo binds to the 5’CAATTA3’ motif; in addition, when Repo was ectopically expressed with this reporter, non-glial cells produced β-galactosidase (Yuasa, et.al, 2003). Interestingly, the strongest amount of β-galactosidase staining was observed in the epidermis of the embryos.
This section of the review has described Gcm followed by Repo because that is how the genes are expressed in the developing glial cells; Gcm activates the expression of Repo (Lee and Jones, 2005). However, this order of expression was not known when these genes were identified; Repo was identified before Gcm. In fact, since the Repo antibody was available to use as a glial marker, it was used to detect the absence of glial cells in the Gcm mutants (Hoysona et.al, 1995; Jones, et.al, 1995; Vincent, et.al, 1996). Only when Gcm mutants failed to show Repo staining was there an indication that Gcm was necessary for the expression of repo.

Akiyama et.al (1996) first discovered that Gcm directly binds to specific DNA sequences (see above), and the authors used the putative regulatory region of the repo gene for these experiments. Therefore, they were the first to show that repo is a direct target of Gcm (Akiyama, et.al, 1996). Once the consensus sequence for the GBS had been identified, the authors identified eleven GBSs in the 4 kb region upstream of the repo gene (Akiyama, et.al, 1996). Lee and Jones (2005) confirmed the 4.3 kb upstream of repo as a direct target of Gcm through the construction of a lacZ reporter construct; when transgenic embryos were made with this artificial gene and stained with an antibody to β-galactosidase (β-gal), the staining pattern duplicated the native pattern of Repo (Figure 1). Upon mutation of the GBSs in this 4.3 kb repo regulatory region, and expression of the repo4.3-lacZ construct in a gcm mutant background, β-gal staining was abolished, further evidence that Gcm is necessary for expression of repo (Figure 2) (Lee and Jones, 2005).
Figure 1. Identification of the 4.3 kb repo regulatory region via a lacZ reporter construct. Embryos shown are dissected with anterior to the top, posterior to the bottom at stage 17. A) A wildtype embryo stained with an antibody to Repo protein, resulting in staining of all lateral glial cells. B) A transgenic embryo carrying the repo4.3-lacZ construct stained with an antibody to β-gal. The pattern of staining is identical to that shown in A; all lateral glia are stained. β-gal is a cytosolic protein, which explains the lack of punctuate staining as compared to Repo staining. Scale bar 20 μm. Adapted from Lee and Jones (2005) and used with permission.
Figure 2. The lack of Gcm interaction with the reporter construct eliminates reporter protein. A) The reporter \textit{repo}4.3-\textit{lacZ} is expressed in a gcm mutant background. Since no Gcm protein is available, β-gal staining is not observed. B) Expression of \textit{repo}4.3-\textit{lacZ}\text{Δ}GBS reporter in a wildtype background. Virtually all of the β-gal staining has disappeared, with the exception of cell body glia. Embryos are stage 17, anterior at the top and posterior at the bottom. Adapted from Lee and Jones (2005) and used with permission.
Recently, Flici, et.al (2014) published results that further illustrate the complex interplay of Gcm and Repo. The authors found that Repo is capable of binding to the $gcm$ promoter, both in S2 cell culture experiments and in vivo with ChIP analysis on stage 11 embryos, suggesting that Repo is necessary to maintain $gcm$ expression. However, upon ectopic co-expression of Gcm and Repo, the authors found a decreased number of glial cells, indicating that Repo can reduce the amount of Gcm (Flici, et.al, 2014). This degradation of Gcm by Repo is mediated, at least in part, by the ubiquitin proteasome; the addition of MG132 proteasome inhibitor to S2 cells co-expressing Repo and Gcm resulted in higher levels of Gcm than without the inhibitor present (Flici, et.al, 2014). Finally, Flici, et.al (2014) show that Drosophila Creb Binding Protein (dCBP) mediates the interaction between Gcm and Repo. dCBP is a histone acetyltransferase, and the human homolog is known to stabilize hGCM1 (Chang, et.al, 2005). Using both S2 cell culture and in vivo data, Flici et.al (2014) provide a model in which Gcm accumulates in newly forming glial cells and reaches a threshold level, stabilized by dCBP. After Gcm activates Repo, Repo contributes to the transcription of Gcm, but when Gcm levels become high, dCBP then acts with Repo to target Gcm for degradation. This physical interaction of Repo with dCBP is confirmed with immunoprecipitation assays (Flici, et.al, 2014). While this new research adds new answers to the story of Gcm and Repo regulation, one key question remains regarding the observed expression pattern for Repo.

