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Transcriptional regulation of the *Drosophila* glial gene *repo*

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Abstract

reversed polarity (*repo*) is a putative target gene of *glial cells missing* (*gcm*), the primary regulator of glial cell fate in *Drosophila*. Transient expression of Gcm is followed by maintained expression of *repo*. Multiple Gcm binding sites are found in *repo* upstream DNA. However, while *repo* is expressed in Gcm positive glia, it is not expressed in Gcm positive hemocytes. These observations suggest factors in addition to Gcm are required for *repo* expression. Here we have undertaken an analysis of the *cis*-regulatory DNA elements of *repo* using *lacZ* reporter activity in transgenic embryos. We have found that a 4.2 kb DNA region upstream of the *repo* start site drives the wild-type *repo* expression pattern. We show that expression is dependent on multiple Gcm binding sites. By ectopically expressing Repo, we show that Repo can regulate its own enhancer. Finally, by systematically analyzing fragments of *repo* upstream DNA, we show that expression is dependent on multiple elements that are responsible for activity in subsets of glia, as well as repressing inappropriate expression in the epidermis. Our results suggest that Gcm acts synergistically with other factors to control *repo* transcription in glial cells.
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1. Introduction

Glial cells maintain and support neuronal function. During neural development, glial cells secrete neurotrophic factors, provide cues for axon guidance, and remove apoptotic cells. These glial functions aid growing axons by directing growth cones to their targets and promoting their survival, thereby allowing the nervous system to establish connectivity. Over the course of neuronal function, glia are important for neurotransmitter recycling, and maintaining homeostatic conditions necessary for neural conduction. Disruption of these glial functions can result in severe neurological defects such as paralysis and neural degeneration. Glial cells have multiple roles, but how these roles are assigned and regulated is poorly understood.

In *Drosophila*, glial cells are diverse but have been classified based on their position and cell morphology (Ito et al., 1995; Klämbt and Goodman, 1991). With the exception of the midline glia originating from the mesectoderm, nearly all glial cells are derived from the neuroectoderm and the peripheral ectoderm lateral to the ventral midline. These 'lateral' glia include the longitudinal glia that run in two parallel anterior/posterior tracts ensheathing the longitudinal axons, the cell body glia that reside within the cortex of the ventral nerve cord (VNC), and the subperineurial glia that surround the VNC surface. In the periphery, lateral glial cells include the peripheral glia that ensheath the motor and sensory axons.

The development of lateral glial cells depends on the expression of the *gcm* gene (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). *gcm* is transiently expressed in all embryonic glia except for the midline/mesectoderm-derived glia. *gcm* mutant embryos lack nearly all lateral

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glial cells, and presumptive glial cells are transformed into neurons. Conversely, when *gcm* is ectopically expressed, presumptive neurons are transformed into glia (Hosoya et al., 1995; Jones et al., 1995). Thus, *gcm* appears to function as a binary genetic switch, with Gcm positive cells becoming glia and Gcm negative cells becoming neurons. *gcm* encodes a novel DNA-binding transcription factor that recognizes an octamer consensus DNA sequence; this sequence is found repeated in the putative regulatory regions of a number of glial-specific genes (Akiyama et al., 1996; Freeman et al., 2003; Schreiber et al., 1997). Taken together, *gcm* appears to act as a master transcriptional regulator of glial cell differentiation.

In addition to acting as a regulator of glial cell differentiation, *gcm*, together with its homolog *gcm2*, is required for the proper differentiation of the plasmatocyte/-macrophage lineage of hemocytes (Alfonso and Jones, 2002; Bernardoni et al., 1997; Lebestky et al., 2000). Both *gcm* and *gcm2* are expressed in the hemocyte primordium and in hemocyte precursors where they act in concert to trigger the maturation of hemocytes into macrophages. Embryos that are double mutants for *gcm* and *gcm2* show abnormal plasmatocyte/macrophage morphology (Alfonso and Jones, 2002), and expression of *gcm* alone is sufficient to induce plasmatocyte/macrophage development within hemocyte lineages (Alfonso and Jones, 2002; Bernardoni et al., 1997; Lebestky et al., 2000). Thus *gcm* has the ability to promote either glial cell differentiation or hemocyte differention in different developmental contexts. How some *gcm* target genes are restricted to glial cells and are excluded from hemocytes is not understood. Because *gcm* is expressed in both hemocytes and glia, and because *gcm* expression in glial cells is early and transient, the transcriptional regulation of glial cell differentiation cannot be explained by *gcm* alone. Additional co-factors must act with Gcm to initiate gliogenesis, and downstream Gcm target genes must act to carry out the differentiation and maintenance of glial cell fate.

To date, several genes have been identified that are targets of Gcm; three of these genes are transcription factors controlling different aspects of the terminal differentiation of glia. The homeodomain transcription factor encoded by the *repo* locus is expressed in all lateral glial cells (Campbell et al., 1994; Halter et al., 1995; Xiong et al., 1994). *repo* expression is *gcm*-dependent; transient expression of *gcm* is followed by maintained expression of *repo*. In contrast to *gcm*, glial cell defects in *repo* mutants appear later during embryonic development, suggesting a role for *repo* in terminal glial cell differentiation. Also dependent on *gcm* expression are the P1 form of the ETS domain transcription factor encoded by the *pointed* (*pnt*) gene (Klaes et al., 1994; Klämbt and Goodman, 1991), and the P69 form of the zinc-finger factor encoded by the *tramtrack* gene (*ttk*) (Giesen et al., 1997). Like *repo*, mutations in these genes do not prevent the initiation of glial cell development, but have terminal differentiation defects. *pnt* promotes aspects of glial cell differentiation, while *ttk* acts to repress neuronal differentiation. Taken together, these data support a model whereby *gcm* promotes glial cell characteristics by initiating the transcription of *repo* and *pnt*, while simultaneously repressing neuronal characteristics by activating the transcriptional repressor *ttk* (Giesen et al., 1997).

Studies of *cis*-regulatory elements of the glial-specific gene *loco* and the expression of other glial markers suggest that *gcm* and its downstream regulators *repo*, *pnt* and *ttk* act cooperatively to initiate and maintain the expression of glial-specific genes (Granderath et al., 2000; Yuasa et al., 2003). Gcm and PointedP1 both bind to a *loco cis*-regulatory element and synergistically control its reporter expression in vivo (Granderath et al., 2000). *repo* also synergizes with *pointedP1* to activate *loco* expression (Yuasa et al., 2003). In another instance, *repo* cooperates with *ttk* to activate the *gcm*-dependent glial marker M84, suggesting that *ttk* may also be an activator in other contexts (Yuasa et al., 2003). Given these interactions, it is likely the regulation of other glial-specific *gcm* target genes involve tiers of regulation and multiple factors.

To further understand how glial-specific gene expression is initiated and maintained in *Drosophila*, we conducted an analysis of the transcriptional regulation of *repo*. There are several reasons why we are interested in studying *repo* regulation. *repo* is expressed exclusively in Gcm positive glia, but not in Gcm positive hemocytes, indicating that other factors must cooperate with Gcm to regulate *repo* expression. Transient expression of Gcm is followed by maintained expression of *repo* mRNA and protein. Multiple Gcm binding sites are found in the putative regulatory region of *repo* suggesting that Gcm is a direct transcriptional regulator of *repo* (Akiyama et al., 1996). Since *gcm* expression is transient, other factors must be responsible for the maintained expression of *repo*. A simple model is that *gcm* initiates *repo* expression, while maintenance is dependent on *repo* autoregulation. It is also possible that *repo* expression is maintained by other factors, or by a combination of autoregulation and other factors.

In this paper we present a systematic dissection of *cis*-regulatory DNA elements controlling the expression of *repo* using *lacZ* reporter activity in transgenic embryos. We have characterized a 4.2 kb DNA fragment that recapitulates *repo* expression. By mutating Gcm binding sites, we show these sites are required for expression in vivo. By ectopically expressing Repo, we show that Repo can regulate its own enhancer. By examining fragments of the *repo* 4.2 kb enhancer, we show that show that expression is controlled by multiple *cis*-regulatory elements. Our data suggest that, in addition to being regulated by Gcm initiation and Repo autoregulation, *repo* expression is maintained by additional glial-specific factors, and inappropriate *repo* expression in other tissues is repressed by negatively acting factors.

