Characterization of Cis-Regulatory Elements Controlling Repo Transcription in Drosophila Melanogaster

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CHARACTERIZATION OF CIS-REGULATORY ELEMENTS CONTROLLING REPO

TRANSCRIPTION IN 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

ROBERT W. JOHNSON

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ABSTRACT

The glial cells missing (gcm) gene has been identified as a “master regulator” of glial cell fate in the fruit fly Drosophila. However, the gcm gene is also expressed in and required for the development of larval macrophages and tendon cells, and lamina neurons in the adult CNS. Thus, the Gcm protein activates the transcription of different sets of genes in different developmental contexts. How the Gcm protein regulates these different outcomes is not known. My long-term goal is to identify proteins that collaborate with Gcm to promote the transcriptional activation of Gcm target genes specifically in glial cells, or prevent their activation in the other tissues in which Gcm is expressed. To address this, I have focused on the transcriptional regulation of a well-characterized glial-specific Gcm target gene, the transcription factor reversed polarity (repo). One of my aims is to understand how the transcription of the glial-specific Gcm target gene repo is regulated by Gcm and other factors. In 2005, Lee and Jones defined a 4.3 kb cis-regulatory DNA region that recapitulates the endogenous Repo expression pattern dependent on a single Gcm binding site. Within that region, are three different cis-regulatory elements that drive cell-specific expression independent of Gcm binding sites: 1) A distal element that promotes expression in dorsolateral epidermis; 2) A repressor element that suppresses expression in the epidermis; 3) A proximal element that promotes expression in a subset of cell body glia. Using lacZ reporter activity in transgenic lines I have further characterized these elements and defined minimal sequences required for expression or repression. Additionally, I have attempted to identify interacting factors using genetic, biochemical and bioinformatic approaches.
DEDICATION

This dissertation is dedicated to all those who have helped me get to where I am today. In particular, my wife, Holly Johnson, who has been extremely supportive, my brother, Wesley Johnson, who encouraged me to further my education, my parents, Darrell and Tojuana Johnson, who taught me how to work hard, and my advisor, Dr. Bradley Jones, for giving me the opportunity to succeed.
<table>
<thead>
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<th>Abbreviation</th>
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<tr>
<td>β-Gal</td>
<td>β-Galactosidase Protein</td>
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<tr>
<td>bp</td>
<td>Base Pairs</td>
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<tr>
<td>CBG</td>
<td>Cell Body Glia</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<td>EPI</td>
<td>Epidermal</td>
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<td>GBS</td>
<td>Gcm Binding Site</td>
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<td>gcm</td>
<td>glial cells missing Gene</td>
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<td>Gcm</td>
<td>glial cells missing Protein</td>
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<td>gof</td>
<td>gain-of-function</td>
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<tr>
<td>lacZ</td>
<td>reporter gene that produces β-Gal</td>
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<tr>
<td>LG</td>
<td>Longitudinal Glia</td>
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<tr>
<td>lof</td>
<td>loss-of-function</td>
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<tr>
<td>M-CBG</td>
<td>Medial Cell Body Glia</td>
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<tr>
<td>MM-CBG</td>
<td>Medial-Most Cell Body Glia</td>
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<tr>
<td>PNS</td>
<td>Peripheral Nervous System</td>
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<td>PG</td>
<td>Periperal Glia</td>
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<td>repo</td>
<td>reversed polarity Gene</td>
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<td>Repo</td>
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1. INTRODUCTION

The development of a functional nervous system requires the correct specification and precise organization of a large number of neural cell types. These cell types fall into two major categories: neurons; cells that transmit information, and glia; cells that maintain and support neurons. In the past, much of the effort spent studying the nervous system was directed toward neurons. Recently, their lesser-understood partners, glial cells, have been the recipients of increased interest and appreciation. Given the variety and importance of roles glial cells participate in, this is no surprise.

The roles that glial cells play in the Drosophila central nervous system (CNS) and peripheral nervous system (PNS) are varied, but are all directed toward neuronal preservation. These roles include, but are not limited to axon guidance, structural support, wrapping and insulation of neurons, establishment of the blood-brain/nerve barriers, nourishment, regulation of growth, ionic homeostasis, and engulfment of dying cells within the nervous system (Jones et al. 1995, Freeman et al. 2003). Disruption or injury of these glial functions can result in severe neurological consequences such as neural degeneration and paralysis.

Interestingly, glial cells have very similar roles in Drosophila and vertebrates. Comparisons can be drawn on developmental, morphological, and functional aspects. In both flies and vertebrates, glia have migration capabilities and interact with neurons in similar ways. For example, one subtype of Drosophila glia, cell body glia (CBG), has characteristics similar to vertebrate astrocytes; another subtype of glia, longitudinal glia (LG), ensheath CNS axons much like their vertebrate counterparts – oligodendrocytes. In the PNS, glial cells known as peripheral
glia (PG), ensheath nerves projecting from the CNS similar to Schwann cells in vertebrates. Additionally, there is an outer CNS sheath composed of several glial subtypes that is analogous to the blood-brain barrier of vertebrates (Freeman et al. 2003).


In *Drosophila*, neurons and glia are found in a stereotypical pattern repeated in each segment. Generally in the abdominal and thoracic CNS, roughly 30 glial cells and 350 neurons can be found per hemi-segment (either side of the midline). This results in a ladder-like appearance along the ventral anterior-posterior axis. The PNS adds to this appearance with what appears to be a single perpendicular line emanating from each CNS hemi-segment. These lines actually are not lines at all, but rather 8 to 10 peripheral glial cells ensheathing axons along the major nerve tracks. Both cell types are easily identified by a large array of markers, and by
The *Drosophila* nervous system is composed of three major categories of glia that can be divided into seven glial subtypes. First, subperineurial, channel, peripheral, and exit glia make up the “surface glia” category. These glial subtypes work together to ensheath the perimeter of the entire nervous system. Second, interface (or longitudinal), nerve root, and midline glial cells compose the “neuropile glia” category. These glial subtypes ensheath the nerve tracks, crossings, and roots. The third category, “cortex glia” is made up of only cell body glia. Residing in the cortex, this glial subtype is intimately associated with neuron bodies (Klämbt and Goodman, 1991, Ito et al. 1995).