As noted previously, Repo is detected during all stages of the Drosophila life cycle (Xiong, et.al, 1994; Campbell, et.al, 1994). Since Gcm expression is diminished by stage 15 embryos, the persistent expression of Repo cannot be due to Gcm. Lee and Jones (2005) provided the first piece of evidence that the sustained expression of repo may be the result of an autoregulatory mechanism. Using the UAS/GAL4 system, Repo was constitutively expressed
throughout the embryo by means of the *daughterless*-GAL4 driver, with the *repo4.3-lacZ* reporter construct in the background. These transgenic embryos showed a patch of β-gal staining in the epidermis of the embryos, which is not seen in wildtype embryos (Figure 3). This was the first indication that Repo can act on its own regulatory sequence.
Figure 3. Ectopic β-gal reporter expression observed with ectopic Repo expression in repo-4.3-lacZ embryos. A) The wildtype staining pattern of transgenic repo-4.3-lacZ embryos, showing β-gal staining in all lateral glia. B) β-gal staining of an embryo that has Repo expressed ectopically with repo-4.3-lacZ in the background. Here, staining is observed in the epidermis of the embryo. Embryos are dissected, stage 17, anterior left, posterior right. Scale bar is 20 μm. From Lee and Jones (2005) and used with permission.
The mechanism of autoregulation is a reasonable hypothesis for the sustained expression of *repo* observed by Lee and Jones (2005). Previous studies have established that Gcm can regulate its own expression through the same mechanism (Miller, et al, 1998; De Iaco, et al, 1998), and others have suggested that Repo can autoregulate (Flici, et al, 2014). In the report that follows, I provide additional experimental evidence to support this hypothesis.
CHAPTER II

EXPERIMENTAL PROCEDURES

2.1. Construction of additional *repo-lacZ* and *-luciferase* reporter genes

I subcloned *repo*-4.3/-2.3ARBS1 as a Sal/Xho fragment into the BamHI/Xho sites of *pCasPeR-hs43-LacZ*. I used the patchΔ136-*luciferase* plasmid (Chen, et.al, 1999), a kind gift from Dr. Stacey Odgen (St. Jude Children’s Research Hospital) to clone selected fragments of the *repo* CRD for expression in S2 cells, creating *repo-luciferase* constructs. Fragments were cloned into the KpnI/HindIII sites of the MCR. For consistency, I also cloned the Xho/Hind III fragment of the *repo* CRD, containing wildtype RBSs 2 and 3 into this vector.

2.2. Mutation of Repo binding sites

I used the Quickchange II Site Directed Mutagenesis kit (Agilent Technologies) to mutate all three RBSs from 5’CAATTA3’ to 5’CCCGTA3’. The following oligonucleotides (reverse not shown) were used for each of the three mutations; underlined base pairs represent the mutations from the wildtype:

RBS1

5’ATGCGGGATTTAAATTGATCTTAACGAAGCTTACGGGGTCGATCTGTATG
TG3’;
RBS2
5’CCTTGAAGCCAGACCCACATACGGGGCACATTGGCTAATGCAAAATAC3’;
RBS3
5’GGAATTCCTCGGCTAGAAGTTACGGGTTCGTCCAACATGTGTGACGATG3’.
Sequencing of clones confirmed successful mutagenesis.

2.3. Drosophila S2 Cell Culture Luciferase Assays

My protocols were based on those used in the laboratory of Dr. Stacey Odgen (St. Jude Children’s Research Hospital). Briefly, 350,000 cells were added to each well of a 24 well dish and grown overnight. Transfections were carried out using 5 μl of Lipofectamine (Invitrogen) with 100 ng each of the following plasmids: pacGal4 (or empty pac vector as a negative control), UAS-repo, the designated repo-luciferase construct, and pacRenilla (for normalization). Transfections were carried out in serum free media. Four hours after transfection, S2 complete media was added to the cells and growth continued for 48 hours. Cells were then assayed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions. For each repo-luciferase construct, assays were done in triplicate and each experiment was repeated three times for a total of nine replicates. Statistical tests include a paired one-tailed T-test to compare Repo-/Repo+, one way ANOVA analysis to compare repo-4.3-luciferase with repo-4.3-luciferase mutations, followed by Tukey’s post-hoc test to compare repo-4.3-luciferase mutated constructs with each other.