2. Results

2.1. *repo* −4.3-*lacZ* reporter gene reproduces the endogenous *repo* expression pattern

The identification of *cis*-regulatory DNA elements that produce the *repo* expression pattern required the isolation of genomic DNA flanking the *repo* transcription unit. The structure of the *repo* locus has been previously described (Adams et al., 2000; Akiyama et al., 1996; Campbell et al., 1994; Halter et al., 1995; Xiong et al., 1994) and is represented schematically in Fig. 1A. Using the plasmid rescue technique for cloning genomic DNA adjacent to P element insertions (Pirrotta, 1986), we isolated an 8.7 kilobase (kb) genomic clone of *repo* upstream DNA (pRPrepo4, Fig. 1A) from flies carrying the *repo* mutant allele *repo*^{4} (Xiong et al., 1994); *repo*^{4} has a P element insertion at nucleotide position −100 relative to the *repo* transcription start site (we define the transcription start site +1 as the position of the 5′ end of the longest reported *repo* cDNA from Halter et al., 1995).

Eleven sequences that match or have one mismatch from a consensus Gcm binding site (GBS)—(A/G)CCCGCAT—have previously been shown to be located within the first four kb upstream of the *repo* transcription unit (Akiyama et al., 1996) (Figs. 1A,3A). Based on this information, we subcloned a 4.2 kb fragment containing all eleven GBSs into a *lacZ* reporter vector (*hs-43-lacZ*, Fig. 1A). This fragment contains sequences from −4307 to −100 base pairs (bp) relative to the *repo* transcription start site and does not include *repo*'s endogenous TATA box located at −30 to −25 bp (TATAAA). However, the *hs-43-lacZ* reporter vector contains a minimal *hsp70* promoter with TATA box. The resulting gene fusion construct, called *repo* −*4.3-lacZ*, was introduced into flies by P element-mediated transformation; five lines were generated.

We assayed reporter protein expression in transgenic embryos using anti-β-gal antibodies; all five lines express β-gal in all lateral glial cells in a pattern identical to endogenous *repo* (Fig. 1B). Embryos from one these lines is shown in Figs. 1C,3C,4A; β-gal is detected in all lateral glial cells, including the longitudinal, cell body, subperineurial, and peripheral glia, as well as in the ligament cells of the lateral chordotonal organs and the glial support cells of the dorsal bipolar dendrite neuron.

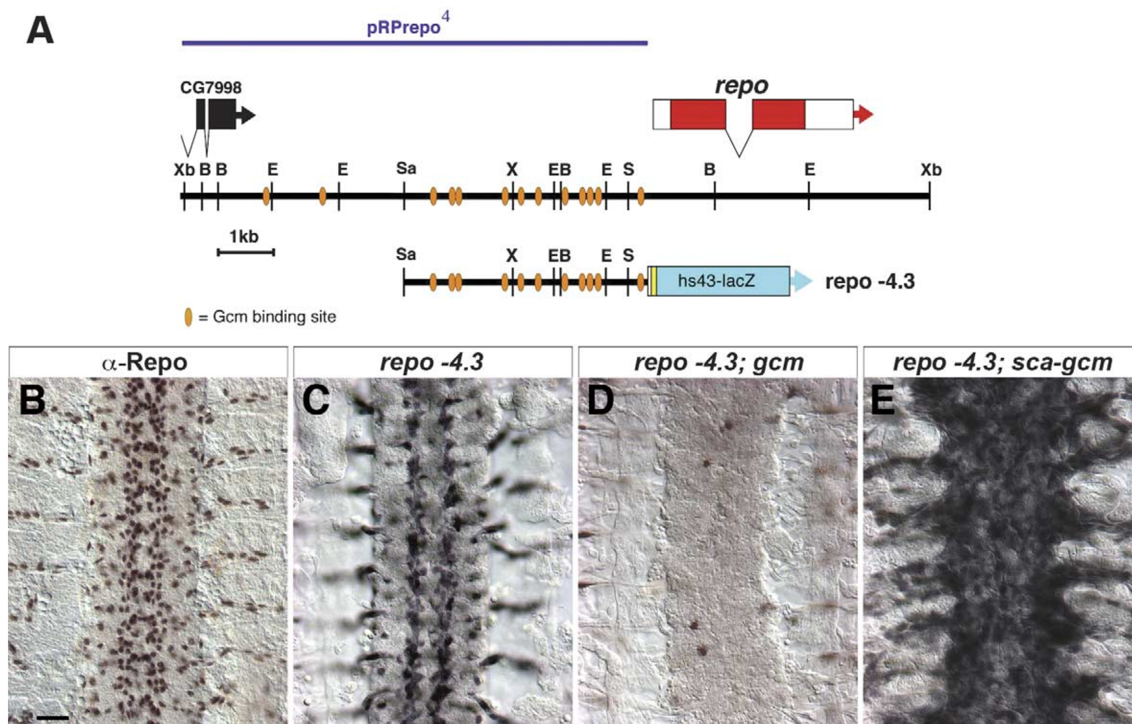


Fig. 1. A 4.2 kb DNA region recapitulates *repo* expression in lateral glia. (A) Genomic map and reporter construct. Predicted transcripts are represented by rectangles with red shaded areas representing *repo* coding sequences. Arrows indicate the direction of transcription and orange ovals represent putative Gcm binding sites (GBS). The purple line above genomic map represents the genomic clone pRPrepo^4. Reporter construct *repo* −*4.3-lacZ* is shown below the genomic map. The overlap of *repo* −*4.3-lacZ* with the genomic map represents the 4.2 kb region used to drive the *lacZ* gene represented by the blue rectangle. Restriction sites are indicated: Xb, *Xba*I; X, *Xho*I; B, *Bam*HI; E, *Eco*RI; Sa, *Sal*I, and S, *Spe*I. (B–E) Dissected stage 16 embryos labeled with antibodies and filleted; nine adjacent segments of the CNS are shown with anterior to the top. (B) Wild-type embryo labeled with anti-Repo antibody. (C–E) Embryos carrying the *repo* −*4.3-lacZ* reporter labeled with anti-β-gal antibody. (C) Wild-type background; *lacZ* reporter is expressed in all lateral glia. (D) *gcm* mutant background; most of the reporter expression is absent. (E) *sca-Gal4; UAS-gcm* background (*sca-gcm*); ectopic expression of *gcm* induces broad activation of *repo* −*4.3-lacZ* throughout the CNS. Scale bar, 20 μm.

2.2. *gcm* is both necessary and sufficient to drive *repo −4.3-lacZ* expression

repo −4.3-lacZ fully recapitulates the behavior of endogenous *repo* expression. Like *repo*, expression is dependent on *gcm*. In *gcm* mutant embryos that also carry the *repo −4.3-lacZ* reporter, β-gal expression is almost entirely absent (Fig. 1D). Expression in the few glia that remain in *gcm* mutants is presumed to be due to the activity of *gcm2* (Alfonso and Jones, 2002; Kammerer and Giangrande, 2001).

Furthermore, ectopic expression of *gcm* is sufficient to induce ectopic *repo −4.3-lacZ* expression. We used the Gal4/UAS binary system (Brand and Perrimon, 1993) to generate *repo −4.3-lacZ* embryos that also express ectopic Gcm. *sca-Gal4* (Klaes et al., 1994) was used to drive Gcm expression throughout the CNS. When *repo −4.3-lacZ; sca-Gal4* flies are crossed with *UAS-gcm*, the resulting progeny show a striking increase in β-gal expression throughout the nervous system in presumptive neurons that have been transformed into glia (Fig. 1E); β-gal expression is identical to Repo expression in the same genetic background (data not shown).

2.3. Rescue of the *repo* mutant phenotype by *repo −4.3* DNA

We were curious find out whether the *repo* −4.3 DNA fragment can direct expression sufficient to rescue the *repo* mutant phenotype. We cloned the *repo* −4.3 DNA fragment into a vector that contains the Gal4 gene under the control of the minimal heat shock promoter (Sharma et al., 2002) and generated transgenic lines capable of driving Gal4 expression (*repo-Gal4*). We also generated transgenic lines that carry a fusion gene of the *repo* cDNA and Gal4 UAS sequences (*UAS-repo*) that allow induced expression of Repo using the Gal4/UAS binary system.

By crossing *UAS-repo* with our *repo-Gal4* driver into a *repo* null mutant background, we can partially rescue the *repo* mutant phenotype. We used the $repo^{rk2}$ null allele (Campbell et al., 1994) for our rescue, and we compared the rescue phenotype to the phenotype of embryos carrying a second *repo* null allele, $repo^{3702}$, which contains a P{*lacZ*} enhancer-trap insertion that labels glial cells in embryos lacking Repo protein (Fig. 2E,F) (Xiong et al., 1994). *repo* mutants are characterized by disorganized lateral glia that are reduced in number (compare Fig. 2E,F to A,B); longitudinal glia are displaced towards the lateral edges of the ventral nerve cord (compare Fig. 2E to A), and most peripheral glia are either missing or displaced (compare Fig. 2F to B). In contrast, mutant embryos expressing *repo* driven by *repo-Gal4* show increased numbers of glia compared to *repo* mutant embryos lacking *repo-Gal4*; rescue is especially apparent in the longitudinal glia (LG) associated with longitudinal connectives (Fig. 2C), and peripheral glia (PG) associated with the peripheral nerves (compare Fig. 2D to F). Rescued embryos still show a slight disorganization and missing glia compared to $repo^{rk2}$ heterozygous embryos carrying *UAS-repo/repo-Gal4*, which show wild-type morphology despite having higher levels of Repo protein than normal (Fig. 2A,B); this partial rescue may be due to a delay in transcriptional activation inherent in the UAS/GAL4 system.