With the exception of midline glia, all other glia, termed “lateral glia,” are derived from the neurogenic ectoderm located in the ventro-lateral region along the anterior-posterior axis of the developing embryo. In the early embryo, a given hemi-segment, within the neurogenic ectoderm, will give rise to 30 neural progenitor cells. Each of these progenitor cells is competent to generate either neurons or glia. Due to different combinations of temporally and spatially expressed proneural genes (e.g. *acheate-scute* complex) and neurogenic genes (e.g. *Notch*) each progenitor will become either a neuroblast (NB), giving rise only to neurons, a neuroglioblast (NGB), giving rise to both neurons and glia, or a glioblast (GB), giving rise only to glia (Bossing et al., 1996, Schmidt et al., 1997, Schmid et al., 1999).

In vertebrates, the mechanism by which glial fate is chosen over neuronal appears to be complex (Tohoku, 2004). However, the mechanism for glial cell fate specification appears to be much simpler in *Drosophila*; the adoption of one fate over the other is primarily due to the action
of a single gene called *glial cells missing (gcm)* (Hosoya et al., 1995, Jones et al., 1995, Vincent et al., 1996). The product of this gene, Gcm acts like a binary switch in that when it is present in a neural progenitor, that cell will differentiate into glia. Conversely, when Gcm is missing those same progenitor cells will differentiate into neurons.

The *gcm* gene encodes a novel 504 amino acid transcription factor that is expressed transiently in all lateral glia. Located at the N-terminus, the DNA binding domain of Gcm is structurally held together with two zinc atoms (Cohen et al., 2002). This creates a protruding β-sheet that has been shown to bind, with very high affinity, to a conserved octameric DNA consensus sequence (AT(G/A)CGGG(T/C) found numerous times within the regulatory regions of many glial specific genes (Akiyama et al., 1996, Schreiber et al., 1997, Granderath et al., 2000, Freeman et al. 2003). Taken with the fact that Gcm shares a highly conserved N-terminal DNA-binding-domain with one *Drosophila* homolog, *gcm2*, and two mammalian homologs, *Gcm1/GCMa* and *Gcm2/GCMb* (Akiyama et al., 1996, Kim et al., 1998), as well as the fact that its C-terminal domain is a potent transactivation domain, there is good evidence that Gcm is a master transcriptional activator (Jones, 2005).

Although we are interested in how Gcm regulates embryonic glial development, it should be noted that *gcm* has been shown to trigger the differentiation of macrophages (Bernardoni et al., 1997, Alfonso and Jones, 2002) and tendon cells within the epidermis (Soustelle et al. 2004). This demonstrates that the actions of *gcm* are context dependent. Furthermore, it shows there must be different cofactors working alongside Gcm to induce either glial, macrophage, or tendon cell differentiation (Alfonso and Jones, 2002). In order to identify the cofactors that function alongside *gcm*, we must first understand the transcriptional control of Gcm target genes that are found specifically in glial cells.
A growing number of genes have been identified as targets of Gcm. Central among them are *repo*, *pointed*, and *tramtrack*. All three are known to encode glial-specific transcription factors. First, *repo* is a homeodomain transcription factor that is expressed in all the lateral glia (Campbell et al., 1994, Xiong et al., 1994, Halter et al., 1995). Gcm first activates *repo*, but *gcm*’s expression is transient. This suggests that the maintenance of *repo* must be regulated by other factors, possibly by autoregulation of *repo* (Lee and Jones, 2005). Mutations in *repo* show up late in embryonic development, thus hinting at a role in terminal glial cell differentiation.

*Gcm* expression is also necessary to initiate the expression of the P1 form of the *pointed* (*pnt*) gene, which encodes an ETS domain transcription factor (Klaes et al., 1994), and the P69 form of the *tramtrack* (*ttk*) gene, which encodes a BTB-zinc-finger factor (Giesen et al., 1997).

*PointedP1* is implicated in several different roles of glial cell differentiation, and mutations in the gene manifest late in development much like *repo* mutants. *Ttk* performs a slightly different role than *repo* and *pointedP1* in that it acts to repress neuronal differentiation rather than promoting glial differentiation (Badenhorst, 2001). All together, a model can be assumed where *gcm* promotes glial cell differentiation by activating transcription of *repo* and *pointedP1* while repressing neuronal characteristics through activation of *ttk* (Jones, 2005).

In order to gain a better understanding of the mechanisms underlying glial differentiation I am focusing on transcriptional regulation of *repo*. To identify the unknown cofactors working in collaboration with *gcm* to either promote glial activity in some tissues, or repress it in others, *repo* gives me the best chance. There are several reasons for this conclusion. First, although hemocytes, tendons, and glia contain *gcm*-positive cells, it is only in the *gcm*-positive glia cells in which *repo* expression is observed. This implies there must be some additional factor(s) regulating *repo* only in glial cells. Second, the regulatory region of *repo* contains several Gcm
binding sites, which implicates repo cis-regulatory DNA as a direct target for Gcm. Third, since gcm expression is transient, and is followed by maintained expression of repo, there must be other factors responsible for repo’s maintenance (Jones, 2005). Alternatively, another model can be assumed where gcm turns on repo, and repo maintains its own expression through auto-regulation. Furthermore, to produce expression in glial cells, and not in tendons or hemocytes, there must be trans-acting factors that associate either directly or indirectly with Gcm. It is possible that each tissue expresses a unique combination of cofactors in addition to gcm in order to attain cell specific expression (e.g., gcm + 1,2 = glia, gcm + 1,3 = macrophage, gcm + 2,3 = tendon cell, etc.). Still, another possibility is that repo expression is repressed in both tendon cells and macrophages.

