repo encodes a glial-specific homeo domain protein required in the *Drosophila* nervous system

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We report the identification of a *Drosophila* locus, *reversed polarity* (*repo*). Weak *repo* alleles were viable but affected glia in the optic lobe, resulting in a reversal in polarity of the electrophysiological response to light in the adult. Strong *repo* alleles caused defects in embryonic glia and resulted in embryonic lethality. Expression of *repo* appeared to be specific to glia throughout development. In the adult visual system, *repo* was expressed in laminal glia, medullar glia, and subretinal cells; in the embryo, *repo* was expressed in nearly all of the identified glia in the central and peripheral nervous systems except midline glia. The *repo* gene encoded a homeo domain protein suggesting that it might be a transcriptional regulator of genes required for glial development.

[Key Words: *Drosophila*; glia; homeo domain; development; vision; neurogenesis]

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Glial cells, the major support cells in the nervous system, have been suggested to have several functions. In vertebrates, these include roles in electrical insulation, ionic homeostasis, and provision of nutrients and factors necessary for neuronal survival and regeneration of peripheral nerves (Roitbak 1983; Vernadakis 1988; Fawcett and Keynes 1990; Barres 1991; Hatten et al. 1991; Buchanan and Benzer 1993; Marchionni et al. 1993; Masu et al. 1993). Glia also appear to play roles in neuronal development (Rakic 1971; Silver and Rutishauser 1982; Silver et al. 1982; Doucette 1990; Stitt et al. 1991; Eccleston 1992).

The morphology of many glial cell types has been described in a variety of insects; however, the functions of the glia are not understood as well as in vertebrates (for review, see Carlson and Saint Marie 1990). Nevertheless, it seems likely that invertebrate glia perform many of the same functions in the nervous system, such as electrical insulation and ionic homeostasis, as in vertebrates (Carlson and Saint Marie 1990). Several studies indicate that glia play important roles in axonal pathfinding in the insect nervous system (Bastiani and Goodman 1986; Fredieu and Mahowald 1989; Jacobs and Goodman 1989). A recent study has shown that a factor secreted by glia is capable of negatively regulating the proliferation of neuronal precursor cells in *Drosophila* (Ebens et al. 1993). In addition, analyses of the *dropdead* locus has led to the

suggestion that disruption of glial function contributes to neural degeneration (Buchanan and Benzer 1993).

Glial development has been characterized in a number of vertebrate systems (for review, see Raff 1989; Cameron and Rakic 1991; Goldman and Vaysse 1991; Le Douarin et al. 1991; Jessen and Mirsky 1992). In *Drosophila*, although many glial cell types have been described (Fredieu and Mahowald 1989; Klämbt and Goodman 1991; Udolph et al. 1993), the origins of the glia are known in only a few cases (Crews et al. 1988; Doe et al. 1988; Jacobs et al. 1989; Klämbt et al. 1991; Winberg et al. 1992; Giangrande et al. 1993; Udolph et al. 1993).

A number of *Drosophila* loci have been identified that are expressed in embryonic glia, in addition to other cell types. These include a group of genes, such as the *spitz* group (*single-minded*, *slit*, *Star*, *spitz*, *rhomboid*, and *pointed*), *breathless* and *orthodenticle* (*otd*), that are expressed in a group of glia, called midline glia, which lie adjacent to the commissures (axon tracts that link the two longitudinal connectives; Crews et al. 1988; Thomas et al. 1988; Bier et al. 1990; Finkelstein et al. 1990; Nambu et al. 1990; Rothberg et al. 1990; Klämbt et al. 1991, 1992; Rutledge et al. 1992; Klämbt 1993). The phenotypes of these mutants indicate that the midline glia play an important role in the separation of the two commissures. Other loci, such as *prospero* (*pros*), *otd*, *pointed* and *fushi tarazu* (*ftz*) are expressed in longitudinal glia as well as in neurons and other cell types (Doe et al. 1988, 1991; Finkelstein et al. 1990; Vaessin et al. 1991; Klämbt 1993). The longitudinal axon tracts are ei-

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ther missing or defective in these mutants, but it is not known whether the phenotypes are attributable primarily to abnormalities in the longitudinal glia or in the neurons that form these pathways. *Drosophila* genes have also been identified that are expressed in glia in the visual system. These include *cut*, a locus regulating sensory organ identity, *tramtrack*, a gene encoding a transcription factor required for eye development and embryogenesis, and *optomotor-blind*, a putative transcription factor required in the optic lobe (Blochlinger et al. 1993; Poeck et al. 1993; Xiong and Montell 1993). Each of these genes is expressed in either photoreceptor cells or cone cells in addition to glia. Recently, the *Drosophila anachronism* locus has been identified and shown to encode a glycoprotein that is secreted by glia and inhibits neuroblast proliferation (Ebens et al. 1993).