2.4. *repo −4.3-lacZ* expression is dependent on multiple Gcm binding sites

Previous in vitro studies examined the ability of Gcm protein to bind to a consensus sequence and to variations from this sequence (Akiyama et al., 1996; Cohen et al., 2003; Schreiber et al., 1997, 1998). Akiyama et al. (1996) reported a consensus sequence (A/G)CCCGCAT, degenerate at position 1. Schreiber et al. (1997) reported a consensus sequence ACCCG(C/T)AT, degenerate at position 6—however, they also showed that there may be a preference for a C over T at position 6. Additional mutational analysis showed that altering base pair sequences at positions 1, 2, 3, 6 or 7 have the most profound affect on Gcm protein binding to the site, with severely reduced or abolished binding in electrophoretic mobility shift assays (Schreiber et al., 1998). In this study we focused on sites that match or have one mismatch from the (A/G)CCCGCAT consensus. Within the *repo* −4.3 genomic DNA fragment, the eight proximal Gcm binding sites (GBSs 4-11, Fig. 3A) are sequences that would predict strong Gcm binding, while the distal three GBSs (GBSs 1-3, Fig. 3A) have at least one mismatched base pair that would predict diminished Gcm binding.

To demonstrate that GBSs are required for *repo* expression, we performed site-directed mutagenesis of GBSs as depicted in Fig. 3A,B. In a first round we mutated the eight 'perfect' binding sites (GBSs 4-11) at nucleotide positions 6 and 7 (*repo −4.3ΔGBS8-lacZ*). β-gal expression was significantly reduced compared to wild-type *repo −4.3-lacZ,* but not abolished (Fig. 3D). β-gal expression in most longitudinal glia and subperineurial glia is lost; however, some glial cells ventral to the commissural axons, as well as many other lateral glia express β-gal. This result suggests that the remaining three wild-type 'imperfect' GBSs confer a moderate amount of reporter activity and/or that the introduced mutations retain some Gcm binding activity.

In a second mutagenesis round, we mutated the remaining three GBSs (GBSs 1–3) at positions 2 and 3. Schreiber et al. (1998) showed that changing C-G to A-T at either site 2 or 3 abolishes binding in vitro; we introduced these mutations at both sites. The resulting construct with mutations in all eleven GBSs (*repo −4.3ΔGBS11-lacZ*) showed greatly diminished glial expression (Fig. 3E); however, β-gal is still expressed in a few glial cells. This β-gal expression is typically in the same 2–3 glial cells in each hemisegment, which we have identified as the cell

body glia M-CBG and MM-CBG by position and morphology (see also Fig. 5A). Expression is variably missing in some segments. The diminished expression observed between *repo −4.3ΔGBS8-lacZ* and *repo −4.3ΔGBS11-lacZ* demonstrates that the 'imperfect' GBSs 1–3 are important for expression, despite having base pair mismatches detrimental to in vitro Gcm binding.

To further test whether the mutations we introduced effectively abolished Gcm dependent activity at these sites, we mutated an additional two base pairs at positions 2 and 3 in the eight 'perfect' GBSs in the construct *repo −4.3ΔGBS11*. This final round of site-directed mutagenesis produced no difference in expression from *repo −4.3ΔGBS11-lacZ* (data not shown) suggesting our original mutations effectively abolished Gcm dependent *cis*-regulatory activity at these sites. We conclude that mutating the eleven Gcm binding sites in the 4.2 kb fragment nearly abolishes its ability to drive *lacZ* expression in glia. While these mutations are effective, we cannot rule out the possibility that Gcm is responsible

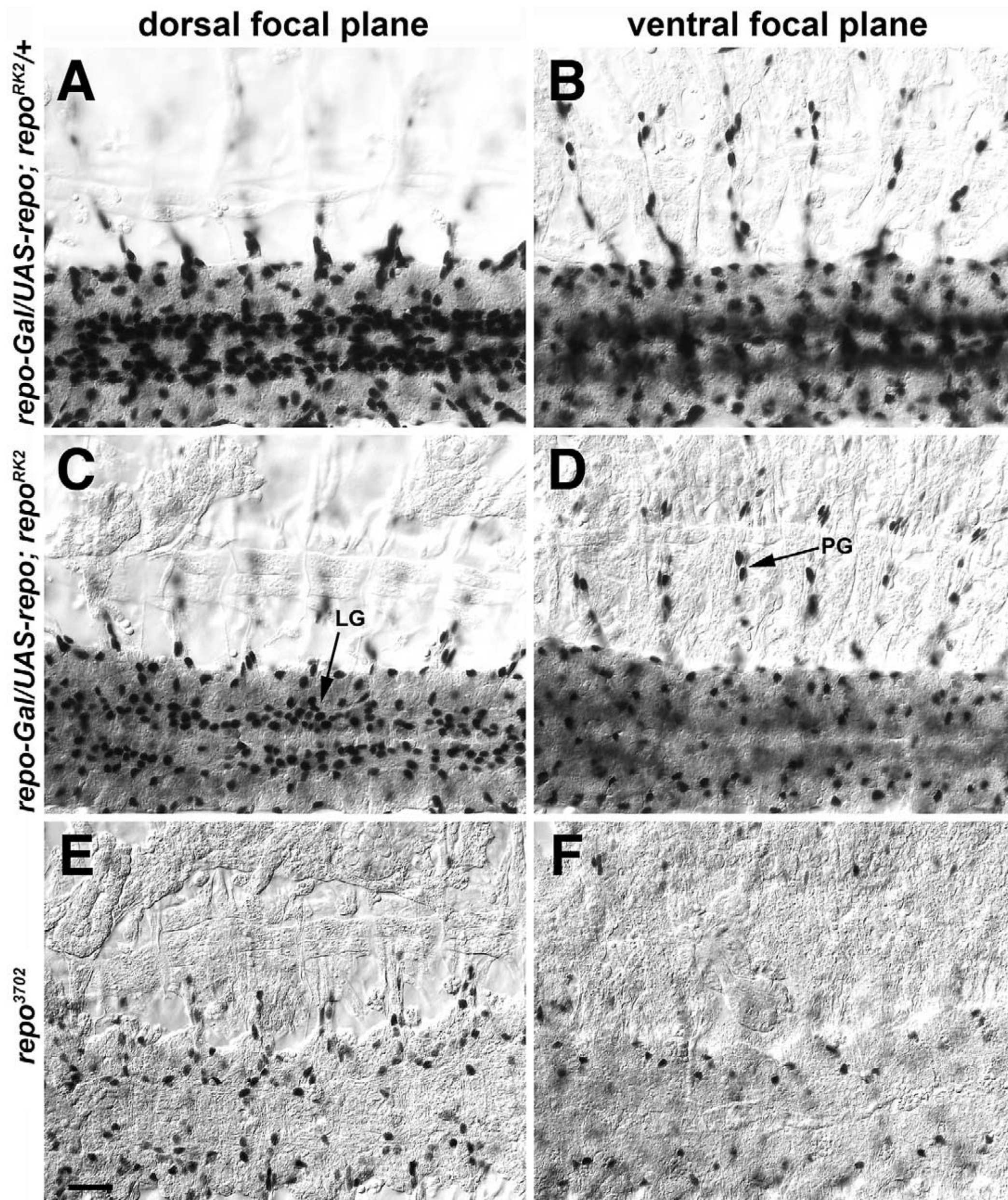
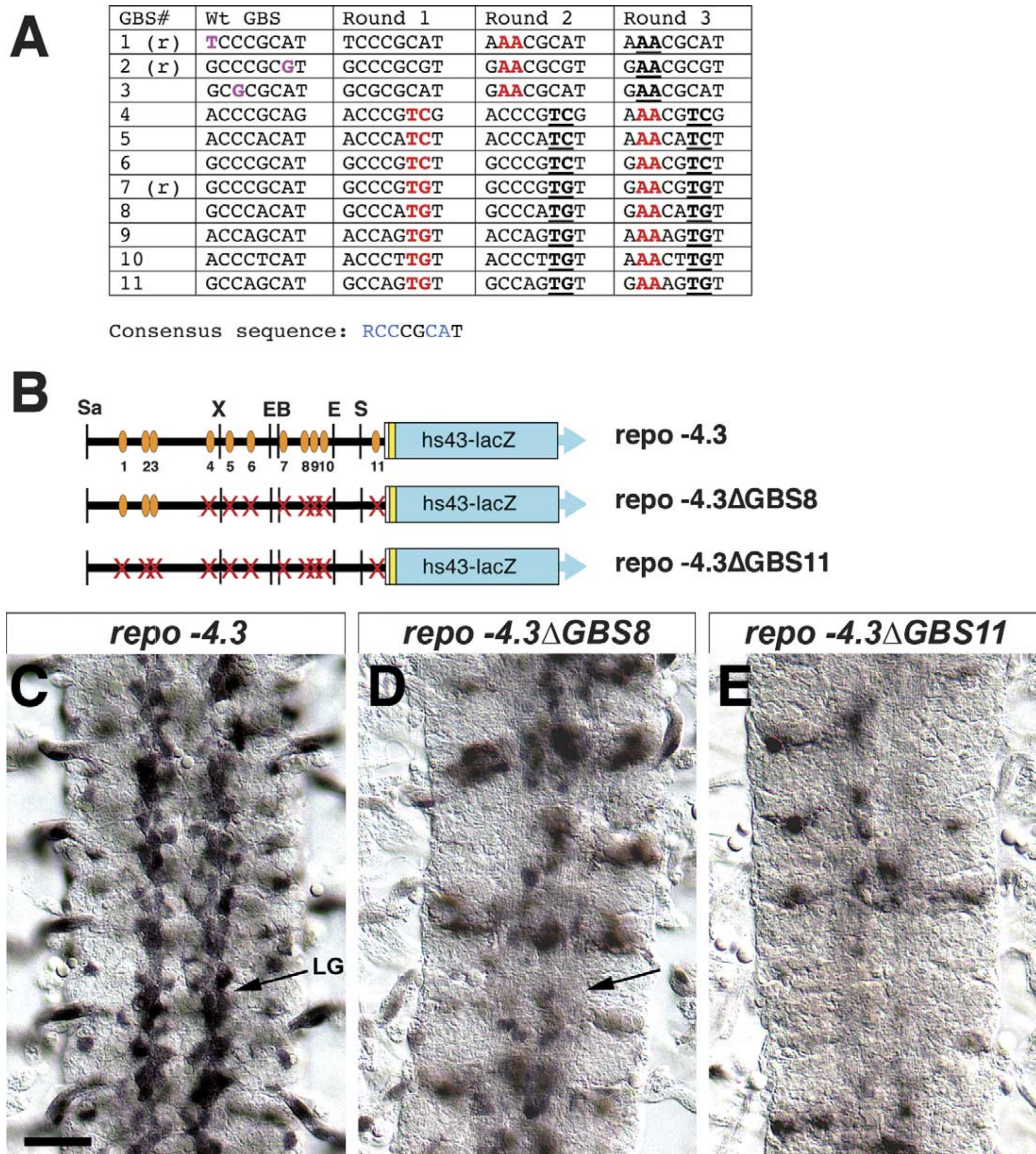