In 2005, Lee and Jones published results of a study in which they systematically dissected 4.2 kilobases (kb) of repo cis-regulatory DNA. By mutating Gcm binding sites (GBS) they showed that these sites were necessary for in vivo expression. Perhaps more importantly, they demonstrated that expression of repo was governed by multiple cis-regulatory elements.

In this study, I extend observations made by Lee and Jones (2005). Using lacZ reporter activity in transgenic embryos, I further characterize three distinct cis-regulatory DNA elements controlling the expression of repo: (1) epidermal enhancer (EPI enhancer), (2) epidermal repressor (EPI repressor), and (3) cell body glia enhancer (CBG enhancer). As well as demonstrating that these three elements are each necessary and sufficient to drive specific expression patterns, I further attempt to define the minimal functional sequences responsible for specific repo reporter activities by introducing small deletions and mutations into evolutionarily conserved sequences. Additionally, I test the functional conservation of two cis-regulatory elements in a closely related species of Drosophila. I also examine the influence of mutated
GBSs on several reporter constructs. Lastly, utilizing a yeast one-hybrid screen and a series of genetic crosses, I identify and test a candidate transcription factor for the ability to both positively and negatively alter repo reporter expression. My data supports earlier findings that repo is a direct target for regulatory factors besides Gcm.
2. RESULTS

The structure of the repo locus and summary of regions promoting specific transcriptional activity has been previously described and is illustrated in figure 1 (Lee and Jones, 2005). This study showed that the 476 base pair (bp) region spanning from restriction site ScaI to XhoI was necessary to promote repo reporter expression in epidermal cells. Concomitantly, it was shown that the adjacent 468 bp region spanning from XhoI to BamHI was necessary to repress repo reporter expression in epidermal cells. Finally, a 350 bp element, located between EcoRI and SpeI, was shown to promote repo reporter expression in a subset of cell body glia. That study did not attempt to further define each regulatory element. This prompted me to inquire whether these regions are not only necessary, but also sufficient to regulate repo transcription in the epidermis and cell body glia; and, if so, to determine the minimal functional sequence in each element. Lastly, does the presence of Gcm binding sites (GBSs) have an effect on expression of these elements?

Fig. 1. Summary. DNA map of the repo gene showing predicted repo transcript is represented by rectangles. Red shaded area represents repo coding regions. Arrow indicates direction of transcription. Orange Ovals represent Gcm binding sites. Three DNA regions necessary for specific expression activities are shown as bars below the map. Restriction enzyme sites: Sa, Sall; Sc, Scal; X, XhoI; E, EcoRI; B, BamHI; S, SpeI (adapted from Lee and Jones, 2005).
2.1 Epidermal Enhancer

The study of the EPI enhancer region originated with a former Jones Lab research associate, Jamie Wood, testing this region for the ability to drive repo-lacZ reporter expression. The 476 bp fragment located between restriction sites Scal and XhoI was subcloned into pCasper-hs43-LacZ to make reporter vector pBJ 100-LacZ (Fig. 2A). The construct was then introduced into Drosophila via P-element mediated transformation. Five separate transgenic lines were generated (a minimum of three lines were generated for each construct presented in this study).

Protein expression was then assayed in transgenic embryos using anti-β-gal antibodies. For pBJ 100-LacZ, all lines displayed β-gal in epidermal patches on the lateral body walls. An embryo from one of these lines is shown in Fig. 2B. β-gal is detected in lateral epidermal cell clusters (unpublished data).

In an effort to define the minimal DNA sufficient to drive expression, Jones and Wood generated a reporter construct, pBJ 111-LacZ, using the 116 bp fragment located between EcoRV and XhoI (Fig. 2A). Transgenic lines were created and then assayed for β-gal expression. No change in expression pattern was observed between embryos expressing the pBJ 100-LacZ reporter construct and the shorter pBJ 111-LacZ reporter construct (Fig. 2B). This evidence suggested that the minimal element responsible for driving repo reporter expression in the epidermis was confined to 116 bp (unpublished data).

Since the EPI enhancer had been reduced to a more manageable size, we generated an alignment of 6 Drosophila species using the UCSC Genome Browser (Kent et al. 2002) (Fig. 2C).
to identify conserved regions. It was observed that there was a high amount of conservation (conserved in > 4 species) in several areas (See Fig. 2C, gray shading). Using PCR site-directed mutagenesis, we targeted the proximal region, which contained the most highly conserved regions (conserved in all 6 species), for deletion. Four deletion reporter constructs were made. The first, \textit{pBJ125-LacZ}, removed a 7 bp sequence from position 106-112 (TTTTGAT) (Del A, Fig. 2A,C). The second, \textit{pBJ123-LacZ}, also removed a 7 bp sequence, slightly upstream, at position 98-104 (TAATTAA) (Del B, Fig. 2A,C). The third, \textit{pBJ174-LacZ}, removed a 13 bp
sequence, again slightly upstream, at position 81-93 (GGTTCGAGGATCA) (Del C, Fig. 2A,C).

The fourth, pBJ175-LacZ, removed a 36 bp sequence, that encompassed the first three deletions, at position 81-116 (Del D, Fig. 2A,C). Embryos carrying any of these constructs show unchanged β-gal expression in lateral epidermal patches (Fig. 2B). The result of the fourth deletion (Del D), which overlaps the three previous deletions, shows that the remaining upstream 80 bp is sufficient to drive repo reporter expression and indicates the functional element must be located in the distal portion of the element (see construct pBJ 175-LacZ, figure 2A).

2.2 EPI Repressor

As with the EPI enhancer region, the EPI repressor region was initially tested for the ability to inhibit repo reporter expression (Woods and Jones, unpublished data). The 468 bp fragment located between restriction sites XhoI to BamHI and the adjacent 476 bp enhancer region were subcloned into pCasper-hs43-LacZ to make reporter vector pBJ 103-LacZ (Fig. 3A). Embryos carrying this construct express β-gal in lateral glial cells, but fail to express β-gal in the epidermis (Fig. 3B). It was concluded that the 468 bp region from XhoI to BamHI is sufficient to inhibit repo reporter expression in the epidermis.