2.4. Drosophila S2 Cell Staining

I performed S2 cell staining according to the protocol published by the Rebay lab (http://web.wi.mit.edu/rebay/wi/protocols/cellculture/S2stainingIR.pdf, 2002) with only
minor changes. S2 cell transfections were carried out with the standard protocol (see above) and cells were resuspended in their media after 48 hours of incubation. 75 μl of the cell suspension was added to each well of a 12-well multitest slide (MP Biomedicals, Cat. No. 096041205) and allowed to incubate in a humid chamber for one hour. The cells were fixed for 15 minutes using the same 3.7% formaldehyde fix solution used to fix embryos. After washing the cells 3X with 2 ml of 1X PBS, 15 μl of 1:5 dilution of anti-Repo monoclonal antibody MAb 8D12 was added to each well and allowed to incubate for one hour at room temperature in a humid chamber. The washing step was repeated and 15 μl of 1:1000 dilution of anti-mouse Cy3 conjugate (Jackson, 115-165-146) was added and allowed to incubate for one hour under the previous conditions. Slides were washed a final time in PBS and mounted using VectaShield with DAPI (Vector Laboratories, Inc.).

2.5. Immunohistochemistry

Horseradish peroxidase (HRP) immunohistochemistry and embryo dissections were carried out as previously described (Patel, 1994). Rabbit anti-β-galactosidase (β-gal) antibodies were prepared at a 1:10,000 dilution (Cappel). HRP-conjugated secondary antibodies (Jackson Immunoresearch) were prepared at a 1:300 dilution. Secondary antibodies were detected via the HRP/diaminobenzidine (DAB) reaction. For consistency, the DAB reactions were stopped after 15 min.

2.6. Drosophila genetics

To observe the effect of ectopic Repo expression on repo-lacZ reporters, Repo was ectopically expressed in embryos by using the UAS/Gal4 system (Brand and Perrimon, 1993). repo-lacZ reporter flies were crossed into a UASrepo line, resulting in
heterozygotes. Heterozygous males were then crossed to virgin Act5CGal4 females, and embryos from this cross were stained for β-gal expression.
CHAPTER III

RESULTS

1. Introduction

In the first chapter I provided a detailed introduction to the Drosophila nervous system and reasons why Drosophila are a useful model organism for study of the nervous system. Briefly, I am interested in how reversed polarity (repo) expression is regulated after initial activation by the master regulatory transcription factor Gcm. Activation by Gcm does not fully explain the observed expression pattern of Repo. Gcm has a relatively short expression time in the newly forming glial cells, disappearing by stage 15 (Jones, et.al, 1995). Repo, once expressed, is maintained throughout the life of Drosophila, throughout all larval stages and adult stages. As Gcm is not continuously acting on the CRD of repo, another mechanism must be in place to sustain the expression of repo. Lee and Jones (2005) previously observed that ubiquitous expression of Repo in repo4.3-lacZ embryos produces ectopic expression of β-galactosidase in epidermal patches. This observation suggested that Repo can interact with its own cis-regulatory DNA (CRD). We hypothesize the mechanism of sustained repo expression is that of autoregulation, whereby Repo protein interacts with its own CRD in a manner of positive feedback to maintain its own expression. Once Gcm is produced in the newly developing glial cell, it will activate the expression of repo. Repo, also a transcription
factor, will act on other genes necessary for the completion of glial cell development, but will also act on its own CRD for maintained expression in the absence of Gcm. This model requires the presence of Repo binding sites (RBSs) within the repo CRD. I have identified three canonical RBSs in this sequence. Extending the observations of Lee and Jones (2005), I present data here that strongly suggest Repo is capable of interacting with at least one of these sites, and that mutation of this site decreases expression of reporter genes, providing strong experimental evidence for Repo autoregulation.

