Characterization of cis-regulatory elements controlling repo transcription in Drosophila melanogaster

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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. The roles that glial cells play in the Drosophila central nervous system (CNS) and peripheral nervous system (PNS) are varied, but are all directed towards 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. Disruption or injury of these glial functions can result in severe consequences such as neural degeneration and paralysis (Freeman et al., 2003; Jones, 2001).

Despite our current knowledge about the functional roles of glial cells, their mechanisms of development remain poorly understood. The fruit fly Drosophila melanogaster provides us with a unique opportunity to examine these mechanisms. We have at our disposal sophisticated classical and molecular genetic tools, such as a short life cycle, a plethora of phenotypic markers, and various genetic manipulation techniques (Adams and Sekelsky, 2002; Blair, 2003; Matthews et al., 2005; Rubin, 1988; St. Johnston, 2002; Venken and Bellen, 2007). Additionally, much is known about the lineages, patterns, and identities of neurons and glia, and about the projections and pathways taken by axons in the developing CNS and PNS (Bassingthwaighte et al., 1996; Campos-Ortega and Harnstein, 1997; Goodman and Doe, 1993; Ito et al., 1995; Jacobs et al., 1989; Jones, 2001; Klambt and Goodman, 1991; Schmid et al., 1999; Schmidt et al., 1997; Sepp et al., 2000; Udolph et al., 1993).

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). In the PNS 8 to 10 peripheral glial cells ensheath axons along the major nerve tracks. Both cell types are easily identified by a large array of markers, and by position (Bossing et al., 1996; Campos-Ortega and Harnstein, 1997; Goodman and Doe, 1993; Ito et al., 1995; Jacobs et al., 1989; Jones, 2001; Klämbt and Goodman, 1991; Schmid et al., 1999; Schmidt et al., 1997; Sepp et al., 2000: Undohl et al., 1993).

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; Schmid et al., 1999; Schmidt et al., 1997).

In vertebrates, the mechanism by which glial fate is chosen over neuronal fates is complex (Takahashi et al., 2004). However, the mechanism for glial cell fate specification is 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, the transcription factor 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.

Although Gcm regulates embryonic glial development, it has also been shown to trigger the differentiation of macrophages (Alfonso and Jones, 2002; Bernardoni et al., 1997) and tendon cells within the epidermis (Soustelle et al., 2004) of the larva. 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. In order to identify the cofactors that function alongside Gcm to promote glial cell differentiation we must understand the transcriptional control of Gcm target genes that are transcribed specifically in glial cells.

A growing number of genes have been identified as targets of Gcm. In glial cells, central among them are repo, pointed, and tramtrack. All three are known to encode glial-specific transcription factors. repo encodes a homeodomain transcription factor that is expressed in all the lateral glia throughout development (Campbell et al., 1994; Halter et al., 1995; Xiong et al., 1994). Gcm first activates repo, but gcm’s expression is transient. The maintenance of repo expression must be regulated by other factors, possibly by autoregulation of repo (Lee and Jones, 2005). Embryos mutant for repo show defects late in embryonic development indicating 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.

As our long-term goal is to identify collaborating factors that act with Gcm to promote the transcriptional activation of one set of Gcm target genes specifically in glial cells, or prevent their activation in other tissues where Gcm is expressed, we chose to focus our analysis on the transcriptional regulation of the glial-specific gene repo. There are several reasons for this focus. repo is expressed exclusively in all Gcm-positive glia, but not in Gcm-positive hemocytes or tendon cells, indicating that collaborating factors act with Gcm to regulate repo expression exclusively in glial cells. Transient expression of Gcm is followed by maintained expression of repo mRNA and protein in glia. Multiple Gcm binding sites with the consensus sequence (ATCGGG(T/C)) are found in the regulatory region of repo suggesting that Gcm is a direct transcriptional regulator of repo (Akiyama et al., 1996; Schreiber et al., 1997). Since Gcm expression is transient, other factors must maintain the expression of repo. A simple model is that Gcm initiates repo expression, while maintenance is dependent on repo autoregulation. repo expression may also be maintained by other factors.