Due to the presence of several unique restriction sites, it was then decided to systematically dissect the 468 bp region. In all, 7 progressively shortened reporter constructs (pBJ 103-109) were generated and transgenic lines assayed (Fig. 3A). Embryos carrying the pBJ 107-LacZ construct show weak β-gal expression in lateral glia (Fig. 3C). By contrast, embryos
Fig. 3. A 98 bp region represses repo-lacZ reporter expression in the epidermis, as well as promotes expression in longitudinal and peripheral glia. (A) Summary of epidermal repressor repo-lacZ reporter constructs and their expression. Restriction sites are indicated: Sc, Scal; EV, EcoRV; X, XhoI; T, Thh111I; Bst, BstBI; P, PpuMI; N, NruI; Bfu, BfuAI; E, EcoRI; B, BamHI. (B–D) Dissected stage 16 embryos labeled with anti-β-Gal antibody (anterior left, dorsal up). (B) pBJ 103-lacZ drives strong repo reporter expression glial cells, but not in the epidermis. (C) pBJ 107-lacZ inhibits reporter expression in the epidermis, but promotes weak glial expression. (D) pBJ 109-lacZ drives reporter expression in the epidermis, but lacks expression in glia. (E) Drosophila species alignment of 98 bp repressor region. Gcm binding site is indicated by yellow shading. Deletions are represented by green shading and Del A-F.
carrying pBJ 109-LacZ, which is shorter than pBJ 107-LacZ by 98bp, show β-gal expression in specific patches within the epidermis, but fail to show expression in CNS glia (Fig. 3D). This data suggests that the 98 bp region, from restriction site BstBI to PpuMI is required for inhibiting epidermal expression (Woods and Jones, unpublished data).

In an attempt to further characterize the 98 bp element, I decided to generate an alignment of 6 Drosophila species using the UCSC genome browser. Upon examination, it was clear that there is a high amount of conservation where the GBS was located, with slightly less conservation observed throughout the element (Fig. 3F, gray shading). Using site-directed mutagenesis, I introduced a series of small deletions into the 98 bp region. This was an attempt to restore EPI reporter expression by eliminating the DNA binding sequence responsible for EPI reporter inhibition. Six deletion reporter constructs were made. The first, pBJ 132-LacZ, removed a 7 bp sequence from position 45-51 (AATTGGC) (Del A, Fig. 3A,F). The second, pBJ 133-LacZ, removed a 7 bp sequence from position 64-70 (GCAAAAT) (Del B, Fig. 3A,F). The third, pBJ 137-LacZ, removed a 7 bp sequence from position 76-82 (CTGATTA) (Del C, Fig. 3A,F). The fourth, pBJ 138-LacZ, removed a 7 bp sequence from position 87-93 (CACGCAA) (Del D, Fig. 3A,F). The fifth, pBJ 153-LacZ, removed a 7 bp sequence from position 20-26 (GCAATCC) (Del E, Fig. 3A,F). The sixth, pBJ 154-LacZ, removed a 7 bp sequence from position 3-9 (AATCCTC) (Del F, Fig. 3A,F).

Embryos carrying any of these reporter constructs fail to express β-gal protein in the epidermis, but do exhibit weak lateral gial staining (Fig. 3C). These results indicate that the region responsible for repression was missed by my deletions and/or hints at the possibility of redundant repressor binding sites.
2.3 Gcm binding sites

We next wanted to examine the influence of both the presence and absence of GBSs on reporter activity in both glial and epidermal cells. Since there are no GBSs in the CBG enhancer element, we focused on the EPI enhancer and repressor region.

Within the 98 bp region is one Gcm binding site (GBS). To determine whether the absence of this GBS affects the ability of this region to repress epidermal reporter expression, a reporter construct was created, \textit{pBJ 117-lacZ}, that contained a mutated GBS (Fig. 4A) (Wood and Jones, unpublished data), in which 4 out of 8 nucleotides of the Gcm binding site had been altered (Lee and Jones, 2005). Mutating the GBS had no effect on the epidermal reporter expression pattern, but did abolish glial expression in the CNS (data not shown). This suggests the repression by the \textit{pBJ 117-lacZ} to be GBS independent. Furthermore, Wood introduced previously mutated GBSs, upstream and downstream, in the \textit{pBJ 110-LacZ} and \textit{pBJ 112-LacZ} constructs, which also had similar effects (Fig. 4A, data not shown) (unpublished data).

To test the effect of the presence of the single GBS located within the 98 bp region, I generated and compared constructs \textit{pBJ 145-lacZ} and \textit{pBJ 146-lacZ} (Fig. 4A). Embryos carrying one copy of the 98 bp region, \textit{pBJ 145-lacZ}, exhibit weak \(\beta\)-gal expression in glial cells (Fig. 3D). Embryos carrying two copies of the 98 bp region, \textit{pBJ 146-lacZ}, exhibit increased expression of \(\beta\)-gal, but do not show ectopic activity (Fig. 4C). This data demonstrates that all the information required to drive cell specific expression in glial subsets, can be derived from a 98 bp fragment containing a single GBS.
2.4 EPI regions from D. pseudoobscura share function with EPI regions from D. melanogaster.