We report the isolation of a mutation in a locus, *reversed polarity* (*repo*) that encoded a glial-specific homeo domain protein. This locus appeared to be required for proper differentiation of glia in the visual system and in the embryonic nervous system. A weak mutation in *repo* resulted in a dramatic electrophysiological phenotype attributable to defects in glia in the adult visual system. Strong *repo* alleles resulted in embryonic lethality.

Results

repo mutant identified on the basis of a reversed-polarity ERG phenotype

The *repo* locus was identified in a screen for recessive autosomal P-element-induced mutations affecting the physiology of the adult visual system by performing electroretinogram (ERG) recordings, an extracellularly recorded, light-evoked mass response of the eye. The wild-type ERG consists of a corneal-negative sustained component and a transient component, the off-transient, which is coincident with the cessation of the light stimulus (Fig. 1A). The sustained component of the ERG corresponds mainly to summed responses of all the cells in the retina and the off-transient originates from the neurons in the lamina (L-neurons; Buchner 1991; Minke and Selinger 1992). The *repo*¹ mutant was characterized by an ERG of reversed polarity (Fig. 1B). No other lines, among 7000 tested, exhibited this phenotype. The *repo*¹ allele was a weak allele (hypomorphic) and strong alleles (*repo*³ and *repo*⁴, identified among a collection of P-element-induced lethal lines (Karpen and Spradling 1992), caused lethality during embryogenesis (Table 1).

Each of the four *repo* alleles (*repo*¹, *repo*², *repo*³, and *repo*⁴) contained a single P-element that mapped to 90F1-2 by in situ hybridization to polytene chromosomes using a P-element specific probe (data not shown). The *repo* ERG and embryonic lethal phenotypes were caused by the P element because, upon excision of the P element from the *repo*¹ or *repo*⁴ alleles, a high percentage of the lines reverted to wild-type (see Materials and methods, Fly stocks and genetics). One of the lines, *repo*^{4e19}, was found to be a null allele (see below).

Figure 1. ERG and intracellular recordings of wild type and *repo*¹. (A) Wild-type ERG (Canton-S); (B) *repo*¹ ERG; (C) wild-type intracellular recording; (D) *repo*¹ intracellular recording. The on-transients were typically not detected in the ERGs because of limitations in the sampling rate of the analog-digital converter. An event marker below the recordings indicates the initiation and cessation of the light stimulus. The duration of the light stimulus was 5 sec for the ERGs and 10 sec for the intracellular recording. The vertical lines adjacent to the left end of the event markers in A and C are 10 mV scales. The recordings shown were representative of flies <7 days old.

The unique repo ERG phenotype was not attributable to a defect in photoreceptor cells

The phenotypes of all previously characterized *Drosophila* mutants that affect the sustained component of the ERG have been shown to be attributable to defects in

Table 1. *repo* Alleles

Genotype	Phenotype
<i>repo</i> ¹ / <i>repo</i> ¹	viable (<i>repo</i> ERG)
<i>repo</i> ² / <i>repo</i> ²	lethal
<i>repo</i> ³ / <i>repo</i> ³	lethal
<i>repo</i> ⁴ / <i>repo</i> ⁴	lethal
<i>repo</i> ¹ / <i>repo</i> ²	semilethal (<i>repo</i> ERG)
<i>repo</i> ¹ / <i>repo</i> ³	lethal
<i>repo</i> ¹ / <i>repo</i> ⁴	lethal
<i>repo</i> ¹ / <i>Df</i> (3R)P14	lethal

photoreceptor cells (for review, see Montell 1989; Smith et al. 1991; Minke and Selinger 1992). To determine whether the *repo* phenotype was attributable to a disruption of a photoreceptor cell function, intracellular recordings on photoreceptor cells were performed. The results demonstrated that the *repo* ERG was attributable to a defect in a cell type other than photoreceptor cells, as the polarity of the receptor potential in wild-type and *repo* photoreceptor cells was the same (Fig. 1C–D).