2. Results

2.1. Ectopic expression of Repo induces ectopic β-gal expression with specific repo-lacZ constructs

After identifying the 4.3 kb cis-regulatory DNA (CRD) of repo, Lee and Jones (2005) created eight reporter constructs containing different segments of the repo CRD coupled to the lacZ gene (collectively referred to as repo-lacZ constructs); in this promoter bash, different restriction enzymes were used to create the various constructs (Figure 4). To determine if Repo could interact with any part of its CRD, I used actinGal4 (actGal4) to drive ubiquitous expression of UASrepo during embryogenesis in individual embryos that carried each of these reporter constructs, as well as the full length repo-4.3-lacZ, creating what I call act-Repo embryos. I compared the β-galactosidase (β-gal) staining pattern in each of these act-Repo embryos to those of the repo-lacZ lines alone. These results are summarized in Figure 4. I observed ectopic β-gal expression in the epidermis of act-Repo embryos in which the Sal/Xho fragment of the repo CRD was present. Figure 5 shows the staining pattern of the full length construct (repo-4.3-lacZ) and the three additional constructs where ectopic expression was observed compared with
wildtype embryos. No ectopic β-gal expression was seen in the epidermis of embryos if the Sal/Xho fragment of the CRD was not present; these act-Repo embryos were identical to wildtype in the staining pattern (Lee and Jones, 2005; data not shown). Because of the strategy used to create act-Repo embryos in the repo-lacZ backgrounds (Figure 6), and given the fact the wildtype chromosomes are not marked, I can only infer a positive result when I see ectopic expression. That is, I make a logical inference that embryos showing ectopic expression contain all three transgenes, actGal4, UASrepo, and repo-lacZ, and that this combination results in a staining pattern that is different from repo-lacZ patterns in a wildtype background. Therefore, in populations of embryos in which no ectopic staining is observed, I again infer that some proportion of the embryos do contain all three transgenes, but this combination is not sufficient to promote any ectopic staining, and these embryos are indistinguishable from repo-lacZ wildtype embryos.
Figure 4. Four reporter constructs from the promoter bash of the repo cis-regulatory DNA (CRD) result in ectopic expression driven by actGal4/UASrepo. Eight transgenic fly lines were created using the lacZ reporter constructs shown here, and each line was crossed into an actGal4/UASrepo background. Ectopic expression was determined by the appearance of β-gal staining in the epidermis of the transheterozygotes. Only four constructs resulted in ectopic expression, and all contained the Sal I/Xho I fragment of the repo CRD. All other constructs resulted in staining patterns indistinguishable between wildtype and transheterozygotes.

Restriction sites indicated: Sa, Sal I; Sca, Sca I; X, Xho I; E, EcoR I; B, BamH I; S, Spe I. The orange ovals represent Gcm binding sites.
Figure 5. Ectopic expression of β-gal with specific repo-lacZ reporters in dissected stage 17 embryos (anterior up, posterior down). (A,B) repo-4.3-lacZ recapitulates the native Repo staining pattern in wildtype embryos, but ectopic patches appear in the epidermis of Act-repo embryos. (C,D) repo-4.3/-0.7 and (E,F) repo-4.3/1.9 also show epidermal ectopic patches in Act-repo embryos where none appear in wildtype. (G,H) repo-4.3/-2.3 shows patches of epidermal staining in wildtype embryos, but these patches are increased in the Act-repo embryos, extending over to the dorsal side of the embryo. Scale bar, 20 μm.
Proportion of embryos are \textit{repo-lacZ/UASrepo/Act5CGal4}

Figure 6. Diagram of crossing strategy used to create heterozygous embryos to study effect of ectopic Repo expression on \textit{repo-lacZ} reporters. Flies carrying the specific \textit{repo-lacZ} reporter were crossed to a \textit{UASrepo} line carrying this gene on the opposite chromosome. In this example, \textit{repo-4.3-lacZ} is carried on the third chromosome; therefore, males from this line are crossed to females that carry \textit{UASrepo} on the second chromosome. Heterozygous male progeny are then crossed to \textit{Act5cGal4} virgin females, which is balanced over \textit{Cyo} on the second chromosome. In the resulting offspring, only 1/8 of the embryos will have all genes necessary to test the effect of ectopic Repo expression on the \textit{repo-4.3-lacZ} reporter.
2.2. Repo CRD contains three canonical Repo binding sites

The canonical DNA binding site for Repo protein has been known for years; in 2003, Yuasa, et.al extended observations by Halter, et.al (1995) that Repo directly interacts with the specific 6 bp sequence 5’TCAATTA3’, and both groups show that the AAT region is the most critical for Repo binding. Recent literature on Repo interactions with other genes continues to cite this motif as the binding domain (Mandalaywala, et.al, 2008; Park, et.al, 2009; Flici, et.al, 2014). The repo 4.3 kb CRD region contains three CAATTA motifs, one located in the Sal/Xho fragment, and two located in the Xho/Spe fragment (Figure 7). I have designated these Repo binding sites (RBSs) 1-3.

I compared the sequence of these RBSs to eleven other Drosophila species using the UCSC Genome browser and found a high level of conservation among all three binding sites (Figure 3; http://genome.ucsc.edu; Kent, et.al, 2002). Complete conservation for RBS1 and RBS2 is seen for all 12 Drosophila species, and RBS3 is lacking in only 4 of the 12 species. Interestingly, all 3 RBSs are located close to a GBS, with RBS2 overlapping a GBS by a single base pair.
Figure 7. The repo CRD contains three highly conserved canonical Repo binding sites (RBSs). RBS1 is located within the Sal I/Sca I fragment and is completely conserved among the Drosophila species examined. RBSs 2 and 3 are located within the larger Xho I/ Spe I fragment. RBS2 shows complete conservation, while RBS3 is only conserved among eight species of Drosophila.
2.3. Luciferase assays in S2 cells show similar results with in vivo repo reporter constructs