The data I have presented so far show that the EPI enhancer and repressor elements are conserved among 6 species of Drosophila. Furthermore, I have demonstrated these two elements have the ability to function independently in melanogaster. Lastly, the functions of both the EPI enhancer and repressor are independent of the presence of GBSs. I was curious to see if, in addition to sequence, the functions of the transcriptional regulatory regions were also conserved in a closely related species of Drosophila, D. psuedoobscura.
To test conservation of the EPI enhancer’s function to drive repo reporter expression in closely related species, I used PCR to generate a 135 bp fragment from D. psuedoobscura genomic DNA that corresponded to the EPI enhancer region in D. melanogaster. D. psuedoobscura was chosen because it was the closest related species outside of the melanogaster group. To test conservation of the EPI repressor’s function to inhibit repo reporter expression in the same closely related species, I also generated a 289 bp fragment corresponding to both the EPI enhancer and repressor region in D. melanogaster from D. psuedoobscura genomic DNA using PCR (see experimental procedures). These fragments were then subcloned into pCasper-hs43-LacZ to make reporter vectors pBJ 134-LacZ and pBJ 135-LacZ, respectively (Fig. 4B). Transgenic lines were then created and assayed for protein expression. Embryos carrying pBJ 134-LacZ expressed β-gal in lateral epidermal patches in a pattern identical to the pattern expressed by pBJ 111-LacZ (Fig. 2A,B), but interestingly, weak peripheral glial staining is observed (Fig. 4D). By contrast, embryos carrying pBJ 135-LacZ do not express β-gal in the epidermis (Fig. 4E). Weak glial staining persists, but is expected due to the presence of a known GBS (Orange Oval, Fig. 4B). I conclude that the EPI enhancer and EPI repressor are shared in sequence and function between Drosophila melanogaster and psuedoobscura.

2.5 CBG Enhancer

The cell body glia (CBG) regulatory element was previously localized to a 350 bp region within a 1.1 kb fragment that induces repo expression in peripheral glia (PG), subperineurial glia (SPG), and CBG, but not longitudinal glia (LG) (fig. 1). The 1.1 kb region was found to also contain a GBS located outside the 350 bp CBG element. Mutation of this site removes
expression in the PG and SPG, but only weakens expression in the CBG. This led to the conclusion that factors in addition to Gcm promote CBG expression and that these unknown factors combine synergistically with Gcm to drive expression in the PG and SPG, and cause increased expression in CBG (Lee and Jones, 2005).

Based on these earlier findings, researchers we were curious as to whether this 350 bp region was not only necessary, but also sufficient to drive CBG expression. In order to test this, a 328 bp fragment corresponding to the CBG element found in repo -4.3 was subcloned into pCasper-hs43-LacZ to make reporter vector pBJ 101-LacZ (Fig. 5A). Transgenic lines were then produced and assayed for β-gal.

Embryos carrying pBJ 101-LacZ displayed β-gal within the abdominal and thoracic CBG cells in a very weak pattern, but not in the PG or SPG. An embryo from one of these lines is shown in Fig. 5B. β-gal is detected in a subset of lateral glial cells known as medial CBG (M-CBG) and medial most CBG (MM-CBG). Due to the weak and incomplete staining observed, it was premature to conclude that the CBG element is necessary and sufficient to drive repo reporter expression (Wood and Jones, unpublished data).

To further test this idea and to see if reporter expression would increase synergistically, I then decided to make a construct that contained tandem copies of the 328 bp fragment used to make pBJ 101-LacZ. The construction of tandem copies yielded a 668 bp fragment that was subcloned into pCasper-hs43-LacZ to make reporter vector pbj 118-LacZ (Fig. 5A).

Again embryos were assayed for β-gal protein expression. I observed a very robust expression pattern of β-gal in both the abdominal and thoracic CBG cells (Fig. 5C). High levels
Fig. 5. A 37 bp is necessary and sufficient to drive repo reporter expression in M-CBG and MM-CBGs. (A) Summary of cell body glia enhancer repo-lacZ reporter constructs and their expression. 5X represents five tandem copies. Restriction enzymes are indicated: E, EcoRI; N, NdeI; S, SpeI. CBG represents cell body glia. (B-C) Dissected stage 16 embryos labeled with anti-β-Gal antibody (anterior left, dorsal up). pBJ 101-lacZ drives weak reporter expression in M-CBG and MM-CBGs. (C) Two tandem copies of the same region, pBJ 118-lacZ, drives increased expression in M-CBG and MM-CBGs. (D) Whole mount stage 16 embryo. Five tandem copies of 37 bp region drives repo reporter expression in M-CBG and MM-CBGs. (E) Alignment of 187 bp CBG enhancer region. Point mutations are represented with red lettering with substituted bases indicated (above also red).
of β-gal are detected in the M-CBG and MM-CBG. I conclude that the 328 bp region is both necessary and sufficient to drive repo expression in a subset of CBG cells and that tandem copies act synergistically to increase reporter expression.

2.5 37bp region sufficient to drive CBG expression

The ability of the 328 bp region to drive CBG expression prompted me to pursue the minimal element required for CBG expression. In order to define this, I first made constructs that reduced the overall size of the 328 bp element by half. These two constructs, pBJ 143-LacZ and pBJ144-LacZ, were composed of tandem copies of the left half (187 bp x 2) and the right half (141 bp x 2) of the original element, respectively (Fig. 5A). Embryos carrying the pBJ 143-LacZ reporter construct show strong β-gal expression in the M-CBG and MM-CBG (Fig. 5C). Embryos carrying the pBJ 144-LacZ completely lack CBG expression (data not shown). Based on these findings, I concluded that the minimal element necessary to recapitulate the CBG expression pattern is localized to the distal 187 bp of the 328 bp region.

Next, I generated an alignment of the (now 187 bp) CBG region using the UCSC genome browser alignment tool. An alignment of 6 species of Drosophila revealed a highly conserved region at the distal end of the fragment (Fig. 5E). Lower conservation was observed in the remaining 150 nt. Based on this evidence I deleted/mutated this region within the 187 bp. Using PCR site-directed mutagenesis, I introduced a deletion that removed 37 bp of the conserved region. Using tandem copies of the region containing the deletion, I made reporter construct pBJ 158-LacZ (Fig. 5A). Additionally, via PCR site-directed mutagenesis, I introduced nine point mutations in the middle of the highest conserved sequences (shared by all species in alignment)
within the 37 bp (Fig. 5A,E). Using tandem copies of the region containing the point mutations, I made reporter construct \textit{pBJ 163-LacZ} (Fig. 5A).