repo appeared to be expressed specifically in glia throughout development

The presumptive spatial localization of *repo* at several stages of development was determined by examining expression of the *lacZ* reporter gene present within the P element in *repo*², *repo*³, and *repo*⁴ heterozygous flies (the P element that induced the *repo*¹ mutation did not contain a *lacZ* reporter gene). The reporter gene appeared to reflect the expression of the *repo* gene accurately because the spatial distributions of the β -galactosidase and *repo* RNA were similar in embryos (see below).

The pattern of *repo lacZ* expression was similar to the spatial distribution of a known glial marker. In the adult stage, *repo* β -galactosidase staining was detected in the laminal and medullar regions of the optic lobes. The stained cells within the lamina were found to be associated with the proximal as well as distal surface of the laminal neuropil (Fig. 2A). The *lacZ*-positive cells in the medulla were distributed around the periphery (Fig. 2A). The nuclei of the *lacZ*-positive cells had a flattened appearance, a morphology distinct from the more rounded nuclei typical of neurons in the optic lobe. The *repo lacZ* distribution resembled the pattern of the glial marker, 3-109 (Figs. 2A and 3A; Klämbt and Goodman 1991; Winberg et al. 1992). Judging from the location of the *lacZ*-positive cells, nuclear morphology, and comparison with the 3-109 expression pattern, it appeared that these cells were glia. The lamina glia appeared to correspond to the marginal glia, epithelial glia, satellite glia, and subretinal cells (Trujillo-Cenoz and Mekamed 1973; Shaw 1977; Saint Marie and Carlson 1983; Cagan and Ready 1989). No *lacZ* expression was detected in photoreceptor cells or any cells in the retina.

We found that *repo lacZ* reporter gene expression was also restricted to glial cells in the third-instar larvae based on the characteristic position, morphology of the *lacZ*-positive cells, and comparison with the 3-109 expression pattern (Figs. 2B and 3C). β -Galactosidase staining was detected in a group of glial cells, subretinal cells, which reside in the eye disc at this stage of development (Fig. 2B; Cagan and Ready 1989). In the larval brain, the highest level of *lacZ* expression was in the region that develops into the optic lobes (data not shown).

In the embryo, the *repo lacZ* reporter was expressed in many nuclei in the embryonic central nervous (CNS) and peripheral nervous system (PNS; Fig. 4A). A similar expression pattern was detected by localizing *repo* RNA by the in situ hybridization technique (Fig. 4B). Many of the

Figure 2. Expression of *repo lacZ* in the wild-type and *repo* visual system. (A) Wild-type (*repo*³ heterozygote) adult head section; (B) wild-type (*repo*³ heterozygote) larval eye-antennal disc; (C) *repo* adult head section (*repo*¹/*repo*²); (D) *repo* (*repo*¹/*repo*²) larval eye-antennal disc. Wild-type tissue (A,B) was stained with an anti- β -galactosidase mouse monoclonal antibody and *repo* tissue (C,D) was stained for *lacZ* activity. (AN) Antennal disc; (ep) epithelial glia; (EY) eye disc; (ma) marginal glia; (mg) medulla glia; (RE) retina; (sa) satellite glia (sr) subretinal cells. *repo*² heterozygotes had an expression pattern similar to *repo*³ heterozygotes (data not shown). The adult head sections were prepared from flies <2 days old.

cells in the CNS were located around the longitudinal connectives and others prefigured the location where the intersegmental nerve root leaves the longitudinal connectives (Fig. 4C). In the PNS, some *lacZ*-positive cells were situated just outside of the CNS at a choice point of the two major nerve roots, the intersegmental and the segmental nerves (exit glia); others surrounded the peripheral nerves (peripheral glia; Fig. 4D). The *lacZ*-positive cells did not express a neuronal marker, ELAV (Fig. 5; Bier et al. 1988; Robinow and White 1988). Therefore, the cells expressing β -galactosidase appeared to be non-neuronal. Based on the positions and non-neuronal identities of these cells, we concluded that the *repo lacZ*-positive cells were glia. Many of these corresponded to previously identified glia: longitudinal, nerve root, exit, and peripheral (Jacobs et al. 1989; Klämbt and Goodman 1991). In addition to these axon-associated glia, other