In 2005, Lee and Jones 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. Furthermore, by comparing expression patterns of overlapping reporter constructs, they inferred that repo expression was governed by multiple cis-regulatory elements (Fig. 1). In this study, we extend observations made by Lee and Jones (2005). Using lacZ reporter activity in transgenic embryos, we characterize three proposed cis-regulatory DNA elements controlling 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, we attempt to define the minimal functional sequences responsible for specific repo reporter activities by introducing small deletions and mutations into evolutionarily conserved sequences. Additionally, we test the functional conservation of two cis-regulatory elements in

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**Fig. 1.** Proposed cis-regulatory elements that promote specific transcriptional activity. 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. The line marked repo – 4.3 above the map indicates the DNA region that was sufficient to recapitulate the endogenous repo pattern in reporter constructs (Lee and Jones, 2005). Three DNA regions inferred to be necessary for specific expression activities are shown as bars below the map with question marks. Restriction enzyme sites: Sa, Sall; Sc, Scal; X, Xhol; E, EcoRI; B, BamHI; S, SpeI (adapted from Lee and Jones, 2005).
a closely related species of Drosophila. We also examine the influence of mutated GBSs on several reporter constructs. Our data support earlier findings that repo is a direct target for regulatory factors besides Gcm. Most significantly, the EPI repressor defines a minimal cis-regulatory element that recapitulates the endogenous repo expression pattern dependent on a single Gcm binding site, indicating that all the regulatory information for driving glial specific expression can be contained in a 98 base-pair DNA fragment.

2. Experimental procedures

2.1. DNA alignments


2.2. 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 CGAGGATCACGAGATGATTTAATACCTTACTCGAGATGTATCAC; for Del. B, forward primer CTGGGCTGAGATCGAGAATTGTGATCTAT; for Del. C, forward primer CCTATACACTACTACCTCAGAGATGTATCAC; for Del. D, forward primer CGAGGATCACGAGATGATTTAATACCTTACTCGAGATGTATCAC; for Del. E, forward primer GAGGACCC; for Del. F, forward primer GCTAATGCAAAATACTGTCTGATTATTCACGAGGATCACGAGATGATTTAATACCTTACTCGAGATGTATCAC. 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 CAATCCCTGAAGCCAGCCCCACATACATGGCGAATCTGAAATA; for Del. B, forward primer CCCACATAATGGCAGATTGGATAACTCTGTGTAACTGTTACAGCCACGAGACCCAG; for Del. C, forward primer TTGGCTAATGGCAGATTGGATAACTCTGTGTAACTGTTACAGCCACGAGACCCAG; for Del. D, forward primer GCTAATGGCAGATTGGATAACTCTGTGTAACTGTTACAGCCACGAGACCCAG; for Del. E, forward primer CCTCCTGCTGAGATGACGCAG; and for Del. F, forward primer CTTAACCTTCTGTGAGATGACGCAG.

Genomic pseudoobscura DNA was obtained from the Drosophila Species Stock Center in Tucson, AZ. DNA fragments homologous to the EPI and EPI repressor regions of D. melanogaster were generated via PCR using the following forward and reverse primer sequences: For EPI region the forward primer was CAAGATCATTCAGATCCCTC and the reverse primer was ATGGCACAATCCAGAATCAG. For EPI region plus repressor the forward primer was CGAGATCATTCAGATCCCTC and the reverse primer was GGAATCTGTGGCCGTTGA. Mutated GBS constructs were subcloned from previously mutated constructs in an earlier study (Lee and Jones, 2005).

All constructs generated by mutagenesis or PCR were sequenced by MacrogenUSA to check for errors. All oligonucleotides were obtained from Integrated DNA Technologies, Inc.

2.3. 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 Spradling, 1982). A minimum of three independent lines were generated for each construct made.

2.4. Drosophila melanogaster stocks

Fly line yw67f23 was used to generate transgenic lines.

2.5. 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 (βGal) 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.

3. Results

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

3.1. Epidermal enhancer

We began our study by testing the EPI enhancer region for the ability to drive repo-lacZ reporter expression. The 476 bp fragment located between restriction sites Scal and Xhol 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. Protein expression was then assayed in transgenic embryos using anti-βGal antibodies. For pBJ 100-LacZ, all lines displayed βGal in epidermal patches on the lateral body walls. An embryo from one of these lines is shown in Fig. 2B.