Embryos carrying either of the two reporters, \textit{pBJ 158-LacZ} and \textit{pBJ 163-LacZ}, completely lack β-gal expression in glia (data not shown). This data suggested that the 37 bp region, or a component within, is necessary to produce \textit{repo} reporter expression in CBG cells. I was curious to see if this small region, 37 bp in length, would be sufficient to drive CBG expression. Due to lack of internal restriction sites, I could not clone tandem repeats, so I had to generate a custom repeating oligonucleotide. I decided to generate an oligo composed of five tandem copies of the 37 bp region. I chose five copies because it would give a reliable expression pattern and the oligo would also be used in another experiment where several copies were needed. This fragment was then used to make a new reporter construct, \textit{pBJ 164-LacZ} (Fig. 5A). Embryos from lines carrying this construct were assayed for β-gal protein expression. Each displayed β-gal expression in the M and MM-CBG cells. Interestingly, additional glial staining was also observed in the longitudinal glial cells, suggesting some CBG specific information had been lost. An embryo from one of these lines is shown in Fig. 5D. Based on these observations, I conclude that the 37 bp region is necessary and sufficient to drive \textit{repo} expression in CBG.

2.7 Yeast One-Hybrid Screen

To this point, I have presented data on my attempts to better define the minimal \textit{cis-}
regulatory elements that can drive or repress the expression of \textit{repo} reporter constructs. The
ulterior motive in doing so was to produce manageable sized fragments that could be utilized in a protein-DNA interaction assay, such as the yeast one-hybrid assay.

In an attempt to identify candidate proteins that may interact in vivo with repo cis-regulatory regions, I set up a yeast one-hybrid assay for both the CBG enhancer and EPI repressor (the EPI enhancer was not yet well defined at this point). For bait, we used the 37x5 CBG fragment (Fig. 5A, pBJ 164-LacZ), and the 98x2 EPI repressor fragment (Fig. 4A, pBJ 146-LacZ). The EPI repressor screen yielded 273 clones, while the CBG enhancer screen yielded 357 clones. The 25 fastest growing clones from each screen were sequenced and subjected to a BLAST search. Results for the EPI repressor screen were inconclusive as none of the cDNAs found to be interacting with our bait were from transcription factors. Conversely, the CBG enhancer screen pulled out the transcription factor Dichaete (D) four independent times.

Dichaete is a transcription factor that contains a SOX DNA binding domain and is expressed in the Drosophila nervous system during gliogenesis (Nambu and Nambu, 1996; Russell et al., 1996). Taken together, this warranted further investigation of the potential Dichaete-repo interaction.

To see if the absence of Dichaete would have an adverse effect on repo reporter expression, I subjected embryos to a Dichaete loss-of-function (lof) test in the presence of the CBG reporter construct. In order to test this, I crossed a fly line containing a Dichaete loss-of-function mutation into a fly line containing the CBG P-element reporter construct pBJ 143-LacZ (Crosses shown in Fig. 6A, refer Fig. 5A for P-element construct). As a result, embryos expressing the deficiency had severely disrupted CNS development (Fig. 6B, compare to Fig. 5C). Many of the abdominal M-CBG and MM-CBG are either missing or out of place, however M-CBG and MM-CBGs retain their normal expression pattern in some segments (Fig 6B,
arrows). Based on these results, it was unclear whether or not *Dichaete* was directly regulating the CBG element.

Next, I wanted to see if *Dichaete* could alter reporter expression in a *gain-of-function* (*gof*) experiment. In order to over-express *Dichaete* in the presence of our CBG reporter construct I utilized the UAS-Gal4 method (Brand and Perrimon, 1993). Figure 7 shows the genetic crosses used to create my UAS-*Dichaete*;143 line. Ultimately, two lines were generated; both lines contained UAS-D;143, but one used a Gal4 driver that is expressed ubiquitously, *daughterless*-Gal4 (da-Gal4) (Wozard et al., 1995), and the other used a Gal4 driver that is expressed solely in neural progenitor cells, *scabrous*-Gal4 (sca-GAL4) (Klaes et al., 1994). Embryos carrying UAS-D and either of the two Gal4 drivers did not display any ectopic expression of reporter constructs, nor did they display any repression of expression, but rather display the normal CBG enhancer pattern seen in Fig. 5C.
Fig. 7. Diagram of genetic crosses. Schematic of six crosses used to create a fly line homozygous for UAS-Dichaete (UAS-D), 2nd chromosome, and p-element 143A, 3rd chromosome. Markers used: w = white; Sco = Scutoid; Cyo = Curly-o; + = wild type; Sb = Stubble. Balancers used: CxD & TM3,Sb. y = male chromosome.
3. DISCUSSION

In this dissertation, I present a description of three different cis-regulatory regions from the DNA regulatory region of repo. I show that all three repo regulatory regions are necessary and sufficient to confer specific activities on reporter genes in subsets of glia and the epidermis. Furthermore, I defined minimal cis-regulatory fragments sufficient to drive repo reporter expression (Fig. 8). Mutation of GBSs only has a minor effect on the level of expression created by each element, but not on the pattern itself. I also demonstrate that sequence and functionality of two elements are conserved across closely related species of Drosophila. Moreover, I have identified the CBG region that may be responsible for interacting with trans-acting factors. A yeast one-hybrid assay using the CBG region produced candidate transcription factor Dichaete, however, lof and gof studies were inconclusive in determining if this interaction occurs in vivo.

