Gene 492 (2012) 167–176

Contents lists available at SciVerse ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

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

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ARTICLE INFO

Article history:
Accepted 11 October 2011
Available online 25 October 2011

Received by Meghan Jendrysik

Keywords:
Drosophila
CNS
Glia
gcm
repo
cis-regulatory elements

ABSTRACT

The *glial cells missing* (*gcm*) gene has been identified as a "master regulator" of glial cell fate in the fruit fly *Drosophila*. However, *gcm* is also expressed in and required for the development of larval macrophages and tendon cells. 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. Our 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, we have focused on the transcriptional regulation of a well-characterized glial-specific Gcm target gene, the transcription factor *reversed polarity* (*repo*). We aim to understand how the transcription of the glial-specific Gcm target gene *repo* is regulated by Gcm and other factors. Previously we defined a 4.3 kb *cis*-regulatory DNA region that recapitulates the endogenous Repo expression pattern dependent on multiple Gcm binding sites. We proposed that there may be multiple *cis*-regulatory sub-regions that drive cell-specific expression independent of Gcm binding sites. Here, using *lacZ* reporter activity in transgenic lines, we have characterized three *cis*-regulatory elements: 1) a distal element that promotes expression in dorsolateral epidermis; 2) a repressor element that suppresses expression in the epidermis; and, 3) a proximal element that promotes expression in a subset of cell body glia. Most significantly, we have defined a minimal *cis*-regulatory element that recapitulates the endogenous *repo* expression pattern dependent on a single Gcm binding site.

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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 (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; 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

Abbreviations: A, adenosine; βGal, β-galactosidase; bp, base pair(s); C, cytidine; CBG, cell body glia; CNS, central nervous system; DAB, diaminobenzidine; EPI, epidermal; G, guanosine; GB, glioblast; GBS, Gcm binding site; Gcm, Glial cells missing; *gcm*, gene encoding Gcm; HRP, Horse radish peroxidase; LG, longitudinal glia; M-CBG, medial CBG; MM-GBG, most medial CBG; NB, neuroblast; NGB, neuroglioblast; PCR, polymerase chain reaction; PG, peripheral glia; PNS, peripheral nervous system; *pnt*, gene encoding Pointed; Repo, Reversed polarity; *repo*, gene encoding Repo; SPG, subperineurial glia; T, thymidine; *ttk*, gene encoding Tramtrack.

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doi:10.1016/j.gene.2011.10.032

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; Udolph 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 (Tohoku, 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 (Soustell 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 (AT(G/A)CGGG(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

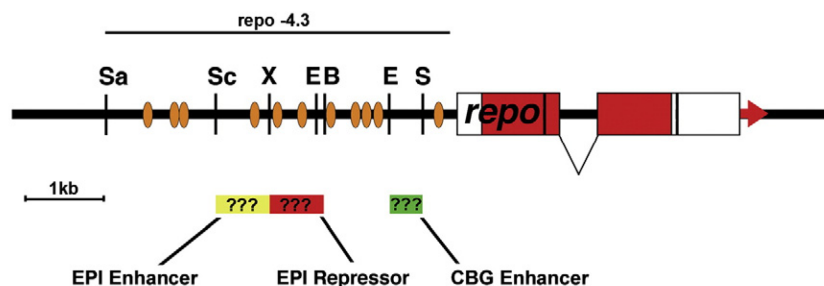

Fig. 1. Proposed *cis*-regulatory elements that promote specific transcriptional activity. DNA map of the *repo* gene showing predicted repo transcript is represented by rectangles. Red shaded area represents *repo* coding regions. Arrow indicates direction of transcription. Orange ovals represent Gcm 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, SalI; Sc, ScaI; X, XhoI; 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

The *Drosophila* species used in alignments of *repo cis*-regulatory regions were *D. melanogaster*, *D. simulans*, *D. sechellia*, *D. yakuba*, *D. erecta*, *D ananassae*, *D. persimilis*, *D. psuedoobscura*, *D. willistoni*, *D. mojavensis*, *D. virilis*, and *D. grimshawi*. We obtained the alignments from the UCSC Genome Browser (http://genome.ucsc.edu, Kent et al., 2002) where multiple alignments were made of the following assemblies to the *D. melanogaster* genome (dm3, Apr. 2006, BDGP Release 5): *D. simulans* (droSim1, Apr. 2005), *D. sechellia* (droSec1, Oct. 2005), *D. yakuba* (droYak2, Nov. 2005), *D. erecta* (droEre2, Feb. 2006), *D. ananassae* (droAna3, Feb. 2006), *D. pseudoobscura* (dp4, Feb. 2006), *D. persimilis* (droPer1, Oct. 2005), *D. willistoni* (droWil1, Feb. 2006), *D. virilis* (droVir3, Feb. 2006), *D. mojavensis* (droMoj3, Feb. 2006), and *D. grimshawi* (droGri2, Feb. 2006). These alignments were last updated on 12-11-2006.