In an effort to define the minimal DNA sufficient to drive reporter expression, we then decided to generate a reporter construct, pBJ 111-lacZ, using the 116 bp fragment located between EcoRV and Xhol (Fig. 2A). Transgenic lines were created and then assayed for βGal expression. The shorter pBJ 111-LacZ reporter construct also promotes βGal expression in lateral epidermal cell clusters (Fig. 2C). However, compared to pBJ 100-LacZ (Fig. 2B) the expression is enhanced and confined to a subset of cells with a distinct morphology within the original epidermal cluster of the parent construct. This evidence suggests that a minimal element responsible for driving repo reporter expression in epidermal clusters is found in the 116 bp
fragment, but also that the DNA to the left of the EcoRV site has some influence in modifying its expression.

Since the EPI enhancer had been reduced to a more manageable size, we obtained an alignment of 12 Drosophila species from the UCSC Genome Browser (Kent et al., 2002) (Fig. 2D) to identify conserved regions. It was observed that there was a high amount of conservation (conserved in >4 species) in several areas (see Fig. 2D, gray shading). In an effort to determine whether the highly conserved sequences are necessary for expression driven from this element, we deleted the most highly conserved sequences. 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, pBJ125-LacZ, removed a 7 bp sequence from position 106–112 (TTTTGAT) (Del A, Fig. 2D). The second, pBJ123-LacZ, also removed a 7 bp sequence, slightly upstream, at position 98–104 (TAATTAA) (Del B, Fig. 2D). The third, pBJ174-LacZ, removed a 13 bp sequence, again slightly upstream, at position 81–93 (GGTTCGAGGATCA) (Del C, Fig. 2D). The fourth, pBJ175-LacZ, removed a 36 bp sequence, that encompassed the first three deletions, at position 81–116 (Del D, Fig. 2D).

Embryos carrying any of these constructs show βGal expression in lateral epidermal patches identical to the parent construct pBJ 111-lacZ shown in Fig. 2C. 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 (Figs. 2A,C).

3.2. EPI repressor

Next, we wanted to test the EPI repressor region for the ability to inhibit epidermal expression driven from the EPI enhancer. The 468 bp fragment located between restriction sites XhoI and 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 βGal in lateral glial cells, but fail to express βGal in the epidermis (Fig. 3B). We concluded that the 468 bp region from XhoI to BamHI is sufficient to inhibit repo reporter expression in the epidermis and drive lateral glia expression.

Using several unique restriction enzyme sites we then systematically dissected the 468 bp region. In all, a nested set of 7 progressively shortened reporter constructs (pBJ 103–109) were generated and transgenic lines assayed; three of the seven are shown (Fig. 3A). Embryos carrying the pBJ 107-LacZ construct show weak βGal expression in lateral glia (Fig. 3C). By contrast, embryos carrying pBJ 109-LacZ, which is shorter than pBJ 107-LacZ by 98 bp, show βGal expression.
in specific patches within the epidermis, but fail to show expression in CNS glia (Fig. 3D). These data suggest that the 98 bp region, from restriction site BstBI to PpuMI is required for inhibiting epidermal expression and promoting expression in lateral glial cells.

In an attempt to further characterize the 98 bp element, we obtained an alignment of 12 Drosophila species from 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. 3E, gray shading). Using site-directed mutagenesis, we introduced a series of small deletions into the highest conserved areas within the 98 bp region. This was an attempt to restore EPI reporter expression by eliminating the DNA sequences 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. 3E). The second, pBJ 133-LacZ, removed a 7 bp sequence from position 64–70 (GCAAAT) (Del B, Fig. 3E). The third, pBJ 137-LacZ, removed a 7 bp sequence from position 76–82 (CTGATTA) (Del C, Fig. 3E). The fourth, pBJ 138-LacZ, removed a 7 bp sequence from position 87–93 (CAGCACA) (Del D, Fig. 3E). The fifth, pBJ 153-LacZ, removed a 7 bp sequence from position 20–26 (GCAATCC) (Del E, Fig. 3E). The sixth, pBJ 154-LacZ, removed a 7 bp sequence from position 3–9 (AATCCTC) (Del F, Fig. 3E).