My in vivo data showing ectopic β-gal expression suggested that Repo was capable of acting on its own regulatory sequence. To further test this possibility, I used S2 cells as a more direct measure of this interaction. I used the pacGal4 plasmid to constitutively drive Gal4 expression along with the UAS-repo plasmid for ectopic S2 Repo expression. Selected fragments of the repo CRD were fused to the firefly luciferase reporter gene (collectively referred to as repo-luciferase) and co-transfected with pacGal4 and UASrepo to determine if autoregulation occurred. To ensure the changes I observed in firefly luciferase expression were due to the induced expression of Repo, I first stained the S2 cells for endogenous Repo protein (Figure 8). The S2 cells did not show endogenous Repo staining with the antibody, but they did show staining with DAPI, an indication that the fixation protocol did not inhibit my ability to detect fluorescent staining and that the nucleus of the cells was intact. Only co-transfected pacGal4/UASrepo cells stained for Repo. As part of the dual-luciferase assay protocol (Promega), firefly luciferase expression was normalized against the renilla luciferase. Transfection with an empty pac vector served as a negative control.

My results indicate that ectopic expression of Repo protein in the S2 cells resulted in an increase in luciferase activity, and this increase was dependent on which repo-luciferase construct is present in the cells (Figure 9). repo-4.3-luciferase resulted in ~27 fold increase in luciferase. However, transfection of repo-4.3/-2.3-luciferase, the construct that contains only RBS1, resulted in ~50 fold increase. repo-2.3-luciferase provided an interesting result; this fragment of the CRD contains RBS2 and RBS3, but resulted in
only ~12 fold increase in luciferase expression, a decrease from both repo-4.3 and repo-4.3/-2.3. Compared to my in vivo Drosophila embryo results, similar variations in reporter protein expression were observed for the same constructs.
Figure 8. Expression of Repo in S2 cells. A) Transfected S2 cells under DIC appear normal in size and shape. B) The same field of cells under UV light showing the DAPI stained nuclei of each cell. C) The same field of cells showing cells expressing Repo protein. Repo is not expressed in S2 cells under normal conditions, and only cells that undergo a successful transfection with the $UAS_{repo}$ plasmid will produce Repo. Scale bar: 20 μm.
Figure 9. Repo in S2 cells can interact with repo-luciferase constructs. A) Construct diagrams showing selected fragments of the repo CRD that were fused to the luciferase reporter gene and used to transfect S2 cells. These repo fragments were chosen based on results from in vivo studies. B) repo-4.3/-2.3 gives the largest increase in relative luciferase activity and has only RBS1, compared to repo-4.3, which has all three RBSs, and repo-2.3, which has RBS2 and RBS3. Luciferase activity was normalized to renilla luciferase.
2.4. **Mutation of canonical RBSs affects reporter expression both in culture and in vivo**

I wanted to test if the canonical RBSs in the repo CRD were the sites of interaction with the induced Repo protein. I started with RBS1 as the repo -4.3/-2.3 gave the strongest increase in reporter expression both in culture and *in vivo*. I mutated RBS1 from the canonical sequence of 5′CAATTA3′ to 5′CCCGTA3′ using the Quikchange site directed mutagenesis kit (Agilent Technologies); using the same Sal/Xho fragment of the repo CRD, I used this new construct, called repo-4.3/-2.3ΔRBS1, in both the S2 cell culture and *in vivo* systems. The amount of luciferase expression in the S2 cells decreased dramatically, with only ~7 fold increase in expression as compared to no Repo protein present in the cells (Figure 10). Examination of act-Repo embryos in the repo-4.3/-2.3ΔRBS1 background found no ectopic expression (data not shown). To determine the effect of the RBS1 mutation in the full 4.3 kb CRD, I made repo-4.3ΔRBS1-luciferase. I also made two additional constructs, repo-4.3ΔRBS12-luciferase and repo-4.3ΔRBS123-luciferase to test the effect of sequential RBS mutations in repo 4.3 kb CRD sequence (Figure 10). These results surprisingly indicated only RBS1 was driving ectopic luciferase expression; RBS2 and RBS3 seem to play no role in Repo autoregulation, at least under these conditions.
Figure 10. Mutation of the canonical RBS1 site significantly decreases luciferase expression in culture. A) Construct diagrams of repo CRD luciferase constructs showing mutations generated to canonical RBSs, indicated by the black “X”. B) Mutation of RBS1 causes a significant decrease in luciferase expression in repo-4.3/-2.3, from ~50 fold to ~7 fold, indicating this is the site of Repo interaction. Mutation of RBS1 also causes a significant decrease in repo-4.3; however, mutation of RBS2 and RBS3 do not result in further decreases in luciferase activity. These results indicate that RBS2 and RBS3 do not interact with Repo. (p<0.01, ns, not significant)
3. Discussion