Fig. 2. *repo −4.3* DNA partially rescues the *repo* mutant phenotype. Dissected stage 16 embryos labeled with Repo monoclonal antibody (A–D) or anti-βgal antibody (E–F). Anterior is to left. (A,C,E) Dorsal focal plane: focus is on the commissural axons. (B,D,F) Ventral focal plane: focus is just below the epidermal layer. (A,B) *Gal4/UAS-repo*; repo^{rk2}/+, (*repo* heterozygote). Embryo shows wild-type morphology and high levels of Repo expression. (C,D) *repo-Gal4/UAS-repo; repo^{rk2}*, (*repo* homozygote lacking endogenous Repo protein). Embryo shows partial rescue of *repo* phenotype. (E,F) *repo^{3702}*, (*repo* homozygote with *lacZ* enhancer trap in *repo* locus). *repo* null mutant embryo shows fewer lateral glia than rescue embryos, with longitudinal glia and peripheral glia missing. LG, longitudinal glia; PG, peripheral glia. Scale bar, 20 μm.

for driving weak reporter expression. Using the doubly-degenerate consensus sequence (A/G)CCCG(C/T)AT (a hybrid of the Akiyama et al. (1996) and Schreiber et al. (1997) sequences), and adding one mismatch, we find two additional imperfect sites that are included in our constructs. These sites as well as others may be responsible for residual expression. Since our experiments conclusively show the direct dependence of *repo* reporter expression on Gcm binding sites, we did not continue with mutations at these additional sites.

2.5. Ectopic expression of Repo induces ectopic repo −4.3-lacZ expression in the epidermis

Our reporter lines allowed us to test whether Repo can regulate its own enhancer sequences. To test whether



GBS#	Wt GBS	Round 1	Round 2	Round 3
1 (r)	TCCCGCAT	TCCCGCAT	AAACGCAT	AAACGCAT
2 (r)	GCCCGCGT	GCCCGCGT	GAACGCGT	GAACGCGT
3	GCGCGCAT	GCGCGCAT	GAACGCAT	GAACGCAT
4	ACCCGCAG	ACCCGTCG	ACCCGTCG	AAACGTCG
5	ACCCACAT	ACCCATCT	ACCCATCT	AAACATCT
6	GCCCGCAT	GCCCGTCT	GCCCGTCT	GAACGTCT
7 (r)	GCCCGCAT	GCCCGTGT	GCCCGTGT	GAACGTGT
8	GCCCACAT	GCCCATGT	GCCCATGT	GAACATGT
9	ACCAGCAT	ACCAGTGT	ACCAGTGT	AAAAGTGT
10	ACCCTCAT	ACCCTTGT	ACCCTTGT	AAACTTGT
11	GCCAGCAT	GCCAGTGT	GCCAGTGT	GAAAGTGT

Fig. 3. Mutation of Gcm binding sites causes reduced expression of *repo-lacZ* reporter constructs. (A) Table of Gcm binding sites at the *repo* locus examined in this study, showing wild-type and mutated sequences. Sequences with reversed orientation with respect to the *repo* promoter are indicated by (r). 'Imperfect' GBSs contain at least one base (purple) differing from the consensus sequence that would predict diminished Gcm binding according to Schreiber et al. (1998). The bases changed during each round of mutagenesis are highlighted in red. Bold and underlined type represent previously mutated sites. The position of each site relative to the *repo* transcription start site are as follows: GBS #1, −3859 to −3852; GBS #2 −3660 to −3653; GBS #3 −3649 to −3642; GBS #4 −2562 to −2555; GBS #5 −2279 to −2286; GBS #6 −2075 to −2068; GBS #7 −1865 to −1858; GBS #8 −1465 to −1458; GBS #9 −1342 to −1335; GBS #10 −1448 to −1141; GBS #11 −155 to −148. Consensus sequence: base pairs in the consensus site with strong influence on Gcm binding according to Schreiber et al. (1998) are marked in blue. R = A or G. (B) *repo-lacZ* reporter constructs. Red Xs represent mutated Gcm binding sites. (C–E) Dissected stage 16 embryos labeled with anti-βgal antibody. Anterior is up. (C) *repo −4.3-lacZ.* (D) *repo −4.3ΔGBS8-lacZ.* Mutation of eight proximal GBSs results in loss of expression in longitudinal glia (LG, arrow) and other glia. (E) *repo −4.3ΔGBS11-lacZ.* Mutation of all 11 GBSs show further reduction in reporter expression. Scale bar, 20 μm.

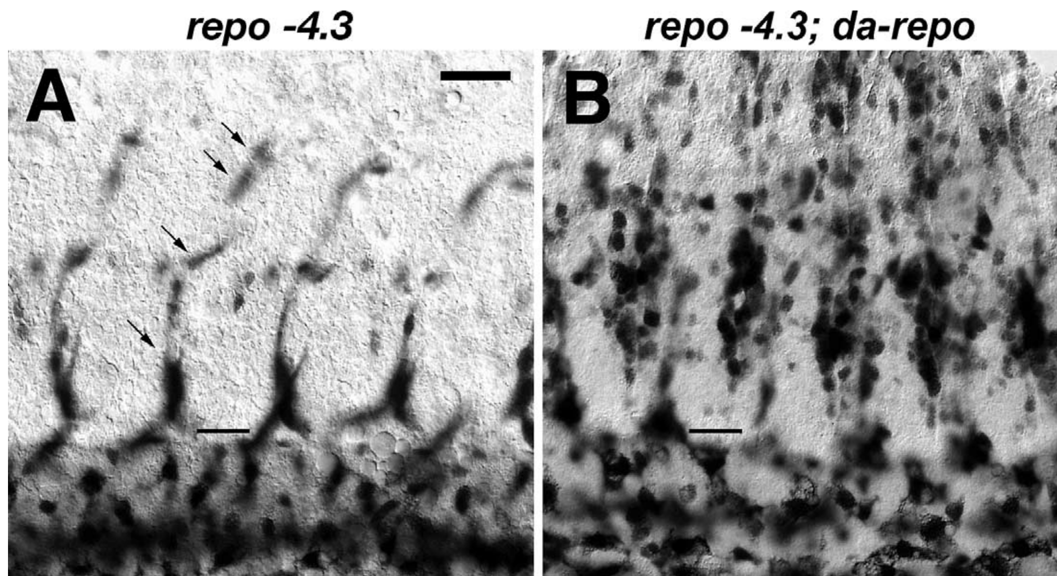


Fig. 4. Ectopic expression of Repo induces ectopic *repo −4.3-lacZ* reporter expression. (A,B) Dissected stage 16 embryo showing four adjacent abdominal segments of the lateral body wall labeled with anti-βGal antibody and focused on the epidermis (anterior left, dorsal up). Thin bar marks peripheral edge of CNS. (A) Wild-type *repo −4.3-lacZ* expression. Arrows point to peripheral glia that are out of focus. (B) *repo −4.3-lacZ; da-Gal4/UAS-repo* (*da-repo*). Ubiquitous expression of Repo induces βGal expression in the epidermis. Scale bar, 20 μm.