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 CGAGGATCACGAGTAATTAACCTTACTCGAGATGGTATCATC; for Del. B, forward primer CTTGGGTTCGAGGATCACGAGCTTTTGATCTTACTCGAGATG; for Del. C, forward primer CATTATACCTTAACCTTCTTGCGAGTAATTAACTTTTGATC; and for Del. D, forward primer CCTTAACCTTCTTGCTCGAGATGGTATCATC.

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 CCCACATAATTGGCACATTGGCTAATACTGTCTGATTATTCACACG; for Del. C, forward primer TGGCTAATGCAAAATACTGTTTCACACGCAACGAGGACCC; for Del. D, forward primer GCTAATGCAAAATACTGTCTGATTATTCACGAGGACCCGACTCC; for Del. E, forward primer TCTCCCTCGGCTGTGAAGCCAGACCC; and for Del. F, forward primer CCCTCTTCCTGCTTTTCGACCCTCGGCTG.

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 ATGGCATCTTGGATAAGATC. For EPI region plus repressor the forward primer was CAAGATCATTCAGATCCCTC and the reverse primer was GGAACTCTTGTTGCGTGTGA. Mutated GBS constructs were subcloned from previously mutated constructs in an earlier study (Lee and Jones, 2005).

All construct 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 y^1w^{67c23} 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 ScaI to XhoI was necessary to promote expression of *repo* in epidermal cells. Concomitantly, it was shown that the adjacent 468 bp region spanning from XhoI 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 ScaI 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. 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 XhoI (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