I present evidence here that clearly demonstrates Repo is capable of interacting with its own cis-regulatory DNA (CRD) and can control its own expression as measured by two different methods with reporter constructs. Levels of reporter gene expression are well correlated between the cell culture and in vivo systems with corresponding fragments of the repo CRD. This includes a decrease in reporter expression when a mutation was made in RBS1. While three canonical Repo binding sites (RBSs) are located within the 4.3 kb CRD of repo, only RBS1 appears critical for autoregulation as mutation of this site significantly diminishes reporter expression in the cell culture and in vivo systems. Despite high levels of conservation among all three canonical RBSs, I cannot find a role in autoregulation for RBS2 and RBS3 in the present study. These conclusions, along with future potential experiments, are discussed further in the following chapter of this manuscript.
CHAPTER IV
CONCLUSIONS AND FUTURE DIRECTIONS

The results of this study provide strong evidence that Repo protein interacts with its own cis-regulatory DNA (CRD), which suggests that autoregulation is a component to the overall regulation of repo. When one of the three canonical binding sites for Repo protein, called RBS1, was mutated, reporter protein expression under ectopic Repo expression was diminished. This reduction was correlated among two different experimental systems, indicating RBS1 is the site of interaction. While two other canonical sites exist within the known regulatory DNA for repo, this study found no role in autoregulation for these sites. Mutation of these sites did not have a significant effect on reporter expression in the experimental systems.

At the time of this writing, a caveat exists with the S2 cell culture data. The vector which contains the luciferase gene used in this study is pGL2-Basic and contains no known basal promoter such as SV40 or heat shock. Without this minimal element, comparisons between constructs are no longer direct as each specific fragment of the repo CRD may be responsible for different levels of promoter activity, resulting in different levels of luciferase activity in the assays. However, there is clearly promoter activity in the repo CRD fragments as luciferase expression is induced, and it is still interesting to observe the correlation between relative luciferase levels and ectopic β-gal expression in embryos for each reporter construct tested. Indeed, the lack of the minimal promoter in the luciferase vector was not noticed due in part to
the correlation in results obtained between the experimental systems. This oversight is now in the process of being corrected; the fragments of the repo CRD are being cloned into a new luciferase vector, pGL4.23, that contains a minimal promoter sequence, and the S2 assays will be repeated in the same manner as before.

My data presented here provide strong evidence that RBS1 is the necessary site of Repo interaction with its own CRD. Mutation of this site decreases the amount of reporter expression in both of our expression systems. However, my data also indicate that in addition to this canonical site, the additional sequence of DNA, previously described as the epidermal enhancer of repo, is required for Repo expression. RBS1 is located in the Sal/Sca fragment, within 23bp of GBS1; however, when the Sal/Sca fragment was tested for its ability to drive ectopic β-gal expression in embryos (repo-4.3/-2.8), no staining was observed. In fact, not only was there no ectopic expression, there is no glial cell staining; these embryos are completely clear (data not shown). This indicates that while the interaction of Repo with the canonical RBS1 is necessary, it is not sufficient to drive expression of the reporter. It is only when the epidermal enhancer sequence, the Sca/Xho fragment, is added that both glial cell staining and ectopic expression are observed in the embryos.

The epidermis as the site of ectopic expression in the embryos remains a puzzle. I have no clear answer as to why these cells express the β-gal protein, both in ectopic expression experiments with four of the repo-lacZ constructs and with repo -4.3/-2.3 expressed in a wildtype background. Furthermore, I observed variability in the expression of the epidermal patch among transgenic lines when repo-4.3/-2.3ΔRBS1 was expressed in a wildtype background. Some transgenic lines showed decreased expression of the epidermal patch while the patch completely disappeared in other lines. These results lead to the intriguing hypothesis
that another factor, possibility a homeodomain transcription factor, can interact with the RBS1 site when repo -4.3/-2.3 is expressed in embryos. This hypothesis also fits the observation of no epidermal expression when the construct repo -4.3/-1.9 is expressed in a wildtype background; this fragment of the CRD contains the sequence referred to as the “epidermal repressor”. If, in fact, another protein can bind to the fragment of the CRD between the Xho I and the BamH I sites, the epidermal repressor region, it may be sufficient to prevent another protein from interacting with the canonical RBS1 site and block epidermal expression.