ectopic expression of *repo* is sufficient to activate *repo −4.3-lacZ* expression, we used *daughterless-Gal4* (*da-Gal4*) (Wodarz et al., 1995) to drive ubiquitous expression of *UAS-repo* throughout embryogenesis in embryos that also carry the *repo −4.3-lacZ* reporter gene. In *repo −4.3-lacZ; da-Gal4/UAS-repo* embryos, broad ectopic β-gal expression is observed in the epidermis (Fig. 4B). Interestingly, ubiquitously expressed Repo was not able to drive ectopic β-gal expression from the *repo −4.3-lacZ* transgene in neurons, nor in any tissue derived from the mesoderm.

2.6. Repo can act as a repressor within the CNS

The ability of *repo* to activate *repo-lacZ* reporter expression in the epidermis implicated an autoregulatory feedback mechanism in *repo* transcriptional regulation. This observation prompted us to enquire whether *repo* is required for normal reporter expression in glia. To test whether *repo* is necessary for reporter expression in glia, we crossed *repo −4.3-lacZ* into a *repo* null mutant background. In *repo* mutant embryos, *lacZ* expression, as detected by both anti-β-gal antibody and in situ hybridization probes to *lacZ* RNA, is initiated in glia in a normal pattern, but appears to diminish prematurely; however, because *repo* mutant embryos have a profound defect in the proliferation and survival of glia, it is difficult to distinguish whether the late diminished expression of *lacZ* we observe is due to the absence of *repo* autoregulation or is a secondary consequence of these glial cell defects (data not shown).

To obtain evidence that *repo* can act on *repo-lacZ* reporter constructs in glia, we decided to test whether ectopic expression of *repo* can act on reporters with mutated GBSs that lack expression in most glial cells. To do this, we crossed *repo −4.3ΔGBS11-lacZ* into a *da-Gal4/UAS-repo* background. Like the wild-type version of the reporter, ubiquitous expression of *repo* is able to activate *repo −4.3ΔGBS11-lacZ* in the epidermis (Fig. 5B). However, we did not find activation in glia, or in other tissues besides the epidermis. Instead, we observed an absence of expression in the cell body glia normally observed in *repo −4.3ΔGBS11-lacZ* (Fig. 5A,B), suggesting that *repo* can repress *repo −4.3ΔGBS11-lacZ* expression in CNS glia.

2.7. repo upstream DNA can be subdivided into multiple cis-regulatory regions

To map elements within the *repo* genomic region, fragments of the *repo* −4.3 genomic DNA were reintroduced in the *hs43-lacZ* reporter vector and transgenic lines were generated; at least four independent lines for each construct were examined for β-gal expression. Constructs and their expression patterns are summarized in Fig. 6. For each construct we scored β-gal expression in different tissues, including subsets of glial cells. Unless otherwise noted, embryos carrying only a single copy of each construct were analyzed. Except for two constructs that showed no activity (*repo −0.7* and *repo −4.3/−2.8*, Fig. 6A), all constructs showed activity in glia, but in different patterns. By comparing constructs we were able to identify *cis*-regulatory regions of DNA that produce consistent expression patterns in glial subsets, and a region required for inhibiting expression in the epidermis

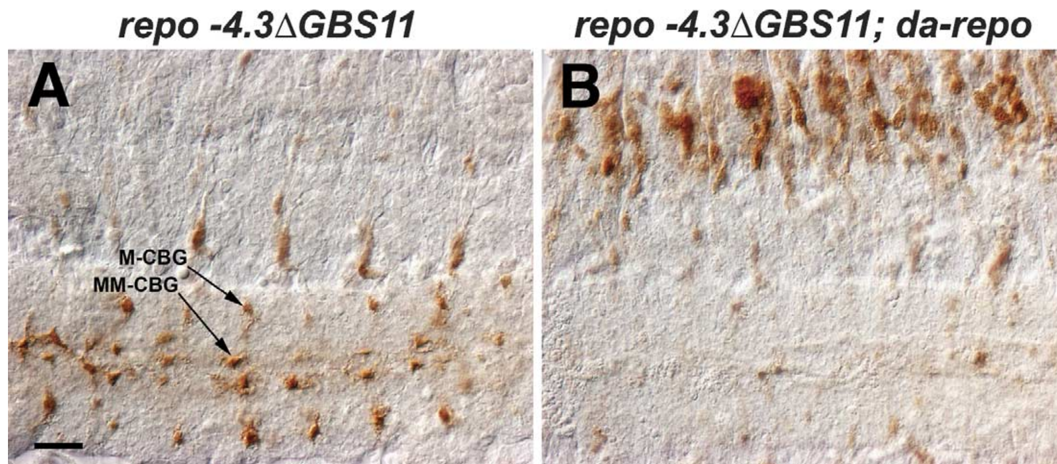


Fig. 5. Ectopic Repo induces GBS mutated reporter expression in the epidermis but represses in CNS glia. Dissected stage 16 embryos labeled with anti-βGal antibody (anterior left, dorsal up). (A) *repo−4.3ΔGBS11* embryo shows expression in M-CBG and MM-CBG. (B) *repo−4.3ΔGBS11; da-Gal4/UAS-repo (da-repo).* Ubiquitous expression of Repo induces βGal expression in the epidermis, but represses glial expression in the CNS. Scale bar, 20 μm.

(summarized in Fig. 6B). In the following sections, we describe our results in more detail.

2.8. A repressor element prevents inappropriate expression in the epidermis

Embryos carrying the *repo −4.3/−2.3-lacZ* construct show β-gal expression in the epidermis in specific patches (Fig. 7C,D). By contrast, embryos carrying *repo −4.3/−1.9-lacZ*, which is longer than *repo −4.3/−2.3-lacZ* by 470 DNA base-pairs (bp), do not show β-gal expression in the epidermis in (Fig. 7A,B). We conclude the 470 bp region (*repo −2.3/−1.9*) is required for inhibiting epidermal expression.

Within the 470 bp region are two GBSs. To determine whether the GBSs affect the ability of this region to repress epidermal expression, we mutated these two sites in our *repo −4.3/−1.9-lacZ* construct. Mutating the GBSs had little overall effect on the expression pattern, suggesting repression by the *repo −2.3/−1.9* region to be GBS independent (data not shown). Furthermore, we mutated all GBSs in our *repo −4.3/−2.3-lacZ* construct, which also had no effect on epidermal expression, even though glial expression in the CNS is reduced (Fig. 8D).

2.9. A distal element promotes expression in longitudinal glia, peripheral glia, subperineurial glia, and epidermis

Embryos carrying the *repo −4.3/−2.3-lacZ* reporter construct show strong β-gal expression in the epidermis (Fig. 7C,D). Embryos carrying the *repo −4.3/−2.8*, which is shorter than *repo −4.3/−2.3-lacZ* by 520 bp at the 3′ end, completely lack expression (data not shown). We conclude a 520 bp region (*repo −2.8/−2.3*), which is contained in *repo −4.3/−2.3-lacZ*, but not covered by *repo −4.3/−2.8,* promotes expression in the epidermis. Since *repo −4.3/−2.3-lacZ* lines also show expression in the LG, PG and SPG, which *repo −4.3/−2.8* also lacks, we also conclude *repo −2.8/−2.3* promotes expression in this glial subset. However, both *repo −2.3* and *repo −1.9,* but not *repo −1.1,* also show LG expression, suggesting that the region promoting LG expression extends through the region *−2.3/−1.1*. Similarly, *repo −2.3, repo −1.9* and *repo −1.1*, but not *repo −0.7*, show PG and SPG activity, suggesting the region responsible for PG, SPG expression extends through the region *−2.3/−0.7*.