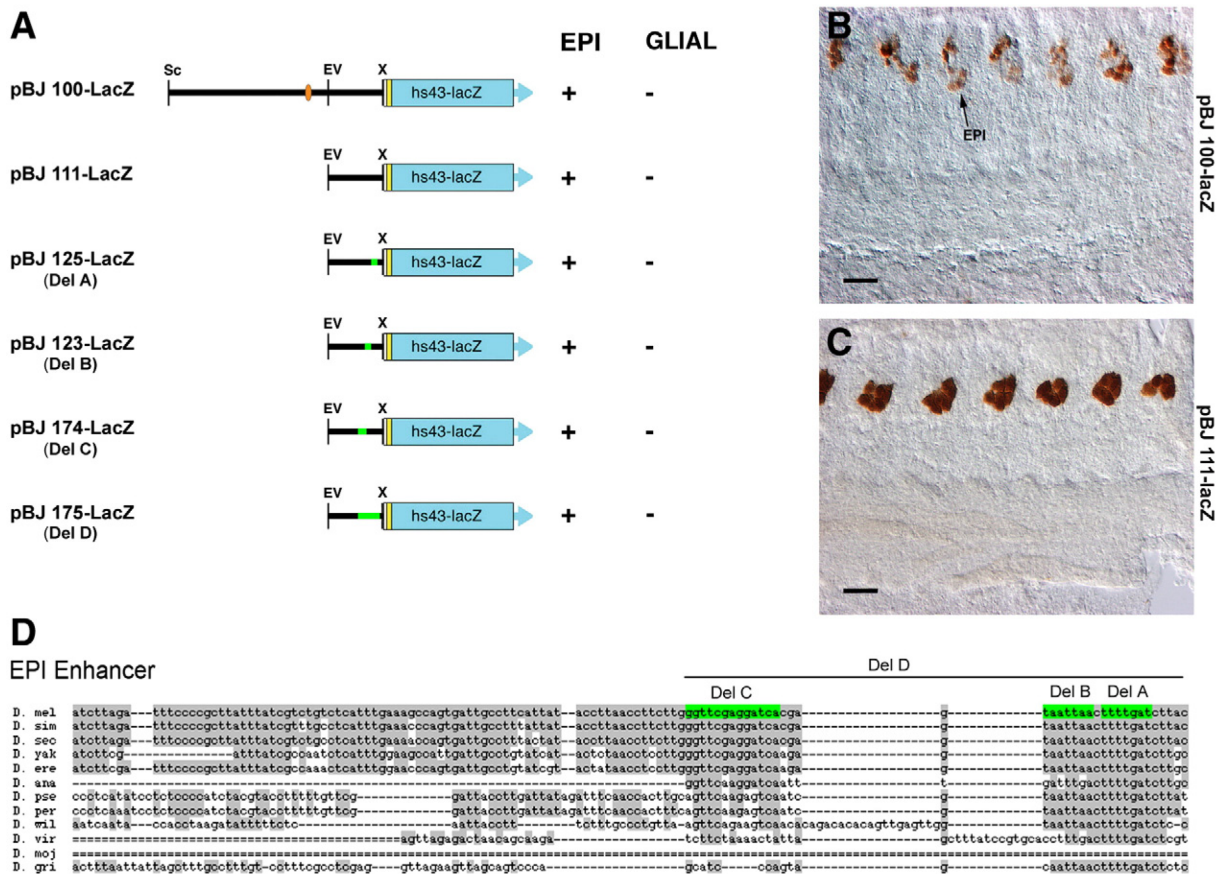

Fig. 2. An 80 base pair region drives *repo* reporter expression in the epidermis. (A) Summary of epidermal enhancer *repo-lacZ* reporter constructs and their expression. Black bar represents *repo* genomic DNA used to drive the *lacZ* gene represented by the blue rectangle. Restriction sites are indicated: Sc, ScaI; E, EcoRI; X, XhoI. + sign represents the presence of reporter expression, − sign represents the absence. EPI stands for epidermal cells and GLIAL stands for lateral glial cells. Bright green shading indicates a deleted region. (B) Dissected stage 16 embryo labeled with anti-βGal antibody (anterior left, dorsal up). *PBJ 100-lacZ* shows expression in the epidermis, black arrow. (C) Dissected stage 16 embryo labeled with anti-βGal antibody (anterior left, dorsal up). *PBJ 111-lacZ* shows enhanced expression in the epidermis in a tight cluster. (D) 12 *Drosophila* species alignment of 116 bp epidermal enhancer region from EcoRV to XhoI. Gray shading represent sequence shared with *D. melanogaster*. Green shading indicates a deleted region, also denoted by Del A–D. Dashes represent sequence gaps. D. mel = *Drosophila melanogaster*; D. sim = *Drosophila simulans*; D. sec = *Drosophila sechellia*; D. yak = *Drosophila yakuba*; D. ere = *Drosophila erecta*; D. ana = *D ananassae*, D. per = *D. persimilis*, D. psu = *D. psuedoobscura*, D. wili = *D. willistoni*, D. moj = *D. mojavensis*, D. vir = *D. virilis*, and D. gri = *D. grimshawi*. Scale bar, 20 μm.