The canonical RBSs exhibit strong conservation among Drosophila species, as shown by the alignment performed using the UCSC Genome Browser. Conservation of sequences often correlates to conservation of function; here this would suggest that all of the RBSs could interact with Repo protein. This is particularly true of RBS2, which is completely conserved among all the Drosophila species tested; however, my results showed no interaction of Repo with RBS2 or RBS3. This observation may be explained by previous results from my lab revealing the different individual cis-regulatory regions for the entire repo CRD. RBS2 lies within the 98 bp region that Johnson et.al (2012) defined as the minimal element necessary for epidermal repression. If a repressor protein is acting on this region, it may block Repo’s access to its binding site (mentioned above). Likewise, RBS3 lies within the 37 bp region that Johnson et.al (2012) defined as necessary for expression in cell body glia. Here, a protein acting as an activator may interact with this sequence and block Repo interaction.

The canonical RBSs may not be the only sites required for Repo interaction. Newly published findings by Crocker et.al (2015) show that clusters of “low affinity” binding sites were required for the Hox protein Ultrabithorax (Ubx) to properly regulate expression of the shavenbaby gene. These sites were found through electrophoretic mobility shift assays (EMSAs)
despite the fact that sequence analysis did not predict Ubx binding to the enhancer in the particular regions tested. Similarly, for my system, Repo contains a homeodomain and may interact with other DNA sequences that contain a similar motif to the canonical 5’CAATTA3’ sequence. I tested this hypothesis through the mutation of three similar sequences, 5’AATTA3’ located within the Sal I/Xho I fragment of the repo CRD. I chose to look for these sequences since previous work to define the canonical RBS never tested if the cytosine residue was critical. My results with this construct, both in embryos and in cell culture, find no significant differences from when this fragment is used with RBS1 mutated alone (data not shown). Therefore, to find other DNA sequences Repo may interact with, similar EMSA experiments as were conducted in Crocker et.al (2015) or similar DNA-protein experiments must be considered.

I believe that autoregulation is a component of the regulatory mechanism of repo, but I cannot rule out the role of chromatin in regulating expression. The entirety of gene regulation cannot be accounted for by the direct action of transcription factors, which makes the field of chromatin regulation an ever growing and complex area for research. I attempted to test the role of chromatin remodeling complexes on the repo CRD through use of two well-known complexes in Drosophila, Polycomb and Trithorax. The Polycomb proteins modify chromatin to repress gene expression, while the Trithorax proteins activate gene expression. Both complexes act through the modification of specific residues in the histones. I was curious to know if Polycomb proteins specifically were needed to repress repo expression in ectopic expression experiments. Using a mutant for PC1, a member of the PRC1 group complex, I made embryos that did not contain PC1 and ectopically expressed Repo in the background of the repo-lacZ constructs. Unfortunately, I did not observe de-repression in embryos, and further experiments will be needed to determine what role, if any, members of the Polycomb complexes have in repo
regulation (data not shown). For example, different members of the complexes could be tested in the same type of experiment.

This analysis focused on the use of reporter constructs to demonstrate that Repo protein interacts with its own CRD and specifically appears to do so at RBS1. This type of analysis does not allow the conclusion that Repo protein directly binds to the repo CRD at this location. An experiment showing the direct interaction of Repo protein and RBS1, such as a gel-shift assay or ChIP, would be needed to conclusively state that a physical interaction occurs. Another option for further testing the effect of an RBS1 mutation is the CRISPR/Cas9 system, a breakthrough technology for endogenous genome editing. CRISPRs (clustered regularly interspaced short palindromic repeat) are sequences that naturally occur in bacteria and play a role in defending the bacterial against invading viruses or other infectious nucleic acids (plasmids) (Gratz, et.al, 2013). This technique has been adapted to many other systems, including mouse, yeast and Drosophila to make endogenous DNA mutations, and Gratz, et.al (2013) show that changes in the Drosophila genome can be passed through the germline. Applying this technique to the questions addressed in this study may provide further evidence that mutation of RBS1 is sufficient to halt repo expression in lateral glial cells.

Glial cells are a vital component of the functioning nervous system in animals, but understanding their functions and development has been vastly outpaced by their neuronal cousins. With this study, I hope to contribute more to the conversation about the genetic controls that lead to the development of these cells and encourage others in our field to ask if this mechanism of autoregulation is important in developing glial genes in other model systems.
REFERENCES


VITA

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EDUCATION
2001-2004 M.S., Clemson University, Genetics
Thesis: Production of Transgenic Tobacco Expressing Novel Fibrous Proteins
Thesis Advisor: William R. Marcotte, Ph.D.