Figure 3. Expression of glial markers in the visual system. X-gal staining (A–D). Anti-OTD antibody staining (E–F). (A) Wild-type adult (3-109 heterozygote) head section; (B) *repo* adult (3-109, *repo*^{1^{ed4}}/*repo*^{1^{ed4}}) head section; (C) wild-type (3-109 heterozygote) larval eye–antennal disc; (D) *repo* (3-109, *repo*^{1^{ed4}}/*repo*^{1^{ed4}}) larval eye–antennal disc; (E) larval eye disc from wild type (focal plane on the subretinal cells); (F) *repo*¹ larval eye disc (focal plane on the photoreceptor cells; there was no staining in the subretinal cells). (AN) Antennal disc; (ep) epithelial glia; (EY) eye disc; (LA) lamina; (ma) marginal glia; (ME) medulla; (RE) retina; (sa) satellite glia; (sr) subretinal cells. Head sections (A,B) were prepared from adult flies <2 days old. *repo*^{1^{ed4}} was generated by mobilization of the *repo*¹ P element and exhibited an ERG phenotype similar to *repo*¹ (data not shown). This line was used because it was *ry*[−] and facilitated crossing the 3-109 *ry*⁺ line into a *repo* background.

glia that expressed the *repo lacZ* marker included glia adjacent to cell bodies in the ventral nerve cord as well as that associated with the surface of the ventral nerve cord (data not shown). The midline glia did not express the *repo lacZ*.

Expression of glial markers altered in *repo*

To examine whether there were defects in glia in the *repo* visual system, we stained *repo* adults with a P-element enhancer trap insertion line, 3-109, which directs

glial-specific *lacZ* expression in subretinal cells, satellite glia, epithelial glia, marginal glia, and medullar glia (Winberg et al. 1992; Fig. 3A). When the 3-109 enhancer trap element was crossed into a viable *repo* allele, *repo*^{1^{ed4}}, there were few *lacZ*-positive cells (Fig. 3B). Furthermore, the level of β -galactosidase staining in the few cells that were still *lacZ* positive was low (Fig. 3B). We also stained wild-type and *repo*¹ adult heads with an antibody to OTD that stained many glia in the wild-type visual system (data not shown). In the *repo* visual system, the glia did not express OTD (data not shown).

Figure 4. Expression of *repo* in embryos. (A) Whole-mount heterozygous *repo*³ embryo stained with an anti- β -galactosidase mouse monoclonal antibody; (B) *repo* RNA expression detected by in situ hybridization to a wild-type whole-mount embryo; (C) higher magnification of the longitudinal glia (lg) and nerve root glia (ng) stained with anti- β -galactosidase; (D) higher magnification of the exit glia (eg) and peripheral glia (pg) stained with anti- β -galactosidase.

Figure 5. *repo lacZ*-positive cells do not express a neural marker. Double labeling of the *repo* heterozygous embryonic nervous system with anti-ELAV (mouse monoclonal) and anti- β -galactosidase (rabbit antiserum). The secondary antibodies were FITC-labeled goat anti-mouse and RITC-labeled goat anti-rabbit antibodies. Shown are the fluorescent images obtained by confocal microscopy. (A) *repo lacZ* expression (red); (B) ELAV (green); (C) *repo lacZ* (red), ELAV (green).