Embryos carrying any of these reporter constructs fail to express βGal protein in the epidermis, but do exhibit weak lateral glia staining (Fig. 3C). These results indicate that the locations responsible for repression were not removed by the engineered deletions. Alternately they indicate the possibility that repressor binding sites are redundant (see discussion).

3.3. Gcm binding sites

We next wanted to examine the influence of both the presence and absence of Gcm binding sites (GBSs) on reporter activity. Since
there are no GBSs in the CBG element, we focused on the EPI enhancer and repressor region.

Within the 98 bp region is one 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, pBJ 117-lacZ, that contained a mutated GBS (Fig. 4A) in four of eight 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 pBJ 117-lacZ to be GBS independent. Furthermore, we also introduced mutated GBSs, upstream and downstream, in the pBJ 110-LacZ and pBJ 112-LacZ constructs, which also had similar effects (Fig. 4A, data not shown).

To test the effect of the presence of the single GBS located within the 98 bp region and its ability to drive lateral glia specific expression, we generated and compared constructs pBJ 145-lacZ and pBJ 146-lacZ (Fig. 4A). Embryos carrying one copy of the 98 bp region, pBJ 145-lacZ, exhibit weak βGal expression in glial cells similar to the expression pattern of pBJ 107-lacZ (Fig. 3C). Embryos carrying two copies of the 98 bp region, pBJ 146-lacZ, exhibit increased expression of βGal, but do not show ectopic activity (Fig. 4C). These data demonstrate that all the information required to drive cell specific expression in lateral glial cells can be derived from a 98 bp fragment containing a single GBS.

3.4. EPI regions from D. pseudoobscura share function with EPI regions from D. melanogaster

The data we have presented so far show that the EPI enhancer and repressor elements are conserved among 12 species of Drosophila. Furthermore, we have demonstrated these two elements have the ability to function independently in melanogaster. Lastly, both the EPI enhancer and repressor functions in the epidermis act independently of the presence of GBSs. We were curious to see if, in addition to sequence, the functions of the transcriptional regulatory elements were also conserved in a closely related species of Drosophila, D. pseudoobscura.

To test conservation of the EPI enhancer’s function to drive repo reporter expression from a closely related species when transferred into D. melanogaster embryos, we used PCR to generate a 135 bp fragment from D. pseudoobscura genomic DNA that corresponded to the EPI enhancer region in D. melanogaster. D. pseudoobscura 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 from the same closely related species, we also generated a 289 bp fragment corresponding to both the EPI enhancer and repressor region in D. melanogaster from D. pseudoobscura genomic DNA using PCR (see Section 2). These fragments were then subcloned into pCasper-hs43-LacZ to make reporter vectors pBJ 134-LacZ and pBJ 135-LacZ, respectively (Fig. 4B). Transgenic D. melanogaster lines were then created and assayed for protein expression. Embryos carrying pBJ 134-LacZ expressed βGal in lateral epidermal patches in a pattern identical to the pattern expressed by pBJ 111-LacZ (Figs. 2A,B), interestingly, weak peripheral glial staining is observed (Fig. 4D). By contrast, embryos carrying pBJ 135-LacZ do not express βGal in the epidermis (Fig. 4E). Weak glial staining persists as expected due to the presence of a known GBS (orange oval, Fig. 4B). We conclude that the EPI enhancer and EPI repressor are shared in sequence and function between D melanogaster and D. pseudoobscura.