![Diagram showing regulatory elements controlling repo expression](image-url)
3.1 Epi Enhancer

In this study I characterized the functional epidermal enhancer down to 80 bp. I show this region to be necessary and sufficient to drive repo reporter expression in dorso-lateral epidermal cells. I also demonstrate that expression is not dependent on Gcm. Furthermore, corresponding regions in Drosophila psuedoobscura retain sequence similarity and function, thereby hinting at the evolutionary importance of this element. Repeated attempts were made, with varying primers, to delete the remaining 80 bp of this region. Unfortunately, I was unable to generate mutated sequences. A remedy for this problem could be to design and synthesize custom oligos that manipulate the region in various ways such as overlapping deletions.

3.2 EPI Repressor

The epidermal repressor provides a glimpse of the complexity and sophistication of gene repression. I show here that 98 bp is both necessary and sufficient to inhibit repo reporter expression. Like the epidermal enhancer, the repressor functions independently of Gcm and is conserved in pseudoobscura. Interestingly, a series of systematic deletions failed to restore epidermal expression, and thus, the identity of the specific DNA sequence necessary for epidermal repression. I attempt to explain this by one of the following four possibilities. First, it is possible I missed the key binding nucleotides because my deletions were not overlapping. Second, this could be a case of redundant repression sites, i.e. multiple sites within my 98 bp fragment could independently be sufficient to inhibit repo reporter expression. This is supported by the expression pattern of construct pBJ 124-LacZ (fig 3A). Although, the 98 bp region has
been deleted, *repo* reporter expression is still not restored. This data suggests that we have identified another viable repressor element. This is not surprising, however, considering mis-expression of a transcription factor causes lethality (Lee and Jones, 2005). Third, I raise the possibility of chromatin-influenced repression. This mechanism has recently been demonstrated between a master regulator protein (like Gcm) and a target gene (like *repo*), where various target genes of the master regulator of intestine development, homeodomain protein CDX2, are regulated via chromatin modifications initiated by CDX2 (Verzi et al. 2010). Finally, it is possible the repressive effect seen on the EPI enhancer is due to the proximity of downstream DNA rather than the specific action of a protein. This scenario could explain why repression was still seen even after introducing an internal deletion (*pBJ 124-LacZ*, Fig. 3A) that removes the 98bp fragment. Further investigation will be necessary to determine the exact mechanism responsible for EPI repression.

3.3 CBG Enhancer

The CBG element that drives *repo* reporter expression in specific subsets of cell body glia, M-CBGs and MM-CBGs, was the most characterized element of this study. Here I have provided direct evidence that a 37bp sequence is sufficient to drive reporter expression in CBGs as well as some other glial subsets. This data suggests I have identified a binding region for a trans-acting factor(s) that is concomitantly expressed in other glial types. I have yet to identify a protein responsible for this expression pattern, but based on sequence and DNA binding motif analysis (data not shown) I believe it could be a homeodomain containing protein.
Homeodomains commonly bind to the core sequence ‘ATTA’ (Florence et al., 1991), which has been shown to be critical for homeodomain binding (Odenwald et al., 1989). Repo, a homeodomain containing protein, has been demonstrated to bind to a CAATTA motif in glial cells (Yuasa et al., 1993). Within the minimal CBG element is the sequence ‘CAATTAAC’. This compelling evidence supports the theory that repo could be autoregulating itself. Still, the possibility remains that a separate homedomain protein is interacting with this sequence.

3.4 Yeast One-Hybrid

My screen for proteins that interact with repo cis-regulatory DNA yielded one promising candidate, Dichaete. Due to the convincing nature of its molecular activity, as well as temporal and spatial expression, I attempted to further characterize it. After lof and gof experiments we can draw some minor conclusions. First, the presence of Dichaete is vital to the correct formation and organization of a fully functional nervous system. Second, Dichaete definitely interacts with the repo CBG cis-regulatory DNA in a heterologous in vivo system, as evidenced by the yeast screen. However, due to lack of any noticeable alteration of expression in our gof experiment, I am not compelled to say that this interaction truly occurs in vivo.

One explanation for my results is the possibility that the protein that actually targets and binds repo cis-regulatory DNA contains a conserved Sox domain like Dichaete. Perhaps I pulled out Dichaete by chance due to a common binding affinity for the bait sequence. One experiment to test this theory would be to cross the CBG P-element into fly lines containing gene deficiencies for all known Sox domain containing proteins and screen for deficiencies that disrupt the repo reporter expression pattern. Candidate proteins from that screen could then be
subjected to \textit{gof} studies. Any protein that passes those two tests could then undergo more rigorous tests such as \textit{in situ} hybridization, gel-shifts, chromatin immunoprecipitation, etc. in order to confirm direct binding.

Another explanation is that the yeast one-hybrid screen is limited in its scope. This type of screen can only pull out a single protein at a time, whereas many transcription factors are engaged in combinatorial regulation via heterodimerization (Alberts et al., 2002). A way around this would be to utilize affinity chromatography, where many copies of an oligonucleotide are chemically connected to a matrix to be used as bait for DNA binding proteins. This technique would provide the opportunity to assay proteins in their native conformations (homo, hetero, etc), thus allowing for the capture of a heterodimerized protein.

\section*{3.5 Conclusion}

This study represents a step toward a thorough understanding of the neural mechanisms underlying glial differentiation. Understanding \textit{repo} regulation by Gcm and other factors will contribute to understanding how context specific regulation of different developmental pathways is under combinatorial control of multiple transcription factors. Based on our current knowledge, we believe that additional glial specific transcription factors reinforce and maintain glial specific expression via cross-regulation after activation by Gcm, which acts as an initiator, not a maintainer, as evidenced by its transient nature (Jones, 2005).

Epidermal expression of \textit{repo} is of interest because we have identified a \textit{cis}-regulatory element that drives reporter expression in a tissue type that \textit{repo} is not normally expressed in. It is possible that the factor(s) acting on the \textit{repo} DNA in the epidermis is also present in the
nervous system. It could be a single factor directing this expression, or it could be a combination of positive and negative inputs. Due to the unique nature of this element, identification of a factor regulating the EPI enhancer could provide valuable insight into the network of regulatory inputs that direct cell specific expression in Drosophila.