2.10. A proximal element promotes expression in cell body glia

Embryos carrying a construct that contains the 1.1 kb genomic region proximal to the *repo* promoter show expression in PG, SPG and CBG, but not in LG (Fig. 8A). The *repo −1.1* region contains a single GBS; mutating this single GBS in the *repo −1.1-lacZ* construct causes a loss of activity in PG and SPG, yet retains activity in CBG (Fig. 8B). Embryos carrying the construct *repo −0.7-lacZ*, which contains the GBS, but lacks the distal part of the *repo −1.1* region by 350 bp, show a complete absence of activity (data not shown). These data demonstrate that the 350 bp proximal region (*repo −1.1/−0.7*) promotes specific activity in CBG that is independent of the GBS, and that a shorter construct containing the GBS is not sufficient to promote glial expression. These data also suggest that other factors besides Gcm promote CBG expression; and that Gcm protein binding to a single GBS acts synergistically with factors in the 350 bp fragment to produce glial-specific expression in PG and SPG, and increased expression in CBG (Fig. 8A,B).

2.11. Gcm binding sites act synergistically with repo cis-regulatory elements

The data we have presented so far show that different fragments of the *repo* upstream region confer different

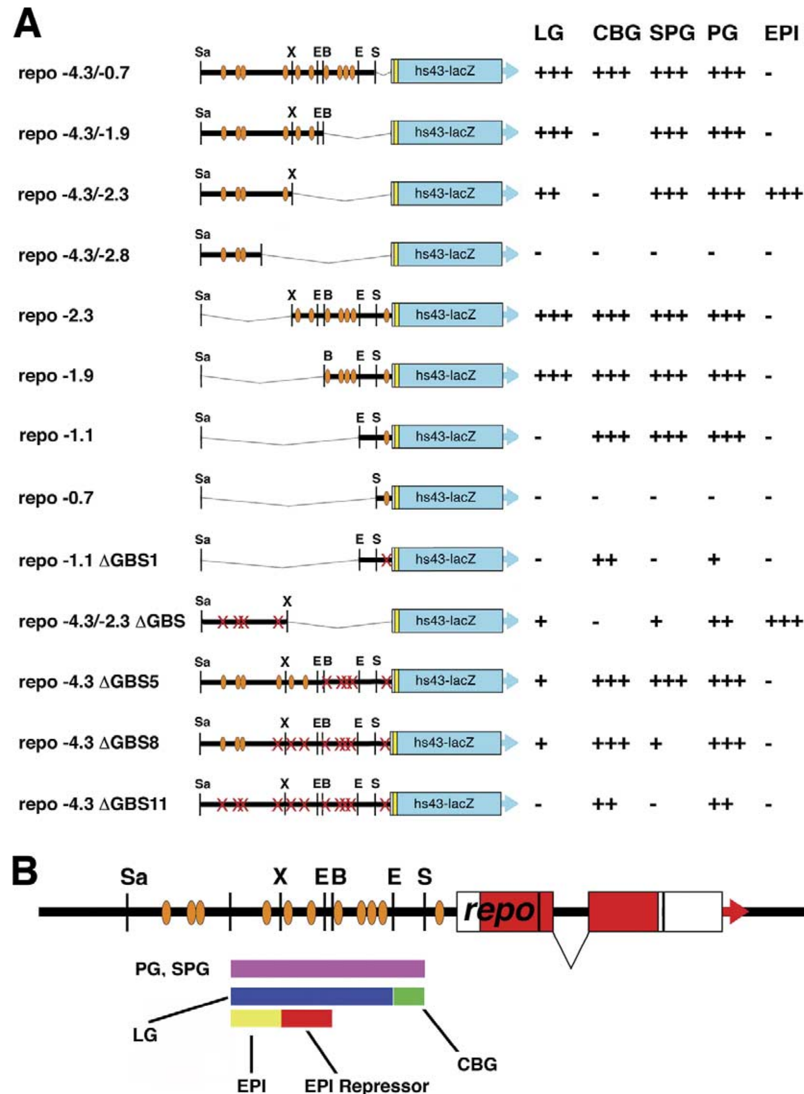


Fig. 6. (A) Summary of *repo-lacZ* reporter constructs and their expression. LG, longitudinal glia; CBG, cell body glia; SPG, subperineurial glia; PG, peripheral glia; EPI, epidermis. +++ represents moderate to strong expression; ++ represents weak expression; + represents very weak expression. (B) Summary: regions promoting specific activities are shown as colored bars below the map of the *repo* genomic region.

specific activities in subsets of glia, and that mutation of GBSs within each fragment reduces the strength of these specific activities, suggesting that Gcm may act synergistically with other *cis*-acting factors at the *repo* locus. To explore the effect of the number and the position of the GBSs on the activity of *repo-lacZ* reporters, we compared two constructs—one which has the proximal −1.9 kb of DNA deleted (*repo −4.3/−1.9-lacZ*), and one which has the full length repo −4.3 genomic region, but with all five GBSs in the proximal −1.9 region mutated (*repo −4.3ΔGBS5*) (see Fig. 6A).

Embryos carrying the *repo −4.3/−1.9-lacZ* construct show strong β-gal expression in LG and SPG (Fig. 7A,B). In contrast, embryos carrying *repo −4.3ΔGBS5-lacZ* have additional expression in CBG (Fig. 7F,H) and diminished expression in LG and SPG (Fig. 7E–H). Doubling the number of copies of *repo −4.3ΔGBS5-lacZ* increases the strength of β-gal expression, but not the overall pattern of specific activity (Fig. 7G,H). An even further weakening of specific activity in LG and SPG is observed when the first eight GBSs are mutated (*repo −4.3ΔGBS8-lacZ*, Fig. 3D). These data are consistent with our previous results showing that the proximal region confers specific expression in CBG. These data also show that GBSs synergize with *cis*-regulatory elements at a distance—mutating proximal GBSs appears to affect the strength of the LG and SPG activity that is conferred by distal elements.

3. Discussion

While glial-specific genes are dependent on *gcm*, synergistic interactions between Gcm and downstream transcription factors are required for regulation of glial