3.5. CBG enhancer

The cell body glia (CBG) regulatory activity was previously localized to a 350 bp region within a 1.1 kb fragment that induces repo

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**Fig. 4.** EPI regions function independently of Gcm and are conserved in D. pseudoobscura. (A) Summary of GBS deletion constructs. Restriction sites are indicated: Sc, Scal; EV, EcoRV; X, XhoI; T, Th111I; Bst, BstBI; P, PpuMI; N, NruI; Bfu, BfuAI; E, EcoRI; B, BamHI. Red Xs represent mutated GBS’s. 2 × represents tandem copies. (B) Summary of PCR constructs generated from pseudoobscura genomic DNA corresponding to epidermal enhancer and repressor regions in melanogaster. (C–E) Dissected stage 16 embryos labeled with anti-βGal antibody (anterior left, dorsal up). (C) pBJ 146-lacZ containing two tandem copies of 98 bp repressor region from BstBI to PpuMI, promotes increased glial expression over a single copy (compare to Fig. 3C). (D) pBJ 134-lacZ containing 135 bp pseudoobscura PCR fragment corresponding to epidermal enhancer region of D. melanogaster from EcoRV to XhoI (see panel A) drives repo reporter expression in epidermal cells. (E) pBJ 135-lacZ containing 289 bp pseudoobscura PCR fragment corresponding to epidermal enhancer and repressor region of D. melanogaster from EcoRV to PpuMI (see panel A) inhibits repo reporter expression in the epidermis, but promotes glial expression in peripheral and longitudinal glia. Scale bar, 20 µm.
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, we were curious to find out 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 pcaspar-hs43-LacZ to make reporter vector pBJ 101-LacZ (Fig. 5A). Transgenic lines were then produced and assayed for β-gal expression.

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 was sufficient to drive repo reporter expression.

To further test this idea and to see if reporter expression would increase synergistically, we 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 pcaspar-hs43-LacZ to make reporter vector pBJ 118-LacZ (Fig. 5A). Again embryos were assayed for β-Gal protein expression. We observed a very robust expression pattern of β-Gal in both the abdominal and thoracic CBG cells (Fig. 5C). High levels of β-Gal are detected in the M-CBG and MM-CBG. We conclude that the 328 bp region is sufficient to drive repo expression in a subset of CBG cells and that tandem copies act synergistically to increase reporter expression.

3.7bp region sufficient to drive CBG expression

The ability of the 328 bp region to drive CBG expression prompted us to pursue the minimal element required for reporter expression. In order to define this, we 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 × 2) and the right half (141 bp × 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 identical to the pattern of pBJ 118-lacZ shown in Fig. 5C. Embryos carrying the pBJ 144-LacZ completely lack CBG expression (data not shown). Based on these findings, we 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, we obtained an alignment of the (now 187 bp) CBG region from the UCSC genome browser. An alignment of 12 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 bp. Based on this evidence we deleted/mutated the highly conserved region within the 187 bp. Using PCR site-directed mutagenesis, we introduced a deletion that removed 37 bp of the conserved region. Using tandem copies of the region containing the deletion, we made reporter construct pBJ 158-LacZ (Fig. 5A). Additionally, via PCR site-directed mutagenesis, we introduced nine point mutations in the middle of the highest conserved sequences (shared by all species in alignment) within the 37 bp (red letters, Fig. 5E). Using tandem copies of the region containing the point mutations, we made reporter construct pBJ 163-LacZ (Fig. 5A). Embryos carrying either of the two reporters, pBJ 158-LacZ and pBJ 163-LacZ, completely lack β-Gal expression in glia (data not shown). These data suggested that the 37 bp region, or a component within, is necessary to produce repo reporter expression in CBG cells.

We were 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, we could not clone tandem repeats, so we had instead generated an oligonucleotide containing tandem repeats of the 37 bp sequence. We decided to design an oligo composed of five tandem copies of the 37 bp region. This fragment was then used to make a new reporter construct, 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, we conclude that the 37 bp region is sufficient to drive repo expression in CBG.

4. Discussion

In this paper we present a characterization of three proposed cis-regulatory regions from the DNA regulatory region of repo. We show that all three repo regulatory regions are sufficient to confer specific activities on reporter genes in subsets of glia and the epidermis. Furthermore, we define minimal cis-regulatory fragments sufficient to drive repo reporter expression (Fig. 6). We also demonstrate that sequence and functionality of two elements are conserved across closely related species of Drosophila. Moreover, we have identified the CBG cis-regulatory element that may be responsible for interacting with trans-acting factors.