The observation that expression of both 3-109 and OTD was absent in most of the *repo* glia suggested that either there was a defect in glial cell differentiation or

that cell death had occurred. To distinguish between these possibilities, we stained *repo* adults with one of the lethal lines, *repo*², which produced a few viable adults in a heteroallelic combination with *repo*¹ (*repo*¹ is not a *lacZ* line). The few adults that emerged displayed an ERG phenotype similar to *repo*¹ (data not shown). The number of *lacZ*-positive cells in the *repo*¹/*repo*² visual system was similar to *repo*²/+, although the spatial distribution of some of the cells was abnormal (Fig. 2C). This result suggested that the lack of 3-109 and OTD staining was not attributable to absence of the *lacZ*-positive cells in young *repo* adults (<2 days old). In addition, the *lacZ*-positive cells in *repo* adults did not appear to be transformed into neurons because they did not express the ELAV neuronal marker (data not shown). However, in older *repo* adults (>7 days old), many of the lamina glia were absent (data not shown). Further experiments will be required to determine whether the disappearance of these cells in older flies was attributable to necrosis or apoptosis.

Developing eye discs and optic lobes from *repo*¹-third-instar larvae were also examined with the glial markers. The number and pattern of *repo lacZ*-positive cells in wild-type and *repo* eye discs was similar (Fig. 2B,D). Expression of 3-109 is detected in wild-type subretinal cells, and satellite, marginal, and epithelial glia early in glial development well before they acquire a fully differentiated phenotype (Winberg et al. 1992). Despite the pronounced change in 3-109 *lacZ* expression in lamina glia and subretinal cells in *repo* adults, similar numbers of cells expressed the 3-109 marker in wild-type and *repo* eye discs (Fig. 3C,D). OTD was expressed in the subretinal cells and all the photoreceptor cells in wild-type eye discs (Fig. 3E; Wieschaus et al. 1992). In *repo*¹ eye discs, OTD was expressed only in the photoreceptor cells (Fig. 3F) and not in the subretinal cells.

To determine whether there were defects in glia in the *repo* embryonic nervous system, we stained the embryos with an antibody to the *repo lacZ* in wild-type and strong *repo* alleles. We found that a similar number of cells expressed *lacZ* in wild-type and mutant embryos (Fig. 6A,B). We also stained wild-type and *repo* embryos with another glia marker, OTD. In wild type, OTD is expressed in the midline cells (including midline neurons and glia), longitudinal glia, exit glia, and peripheral glia (Finkelstein et al. 1990; Fig. 6C,E). In *repo* embryos, only the midline cells, which do not express *repo*, expressed OTD (Fig. 6D). No expression of OTD was detected in the longitudinal glia, exit glia, or peripheral glia (Fig. 6D,F).

Examination of CNS and PNS axon tracts in *repo* embryos

The *repo* embryonic nervous system was examined for morphological defects because *repo* was expressed specifically in the nervous system. The longitudinal axon tracts in wild-type embryos, stained with BP102, appear as two continuous parallel bundles extending the length of the embryo. Between stages 13 and 15, there is con-

Figure 6. Expression of glial markers in embryos and the *repo* embryonic CNS axon pattern. Staining performed with whole-mount embryos and HRP-conjugated secondary antibodies. (A) *repo*⁴ heterozygous embryo stained with a mouse anti-β-galactosidase antibody; (B) *repo*^{4e25} homozygous embryo stained with a mouse anti-β-galactosidase antibody [similar staining was obtained with the null allele *repo*^{4e19} (data not shown)]; (C) longitudinal glia (lg) and midline cells (mc) in wild-type embryo stained with anti-OTD; (D) *repo*³ embryo stained with anti-OTD did not show longitudinal glia expression; (E) wild-type embryo stained with anti-OTD showed exit glia (eg) expression; (F) *repo*³ embryo stained with anti-OTD did not show expression in exit glia; (G) wild-type stage 13 and stage 15; (H) CNS axons stained with BP102; (I) *repo*³/Df(3R)P14 axons stained with BP102.

condensation of the CNS resulting in thickening of the longitudinal tracts (Fig. 6G, H). This condensation appeared normal in the strongest *repo* alleles, *repo*³ and *repo*⁴. In 30–50% of *repo*³/Df(3R)P14 and *repo*⁴/Df(3R)P14 stage 15 embryos there was thinning in the longitudinal tracts in some segments apparently due to poor condensation

(Fig. 6I). Thus, it was unclear whether the strongest *repo* alleles, *repo*³ and *repo*⁴, represented the null phenotype. Another *repo* allele, *repo*^{4e19}, obtained by mobilization of the P element from the *repo*⁴ allele, appeared to be a null allele because there was an imprecise excision of the P element resulting in deletion of part of the *repo* protein-coding region (see below). *repo*^{4e19} embryos exhibited a phenotype similar to *repo*³ and *repo*⁴ (data not shown). These results suggested that there may be another gene within the deficiency whose absence contributed to the improper condensation of the longitudinal axon tracts. No defects in PNS axons were detected in *repo* embryos stained with 22C10 (data not shown).