Repression in Drosophila is a difficult circumstance to study due to the fact that a positive input is required to test against. The epidermal repressor in conjunction with the epidermal enhancer provides us with a fortuitous opportunity for understanding such mechanisms. Characterization of this element will not only provide important knowledge concerning the regulation of repo transcription, but can also shed light on similar mechanisms found elsewhere in Drosophila.

The CBG enhancer offers an excellent opportunity to identify glial specific regulators. Initially, the CBG element only directed expression in a subset of cell body glia. However, when a 37 bp multimer was introduced into fly lines, reporter expression was also seen in other lateral glial cell types. This supports a scenario where there is a common set of developmental transcription factors, and due to different combinatorial inputs, different cell types are specified. Characterizing this particular element is of great interest for both understanding how repo expression is maintained and how glial subtypes are specified.

Finally, the EPI repressor fragment is of additional interest beyond its ability to inhibit reporter expression in the epidermis. This 98 bp fragment contains a single conserved Gcm binding site that is sufficient to drive reporter expression in lateral glia. This is significant because if any factors are working alongside Gcm to drive this pattern, then they must be acting within this 98 bp piece. This new ability to focus efforts provides an excellent opportunity to dissect mechanisms of glial specification in Drosophila.
4. EXPERIMENTAL PROCEDURES

4.1 DNA Alignments

The *Drosophila* species used to align *repo cis*-regulatory regions were *D. melanogaster*, *D. simulans*, *D. sechellia*, *D. yakuba*, *D. erecta*, and *D. pseudoobscura*. The *Drosophila* species alignments were generated using the UCSC Genome Browser (Kent et al., 2002).

4.2 PCR Generation of Fragments and Verification

Site-directed mutagenesis and deletion was performed using the Quick Change Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Sequences were chosen for deletion or mutation by analyzing DNA alignments, and locating the highest conserved regions for each *cis*-regulatory element. EPI enhancer fragments were deleted using the following oligonucleotides as forward primers and their complements (not shown) as reverse primers: For Del. A, we used forward primer CGAGGATCACGAGTAATTAACCTTACTCGAGATGGTATCATC; for Del. B, forward primer CTTGGGTTCGAGGATCACGAGCTTTTGATCTTACTCGAGATG; for Del. C, forward primer CATTATACCTTAACCTTCTTGTGAGTAACTTTTGATCATC; for Del. D, forward primer CCTTAAACCTTCTTGTGCTCGAGATGGTATCATC.
EPI repressor fragments were deleted using the following oligonucleotides as forward primers and their complements (not shown) as reverse primers: for Del. A, forward primer CAATCCTTGAAGCCAGACCCACATACATTGGCTAATGCAAAATA; for Del. B, forward primer CCCACATAATTGGCACATTTGGCTAATTACTGTCTGATTATTCACACG; for Del. C, forward primer TGGCTAATGCAAATACTGTTCACACGCAACGAGGACCC; for Del. D, forward primer GCTAATGCAAATACTGTCTGATTATTCACGAGGACCCGACTCC; for Del. E, forward primer TCTCCCTCCTGCTGTGAAAGCCAGACCC; for Del. F, forward primer CCCTCTTCCTGCTTTTCGACCCTCGGCTG.

Genomic *psuedoobscura* DNA was obtained from the Drosophila Species Stock Center in Tucson, AZ. 5’-3’ and Rep-3’ fragments were generated via PCR using the following forward and reverse primer sequences: For 5’-3’ the forward primer was CAAGATCATTCAGATCCCTC and the reverse primer was ATGGCATCTTGGATAAGATC. For Rep-3’ the forward primer was CAAGATCATTCAGATCCCTC and the reverse primer was GGAACCTCCTGTGCAGTGTA. Generated fragments were then sequenced (MacrogenUSA) to check for errors. Mutated GBS constructs were subcloned from previously mutated constructs in an earlier study (Lee and Jones, 2005).

4.3 Generation of repo-LacZ Reporter Lines

In order to generate repo-LacZ reporter lines, genomic fragments were cloned into the P-element reporter vector pCasPeR-hs43-LacZ (Thummel and Pirrotta 1992). Casper contains a minimal hsp70 heat shock promoter, *lacZ* gene, and the mini-white eye color gene. Reporter
constructs were incorporated into flies via P-element mediated germ line transformation (Rubin and Sprading, 1982).

4.4 *Drosophila melanogaster* Stocks and Genetics

Fly line **y**1w67c23 was used to generate transgenic lines. A *lof* study was performed by crossing *Dichaete* *lof* allele D[r72](FBgn0000411) with P[143A]. *gof* studies were achieved by first crossing *UAS-Dichaete* with P[143C] followed by either *sca-Gal4* (Fba10040466) or *daughterless-Gal4* (*da-Gal4*) (Fbti0013991). The UAS-GAL4 procedure was carried out as previously described (Brand and Perrimon, 1993).

4.5 Immunohistochemical Detection of Proteins in Embryos

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.

4.6 Yeast One-Hybrid Assay

Matchmaker Gold Yeast One-Hybrid Library Screening System was used to perform the screen for candidate proteins. Yeast one-hybrid assays were carried out according to
manufacturers instructions (Clontech). The bait sequence used for the CBG enhancer screen was the 37x5 oligonucleotide (see fragment, Fig. 5A). The bait sequence used for the EPI repressor was the 98x2 cis-regulatory fragment (see fragment, Fig. 4A). cDNAs were generated using *Drosophila* total mRNA. A 50/50 mixture of cDNA libraries generated from oligo-DT and random oligo primers was used for transformation. Candidate colonies for plasmid isolation were selected based on their ability to overcome 100ng of Aureobasidin A. All sequencing was outsourced to MacrogenUSA. In-frame fusion proteins were confirmed with Lasergene DNA software. Flybase BLAST search engine was used to identify proteins.


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Presentations

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