4.1. EPI enhancer

In this study we characterized the functional epidermal enhancer down to 80 bp. We show this region to be sufficient to drive repo reporter expression in dorso-lateral epidermal cells. We also demonstrate that expression is not dependent on Gcm. Furthermore, corresponding regions in Drosophila pseudoobscura retain sequence similarity and function, thereby demonstrating the evolutionary conservation of this element.

4.2. EPI repressor

The epidermal repressor provides a glimpse of the complexity and sophistication of gene repression. We show here that 98 bp is sufficient to inhibit repo reporter expression in epidermis. Like the epidermal enhancer, repressor functions act independently of Gcm and are conserved in D. pseudoobscura. Interestingly, a series of systematic deletions failed to restore epidermal expression, and thus, we failed to identify specific DNA sequences necessary for epidermal repression. We attempt to explain this by one of the following four possibilities. First, it is possible we missed the key binding nucleotides because our deletions were not overlapping. Second, this could be a case of redundant repression sites, i.e. multiple sites within our 98 bp fragment could independently be sufficient to inhibit repo reporter expression. In support of this possibility, we observe a repeat sequence motif within the 98 base pair region – AATCTT – covered by our deletions E and F in Fig. 3E. Third, redundant repressor sites 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 an artifact of the reporter system and is due to the proximity of downstream DNA to the promoter in the reporter construct rather than the specific action of any protein. Further investigation will be necessary to determine the exact mechanism responsible for EPI repression.
positive input is required to test against. The epidermal repressor in (Jones, 2005).

Due to the unique nature of this element, identification, or it could be a combination of positive and negative inputs. the nervous system. It could be a single factor directing this expres-

sion via cross-regulation after activation by Gcm, which acts

pathways is under combinatorial control of multiple transcription factors. Our results are consistent with a model where Gcm-dependent transcription factors cross-regulate each other to maintain glial-specific expression. 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 on this 98 bp fragment. Whatever makes Gcm glial specific, and not macrophage or tendon cell specific must be acting alongside Gcm on this small cis-regulatory module. Identifying such factors that interact with this cis-regulatory module will go along way to explaining the context dependent transcription driven by Gcm.

4.3. 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. We have provided direct evidence that a 37 bp sequence is sufficient to drive reporter expression in CBGs as well as some other glial subsets. Mutation of the most conserved nucleotides in this sequence abolishes expression. These data suggest we have identified a binding region for a trans-acting factor(s) that is concomitantly expressed in other glial types. We have yet to identify interacting proteins responsible for driving this expression pattern, but based on sequence and DNA binding motif analysis (data not shown) one of them could possibly be a homeodomain containing protein. Homeodomains commonly bind to the core sequence ‘ATTAA’ (Florence et al., 1991), which has been shown to be critical for homeo-domain 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., 2003). Within the minimal CBG element is the sequence ‘CAATTAAC’ (the reverse complement is shown in Fig. 5E); the core TT sequences were mutated in our mutant constructs. One possibility is that repo could be autoregulating through this element; however, although we have attempted, we have not demonstrated that ectopic Repo expression can influence expression from this element (data not shown). Still, the possibility remains that a separate homeodomain protein or a protein with similar binding preferences is interacting with this sequence.

4.4. Conclusions

This study represents a step towards a thorough understanding of mechanisms underlying glial cell differentiation. Understanding 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 in the initiation, but not the maintenance of glial specific transcription (Jones, 2005).

Epidermal expression of repo is of interest because we have identified a cis-regulatory element that drives reporter expression in a tissue type that repo is not normally expressed. It is possible that the factor(s) acting on the 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 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 fortui-tous 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 and other species.

The CBG enhancer offers an excellent opportunity to identify glial specific regulators. Initially, we found that 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 suggests that there are important regulatory elements outside the 37 bp fragment identified as the CBG enhancer. Together these data support a scenario where the maintenance of repo expression in different subsets of glial cells is reinforced through regulation by other glial-specific transcription factors. Our results are consistent with a model where Gcm-dependent transcription factors cross-regulate each other to maintain glial-specific expression. Characterizing this particular element is of great interest for both understanding how repo expression is maintained and how glial subtypes are specified.

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