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

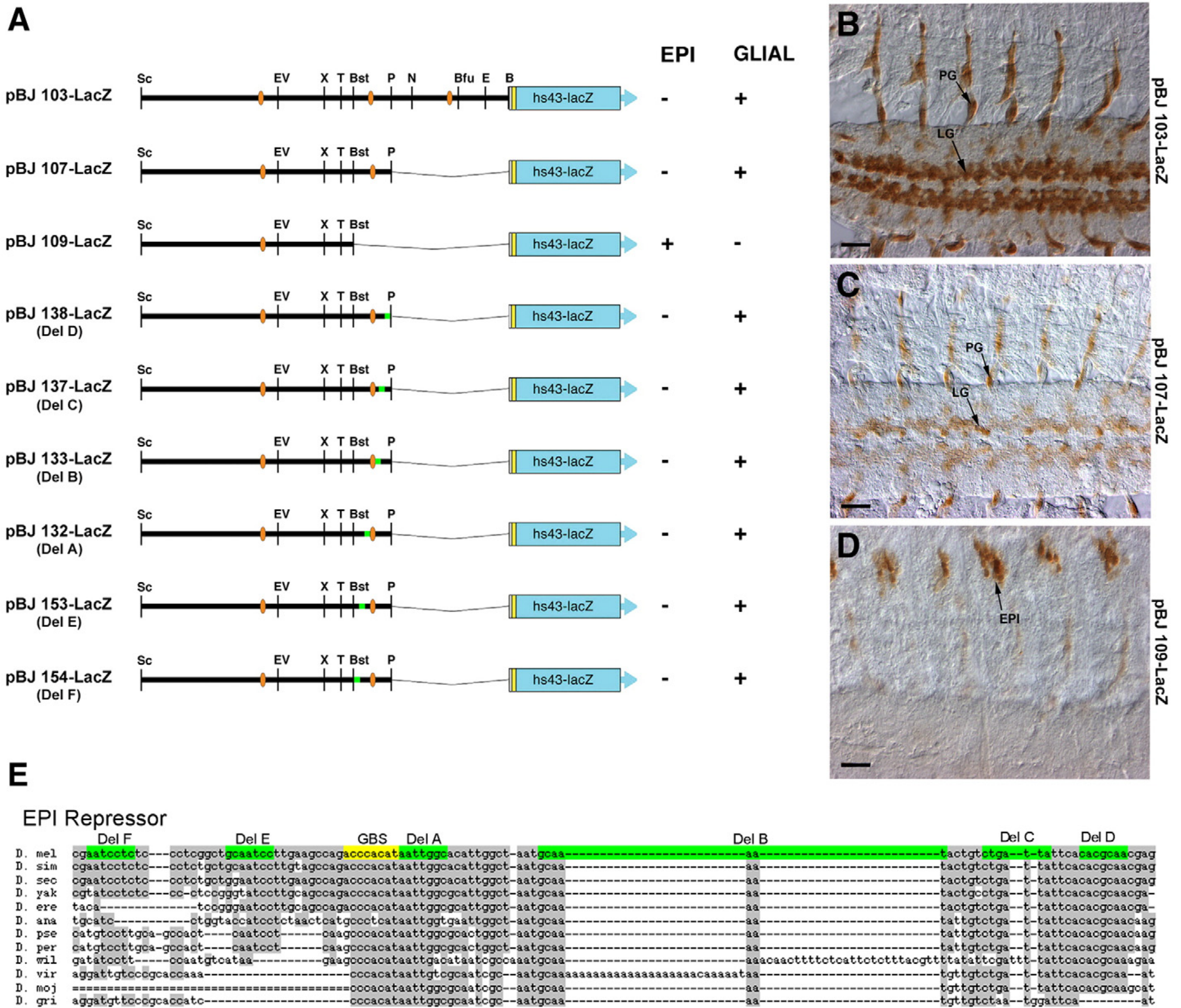

Fig. 3. A 98 bp region represses *repo-lacZ* reporter expression in the epidermis, as well as promotes expression in longitudinal and peripheral glia. (A) Summary of epidermal repressor *repo-lacZ* reporter constructs and their expression. Restriction sites are indicated: Sc, ScaI; EV, EcoRV; X, XhoI; T, Tth111I; Bst. BstBI; P, PpuMI; N, NruI; Bfu, BfuAI; E, EcoRI; B, BamHI. (B–D) Dissected stage 16 embryos labeled with anti-βGal 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) 12 *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. Scale bar, 20 μm.

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 (GCAAAAT) (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 (CACGCAA) (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 gia 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 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 *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 regions 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*