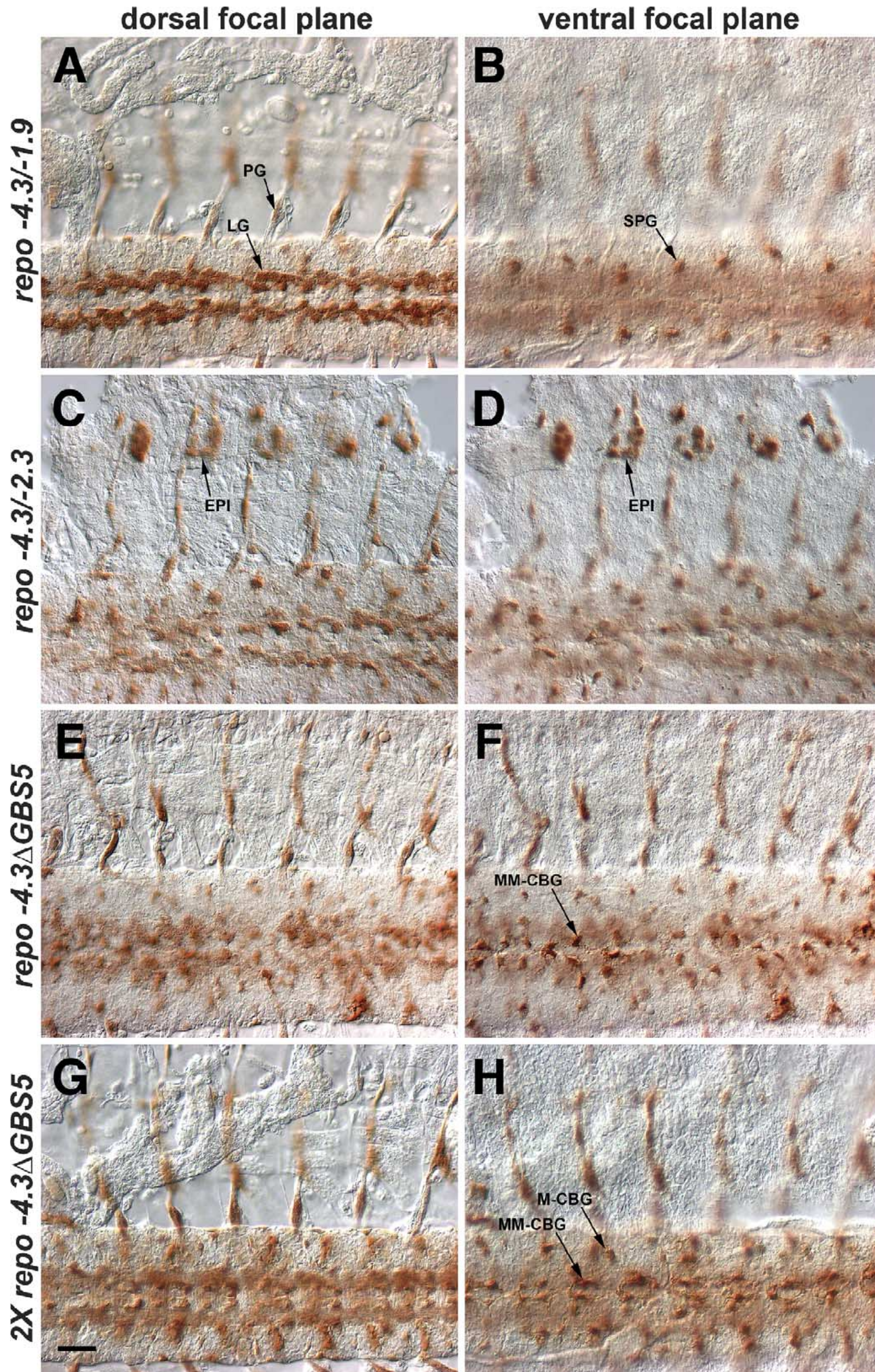


Fig. 7. Expression of *repo-lacZ* reporter fragments. Dissected stage 16 embryos labeled with anti-βGal antibody (anterior left, dorsal up). (A,C,E,G) Dorsal focal plane focuses on the longitudinal commissures. (B,D,F,H) Ventral focal plane focuses above the epidermal layer where SPG and CBG are located. (A,B) *repo −4.3/−1.9-lacZ* shows expression in longitudinal glia (LG) peripheral glia (PG) and subperineurial glia (SPG). (C,D) *repo −4.3/−2.-lacZ* shows expanded expression in the epidermis (EPI) as well as LG and SPG staining in the CNS. (E–H) *repo −4.3ΔGBS5-lacZ* contains mutated GBSs at sites 7–11 (see Fig. 3B) and the same number of wild-type GBSs as *repo −4.3/−1.9-lacZ. repo −4.3ΔGBS5-lacZ* shows expression in CBG as well as LG and SPG, which becomes more apparent in lines containing two copies of the reporter (G,H). Scale bar, 20 μm.

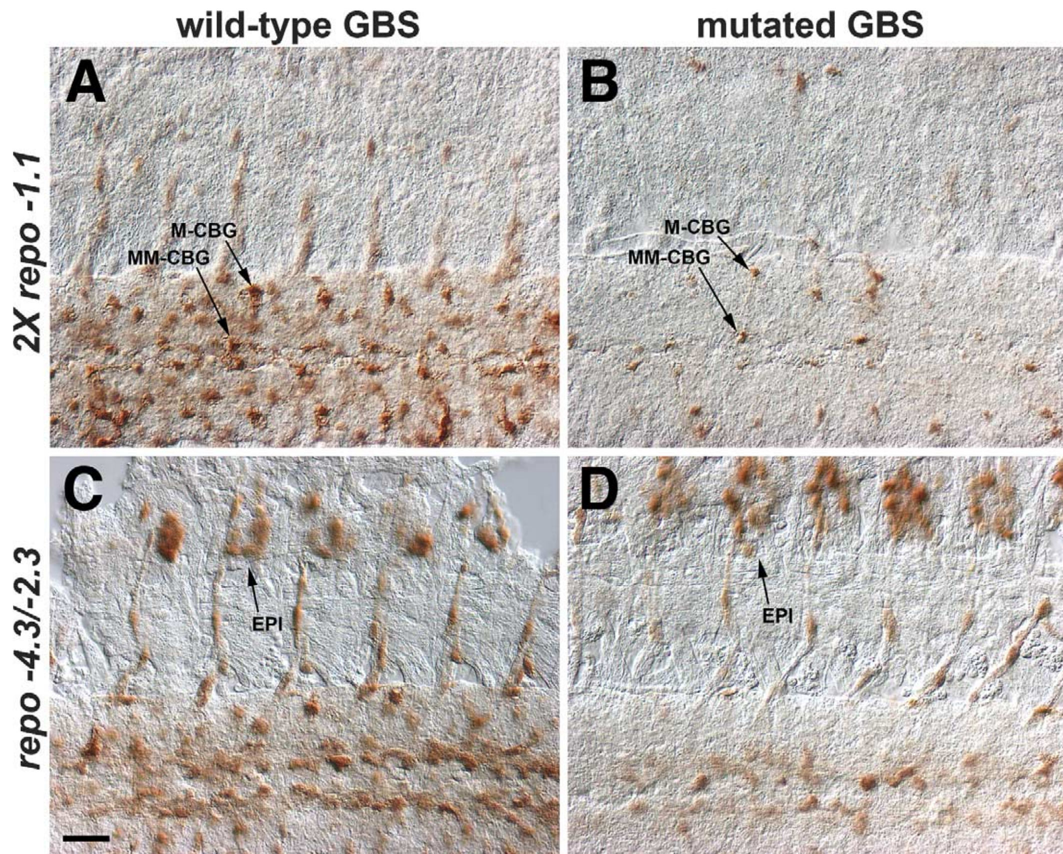


Fig. 8. *repo −1.1* and *repo −4.3/−2.3* still show expression in glial cells when GBSs are mutated. Dissected stage 16 embryos labeled with anti-βGal antibody (anterior left, dorsal up). (A) Embryo carrying two copies of *repo −1.1-lacZ* shows expression in CBG, SPG and PG. (B) Embryo carrying two copies *repo −1.1ΔGBS1-lacZ* where the single GBS is mutated shows expression in CBG only. (C) *repo −4.3/−2.3* shows expanded expression in the epidermis (EPI) as well as LG and SPG. (D) *repo −4.3/−2.3ΔGBS* where GBSs are mutated shows diminished expression in the CNS glia, but expanded expression in the epidermis is maintained. Scale bar, 20 μm.

genes as well as suppression of neuronal differentiation (Giesen et al., 1997; Granderath et al., 2000; Yuasa et al., 2003). One such factor is the homeodomain factor encoded by the *repo* locus. *repo* is expressed exclusively in Gcm positive glia, but not in Gcm positive hemocytes; transient expression of *gcm* is followed by maintained expression of *repo*. Maintained *repo* expression and lack of expression in hemocytes cannot be explained by *gcm* regulation alone. Here, we attempt to address these observations by examining *cis*-regulatory DNA involved in *repo* transcription.

3.1. repo is regulated by multiple factors

We examined the activity of a 4.2 kb fragment 5′ to the *repo* start codon that contains multiple Gcm binding sites, whose consensus sequence has been determined in detail by in vitro and structural studies (Akiyama et al., 1996; Cohen et al., 2002, 2003; Schreiber et al., 1998; Shimizu et al., 2003). Based on the presence of these binding sites, *repo* is predicted to be a target of Gcm. Mutation of eleven binding sites, as defined by Akiyama et al. (1996), results in significant loss of reporter expression in glia, demonstrating the direct regulation by Gcm. Mutation of these sites also demonstrates that 'imperfect' GBSs are responsible for a moderate level of *repo* expression. Because of two additional imperfect binding sites not included in our mutations, our result do not discount the possibility that Gcm activates a residual level of expression of *repo −4.3ΔGBS11*. However, we find that a smaller −1.1 kb region driving expression in CBG and SPG still retains CBG expression even when all identifiable GBSs are mutated. This last result suggests other factors in addition to Gcm activate *repo* expression in glia.

One of these factors may be *repo* itself. We found that ubiquitously expressed Repo activates our reporter constructs. Interestingly, we found strong reporter activation in the epidermis but not in neurons, glia, or in any mesodermal tissue, and we also found that expression of *repo* actually represses the glial expression driven by *repo −4.3Δ11GBS-lacZ*, suggesting Repo can act as a repressor in some contexts. *repo*'s ability to act as a repressor was surprising given that previous studies have shown Repo to be a transcriptional activator, acting through ATTA DNA motifs (Yuasa et al., 2003). However, our studies do not address whether or not Repo regulation of *repo* enhancer constructs is direct. While our studies show Repo can repress activation, they do not

discount a scenario by which Repo auto-activates in the presence of unmutated Gcm binding sites.

The above observations and several lines of evidence suggest that negative factors are acting on the *repo* enhancer region to regulate its expression. First, despite expression of Gcm in hemocytes, neither endogenous *repo* nor our reporter constructs are ever expressed there. Second, ectopic expression of *repo-lacZ* reporters by *UAS-repo* is permissive in the epidermis, but not in neurons. One possibility is that a pan-neural repressor prevents *repo* activation in neurons as well as glia in the absence of *gcm*. We believe *gcm* would be able to displace the repressor to allow for activation by *repo*. If this model is correct, it may explain why *repo* reporters do not activate in glia in when GBSs are mutated, as repressors would still be present.