1997-2000 B.S., Clemson University, Biochemistry
Psychology Minor
Cum Laude

PROFESSIONAL EXPERIENCE

Academic Appointments
2008-2011 Adjunct Instructor of Biology, TriCounty Technical College, Pendleton SC

Research Experience
2010-2011 Research Technician, Clemson University Genomics Institute, Clemson SC
Large scale genomic sequencing; cacao and chestnut BAC clones sequenced for collaborators

2007-2010 Research Associate, Department of Biological Sciences, Clemson University, Clemson SC
Worked with Dr. Bradley Hersh to examine the role of Hox genes in wing development of Drosophila melanogaster
Worked with Dr. Susan Chapman to observe patterns of gene expression in the developing middle ear region of Gallus gallus
Trained and mentored undergraduate students

2005-2007 Research Associate, Department of Biology, University of Mississippi, Oxford MS
Worked with Dr. Bradley Jones to characterize the regulatory DNA of the repo gene in Drosophila melanogaster
Trained and mentored undergraduate students

2004-2005 Biological Sciences Technician, USDA-ARS-NPURU, Oxford MS
Used Affymetrix microarray to determine effects on gene expression in *Saccharomyces cerevisiae* when treated with different fungicides

**Teaching Experience**

01/2015-05/2015 Teaching Assistant for BISC 207 Anatomy & Physiology II Course, University of Mississippi  
Teaching 4 lab sections during the spring semester of 2015, including grading and lab prep; lecture professor Dr. Carol Britson

06/2014-07/2014 Teaching Assistant for BISC 330 Physiology Course, University of Mississippi  
Assisted lecture professor during one section and taught second lab section over two summer sessions of 2014; lecture professor Dr. Carol Britson

01/2014-05/2014 Teaching Assistant for BISC 336 Genetics Course, University of Mississippi  
Have been teaching 3 lab sections during the spring semester of 2014 and assisted course coordinator John Romanowski with student manual creation and test writing; lecture professor Dr. Ryan Garrick

01/2013-12/2013 Teaching Assistant for BISC 336 Genetics Course, University of Mississippi  
Taught 2 lab sections during the spring and fall semester of 2013; assisted course coordinators with lab prep, student manual creation and test writing; lecture professors Dr. Sarah Liljegren and Dr. Ryan Garrick

01/2012-12/2012 Teaching Assistant for BISC 336 Genetics Course, University of Mississippi  
Taught 2 lab sections during the spring and fall semester of 2012; assisted course coordinators with lab prep, student manual creation and test writing; lecture professors Dr. Sarah Liljegren and Dr. Ryan Garrick

08/2011-12/2011 Teaching Assistant for BISC 336 Genetics Course, University of Mississippi  
Taught 3 lab sections during the fall semester of 2011; lecture professor Dr. Sarah Liljegren

2008-2011 Adjunct Instructor of Biology, TriCounty Technical College  
Taught Biology 101 and Biology 102, introductory Biology courses; created exams, assignments, and revised lectures from standard course material to enhance flow of instruction

**MEMBERSHIPS**

Genetics Society of America (2013-present)  
Society for Developmental Biology (2014-present)

**PUBLICATIONS**

**Journal Publications**


**Conference Presentations (past 3 years)**

2015 56th Annual Drosophila Research Conference – Poster presentation
2014 Midwest Society for Developmental Biology Regional Meeting – Poster Presentation
2014 Southeast Society for Developmental Biology Regional Meeting – Poster Presentation
2014 UM Graduate Student Council Research Forum – Oral Presentation, Awarded Best Overall Oral Presentation
2014 Mississippi Academy of Sciences Meeting – Poster Presentation
2013 54th Annual Drosophila Research Conference – Poster presentation, panelist for the Undergraduate Plenary Session and Workshop
2012 UM Graduate Student Council Research Forum – Poster presentation

RESEARCH FUNDING

**Awarded**

UM Graduate School Dissertation Fellowship 08/15/14-12/15/14
Awarded to Ph.D. Candidates to provide financial assistance and relief of service responsibilities to allow completion of dissertation
Amount: $6500 with tuition waiver

UM Graduate Student Council Research Grant 08/05/13-08/04/14
“Methods to Identify Regions of Repo Autoregulation in the repo cis-regulatory element”
Amount: $1000

UM Graduate Student Council Research Grant 01/01/12-12/31/12
“Methods to Identify Regions of Repo Autoregulation in the repo cis-regulatory element”
Amount: $1000

HIGH SCHOOL & UNDERGRADUATE STUDENT RESEARCH MENTORING (past 3 years)

**Current:** Sarah Bugg, B.A. Psychology, University of Mississippi

**Previous:**
Mary Frances Dukes, B.S. Biology, University of Mississippi, 2013 (University of Mississippi Medical School)