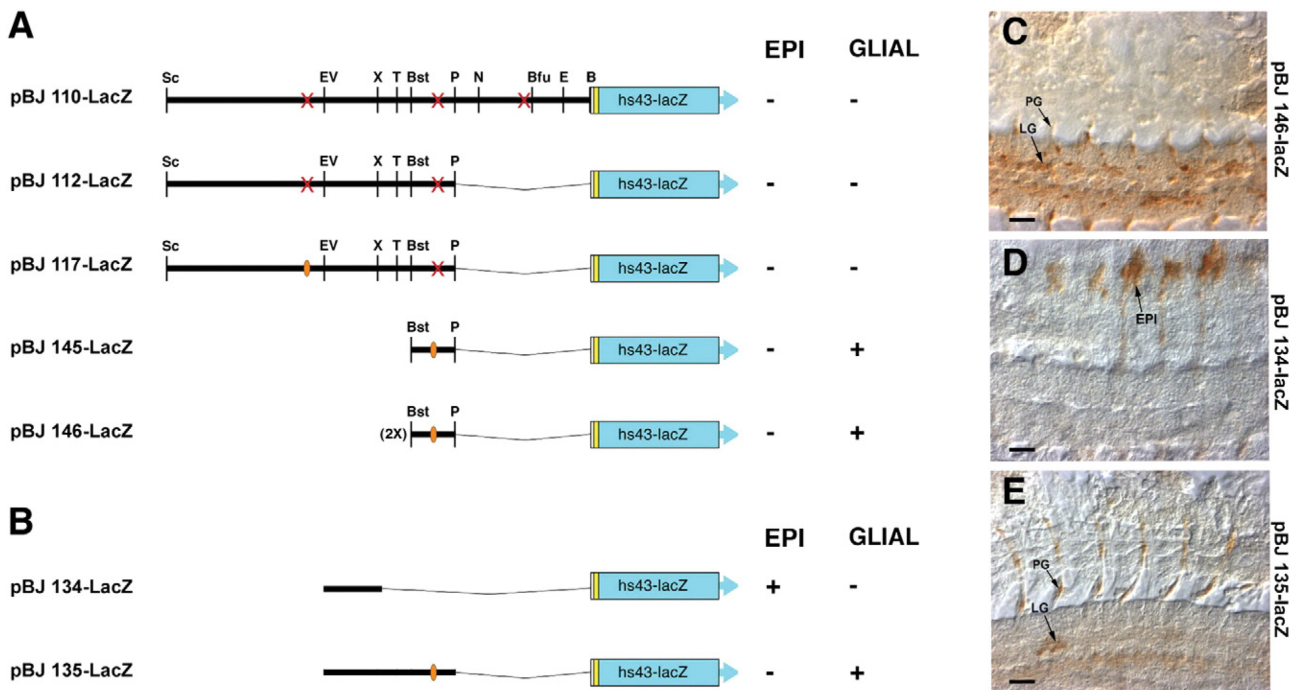

Fig. 4. EPI regions function independently of Gcm and are conserved in *D. pseudoobscura.* (A) Summary of GBS deletion constructs. Restriction sites are indicated: Sc, ScaI; EV, EcoRV; X, XhoI; T, Tth111I; Bst. BstBI; P, PpuMI; N, NruI; Bfu, BfuAI; E, EcoRI; B, BamHI. Red Xs represent mutated GBS's. 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 pCasper-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 βGal 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. βGal 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 pCasper-hs43-LacZ to make reporter vector *pBJ 118-LacZ* (Fig. 5A). Again embryos were assayed for βGal protein expression. We observed a very robust expression pattern of βGal in both the abdominal and thoracic CBG cells (Fig. 5C). High levels of βGal 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.

3.6. 37bp 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 CBG 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 βGal 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 βGal protein expression. Each displayed βGal 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 – AATCCT – 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