Our studies show that regulation of *repo* by transcriptional repression is not limited to the CNS. Characterization of smaller regions spanning *repo −4.3* reveals a repressor element (within *repo −2.3/−1.9)* that prevents inappropriate expression in the epidermis. *repo −4.3/−2.3*, which lacks the region containing this repressor element, shows expanded expression in the epidermal layer and also reveals the presence of an element (within *repo −2.8/−2.3*) that promotes epidermal expression. Activity is GBS independent, consistent with the observation that *gcm* is not expressed in the epidermis. These results show that the activity of factors expressed in the epidermis need to be repressed to maintain glial-specific expression of *repo*. This epidermal repression may also represent a mechanism to prevent activation of *repo* by factors shared between the epidermis and the CNS. Tight regulation and appropriate expression of *repo* is essential, since ectopic expression of *repo* in the epidermis results in lethality (Lee and Jones, unpublished observations).

Our analysis reveals that expression of *repo* in different glial subsets is promoted by other factors in addition to Gcm. We found regions that promote expression in LG, PG, SPG, and epidermis, and a proximal region that promotes expression in CBG. Activity is GBS dependent since mutation of GBSs reduce the strength of these specific glial activities. Despite having one or more GBSs, subfragments of the 4.2 kb region promote reporter expression in subsets of glia, rather than in all lateral glia where Gcm expression is found. This observation suggests Gcm acts with other factors to regulate spatial *repo* expression. Furthermore, our experiments show that mutating proximal GBSs affected the strength of glial-specific activities conferred by distal elements, suggesting that synergistic interactions between Gcm and other *cis*-acting factors can occur at some distance from one another on the DNA sequence.

Our results extend observations by Granderath et al. (1999) that Gcm acts synergistically with glial-specific factors to control downstream genes. We show *repo* regulation is dependent on several *cis*-regulatory elements that synergize with Gcm for activation and repression. Collectively, our results show that multiple factors promote *repo* expression in specific subsets of glia. Since Repo protein, to the best of our knowledge, is expressed at equal levels in all Gcm positive glia, the question of why *repo* transcription depends on additional regulatory factors is subject to speculation. Glial cells have multiple functions that require transcriptional complexity for assignment and regulation. Moreover, failure to tightly regulate the expression of glial genes can result in neural dysfunction and lethality. While *repo* is involved in the terminal differentiation of all lateral glial cells, whether or not *repo* may contribute to the specification of glial cell diversity is not clear. This study represents a step towards understanding Gcm dependent glial-specific gene regulation and how expression is controlled in subsets of glial cells through multiple *cis*-regulatory elements and factors.

4. Experimental procedures

4.1. Isolation of genomic DNA and construction of repo-lacZ reporter genes

Genomic clone pRPrepo^{4} (Fig. 1A) containing 8.7 kilobases (kb) of DNA flanking the P-element insertion associated with the *repo* allele *repo^{4}* (Xiong et al., 1994) was recovered by the plasmid rescue technique (Pirrotta, 1986). A 4.2 kb fragment of DNA from pRPrepo^{4}, containing eleven reported Gcm binding sites (Akiyama et al., 1996), was subcloned into pBluescript SK+ (Stratagene) to create plasmid p*repo* −4.3-SK+; this plasmid was used for site-directed mutagenesis, sequencing, and shuttling into *lacZ* reporter vectors.

Site-directed mutagenesis of Gcm binding sites (GBS) was performed using the Quick Change Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions. For nomenclature and wild-type sequence of GBSs, and the specific mutations introduced in each GBS see Fig. 3A. GBSs were mutated using the following oligonucleotides as forward primers and their complements (not shown) as reverse primers: for GBS 1 we used forward primer GATATTTATGCGTTATTTAAATTGATCTTAACGAAGC (where underlined nucleotides are mutated from wild-type); for GBS 2, forward primer GGGAATGGGCAACGCGTTCATGGCG CGCATCC; for GBS 3, forward primer GGAATGGGCAACGCG*TT*CATGGAACGCATCCACATTGCT (where previously mutated bases in neighboring GBS 2 are shown in italics), for GBS 4, forward primer GAAAACCATACCCGTCGTTTGCCAACTCTTTCTTTCTGGC; for GBS 5, forward primer CCTTGAAGCCAGACCCATCTAATTGGCACATTG GC; for GBS 6, forward primer GACTTCGACTTTGCCCGTCTTTCGGAACC TGC CAGG; for GBS 7, forward primer GTTTTTCATTCGATCAACACGGGCCGGTCTTTGA GG; for GBS 8, forward primer CGATTGCAATGCCCATGTGATTCGGCAGCC; for GBS 9, forward primer GCGATCGAA

GTACCAGTGTCTGGCCAAAAGGTC; for GBS 10, forward primer CGAATGGAACCACCCTTGTGAGTC-CAATTGGAACC; and, for GBS 11, forward primer GTCACACACGCCAGTGTGAAACGCACAGAGCG. An additional round of mutagenesis was performed on the mutated forms of GBSs 4-11 using the following forward primers and their complements (not shown) as reverse primers: for GBS 4, forward primer GAAAA CCATAAACG*TC*GTTTGCCAACTCTTTCTTTCTGGC (where previously mutated bases from first mutagenesis round are shown in italics); for GBS 5, forward primer CCTTGAAGCCAGAAACA*TC*TAATTGGCACA-TTGGC; for GBS 6, forward primer CCCAGAGACTTC-GACTTTGAACG*TC*TTTCGGAACC; for GBS 7, forward primer CATTCGATCAA*CA*CGTTCCGGTCTTTGAG-GACAG; for GBS 8, forward primer CCCTCTTCGATTG-CAATGAACA*TG*TGATT CGGCAGC; for GBS 9, forward primer CCTCGGCGCG ATCGAAGTAAAA G*TG*TCTGGCCAA AAGG; for GBS 10, forward primer GCAACCTCAAATGGAACCAAACT *TG*TGA GTCCAA TTGG; for GBS 11, forward primer GGGCAAGCGGAAAAGTCACACACGAAAG*TG*TGAA ACGC. Mutated constructs were sequenced to check for accuracy.

To construct *repo-lacZ* transgenic reporter lines, wild-type and mutated genomic fragments were cloned into the P element reporter vector pCasPeR-hs43-lacZ (abbreviated *hs43-lacZ*) (Thummel and Pirrotta, 1992). The *hs43-lacZ* vector contains the *lacZ* gene and a minimal *hsp70* promoter. Reporter constructs were introduced into flies by P element-mediated germ line transformation (Rubin and Spradling, 1982).

4.2. *D. melanogaster* stocks and genetics

w^{1118} and $y^{1}w^{67c23}$ were used for generating transgenic lines. The *gcm* null allele was $gcm^{\Delta P1}$ and the *UAS-gcm* lines were *P{UAS-gcm.J}2* and *P{UAS-gcm.J}3* as described previously (Jones et al., 1995). *UAS-repo* lines were generated by inserting the *repo* cDNA *repo*-2.6 (Xiong et al., 1994) into pUAST, a UAS reporter P element vector (Brand and Perrimon, 1993) to generate P{UAS-*repo*}. *repo-Gal4* lines were generated by inserting the 4.2 kb *repo* genomic fragment into the pPTGAL Gal4 P-element vector (Sharma et al., 2002) to make P{*repo-Gal4*}. Several lines carrying P{*repo-Gal4*} or P{*UAS-repo*} insertions were generated. Ectopic expression of *gcm* or *repo* was achieved by crossing *UAS-gcm* lines or *UAS-repo* lines with *sca-Gal4* (drives expression in neuroblasts and their progeny) (Klaes et al., 1994), *daughterless-Gal4* (*da-Gal4*) (drives ubiquitous expression throughout embryogenesis) (Wodarz et al., 1995), or our *repo-Gal4* (drives expression in all lateral glia). Standard genetic techniques were used to combine *repo-lacZ* reporter lines with *sca-Gal4, da-Gal4* and *repo-Gal4* for ectopic expression experiments.

4.3. Immunohistochemistry

Horseradish peroxidase (HRP) immunohistochemistry and embryo dissection were done as previously described (Patel, 1994). Rabbit anti-β-galactosidase (β-gal) antibody (Cappel) was used at 1:10,000 dilution. Anti-Repo Monoclonal antibody MAb 8D12 (Alfonso and Jones, 2002) was used at a 1:5 dilution. Secondary antibodies conjugated to HRP (Jackson Immunoresearch) were used at 1:300 dilutions. Secondary antibodies were detected using HRP/Diaminobenzidine (DAB) reaction.

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