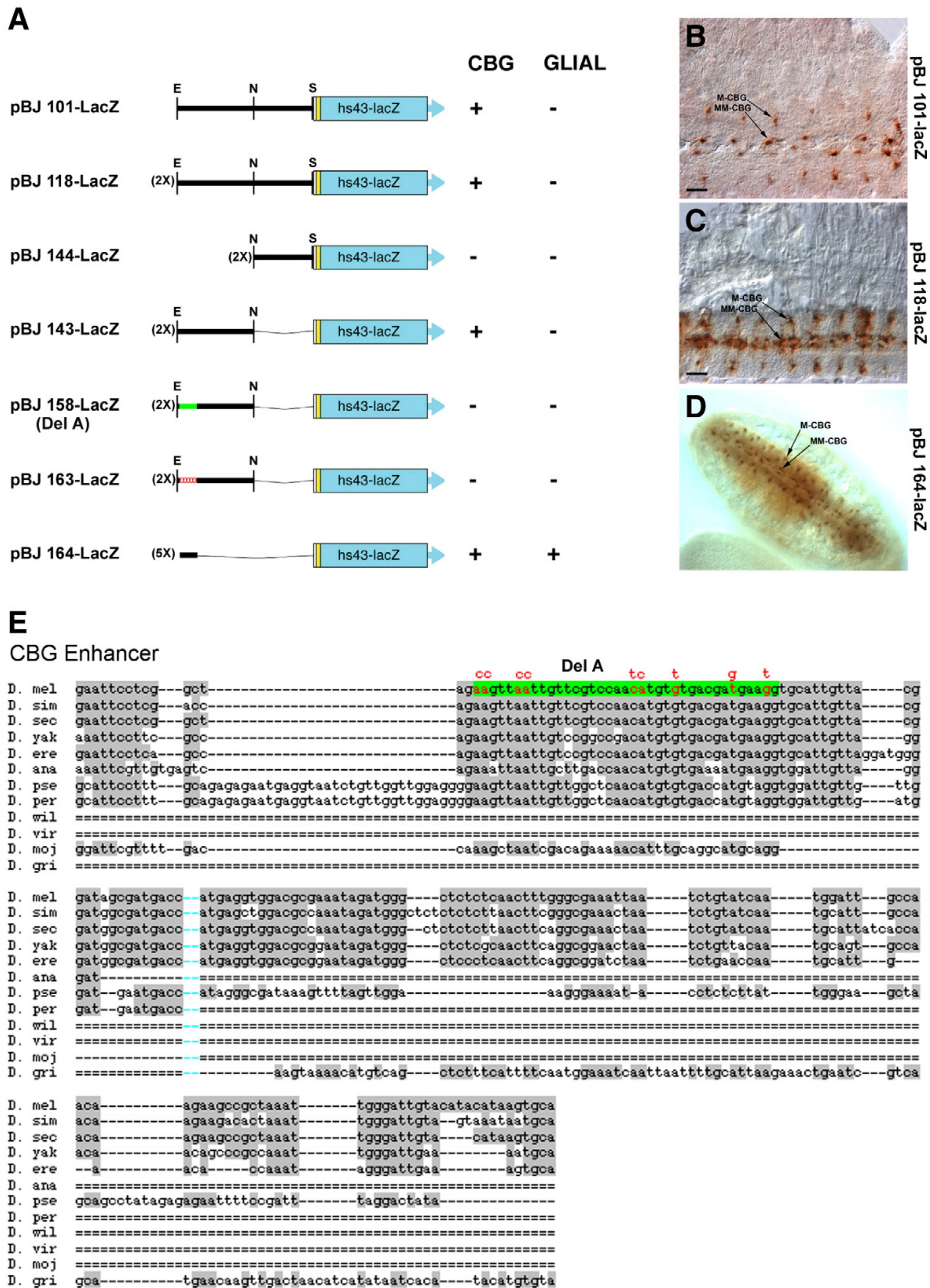

Fig. 5. A 37 bp region is necessary and sufficient to drive *repo* reporter expression in M-CBG and MM-CBGs. (A) Summary of cell body glia enhancer *repo-lacZ* reporter constructs and their expression. 5× represents five tandem copies. Restriction enzymes are indicated: E, EcoRI; N, NdeI; S, SpeI. CBG represents cell body glia. (B–C) Dissected stage 16 embryos labeled with anti-βGal antibody (anterior left, dorsal up). (B) *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, *pBJ 164-lacZ* drives *repo* reporter expression in M-CBG and MM-CBGs. (E) 12 *Drosophila* species alignment of 187 bp CBG enhancer region. Point mutations are represented with red lettering with substituted bases indicated above (also red). LG, longitudinal glia; PG, peripheral glia. Scale bar, 20 μm.

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.

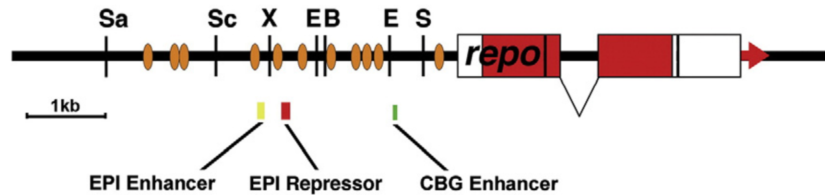

Fig. 6. Updated description of *cis*-regulatory elements controlling *repo* expression. Epidermal enhancer has been shown sufficient to drive *repo* reporter expression in epidermal cells and is reduced to 80 bp. Epidermal repressor has been shown sufficient to inhibit *repo* reporter expression in epidermal cells and is reduced to 98 bp. Cell body glia enhancer has been shown sufficient to drive *repo* reporter expression in CBGs and is reduced to 37 bp.

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 '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., 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 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* 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.

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.

Acknowledgments

We would like to thank lab members Kathy Nipper, Clif Colley, and Steven Clark for their contributions to this study. This research was supported by the National Science Foundation Grant IOB-0615658. R.J. received additional support from the University of Mississippi Graduate Student Council.

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