Identification of *repo* mRNA

The *repo* genomic region was cloned by virtue of P-element insertions (see Materials and methods). The positions and orientations of the P-element insertions in the four *repo* alleles are shown in Figure 7. In addition, the approximate segment of *repo* genomic DNA deleted in *repo*^{4e19}, which removed the initiating ATG codon (see Materials and methods), is indicated.

Several lines of evidence indicated that *repo* encoded a 3.5-kb mRNA. First, a 3.5-kb mRNA was identified on RNA blots using probes flanking the P-element insertion sites. Second, each of the P elements in the four *repo*

Figure 7. Physical map of *repo* locus. A 17-kb portion of the *repo* genomic region is represented by the bottom horizontal line demarcated in kilobase pairs and by the restriction sites *Bam*HI (B), *Eco*RI (E), *Sal*I (S), *Xba*I (X). The insertion sites of the mutator P elements from *repo*¹, *repo*², *repo*³ and *repo*⁴ are indicated by the vertical arrows above the genomic DNA. The orientations of the *lacZ* genes in the P elements are indicated by the horizontal arrows. The middle horizontal line represents *repo*^{4e19} genomic DNA. The break in this line indicates the region deleted in *repo*^{4e19} genomic DNA. The approximate transcribed region, determined by probing Southern blots containing *repo* cloned genomic DNA with ³²P-labeled *pcrepo*-2.6 and *pcrepo*-2.2 cDNAs, is indicated by the boxes. The horizontal lines extending from the boxes indicate the extent of uncertainty in the mapping. The translated region is indicated by the portion of the box marked with a hatched box. The 5'- and 3'-untranslated regions are represented by the open boxes.

alleles interrupted the transcription unit encoding the 3.5-kb mRNA. The *repo*¹, *repo*², and *repo*⁴ P elements inserted into introns, and the *repo*³ P element inserted into the 5'-untranslated region (Fig. 7). Third, the concentration of the 3.5-kb mRNA was reduced in the *repo*¹ hypomorphic allele (Fig. 8). A heterogeneous population of higher molecular weight RNAs was also detected in *repo*¹ RNA from adults. The heterogeneous RNAs were not detected upon reprobing *repo*¹ RNA with another *Drosophila* gene expressed in the adult (Fig. 8). Fourth, the spatial expression pattern of the 3.5-kb mRNA was similar to the *lacZ* pattern described above (see below).

Expression of *repo* RNA during *Drosophila* development was investigated by probing a developmental RNA blot with the *repo* cDNA (data not shown). The highest levels of expression were in embryos and adult heads.

repo encodes a homeo domain protein

The nearly full-length sequence of the *repo* mRNA was obtained from two overlapping cDNAs (Fig. 9A). A single long open reading frame, encoding a putative 612-amino-acid protein, with a predicted molecular mass of 66 kD was identified. Comparison of the deduced amino acid sequence with the sequence data banks revealed the presence of a homeo domain, a 60-amino-acid motif conserved among many transcription factors important in the development of a wide variety of vertebrate and invertebrate organisms (Scott et al. 1989).

Homeo domains have been divided into a number of classes based on amino acid sequence (Scott et al. 1989). The REPO homeo domain was most similar to the paired-(PRD)-like group, which includes *paired* (*prd*), *aristaleless* (*al*), *gooseberry* (*gsb*), *gooseberry-neuro* (*gsbn*),

pax-3, *pax-6*, and *pax-7* (Bopp et al. 1986; Frigerio et al. 1986; Goulding et al. 1991; Jostes et al. 1991; Walther and Gruss 1991; Schneitz et al. 1993). Within this class, the REPO homeo domain was related most closely (~70%) to murine PAX-3, *Caenorhabditis elegans* UNC-4 and *Drosophila* ARISTALESS (*AL*) (Fig. 9B; Goulding et al. 1991; Miller et al. 1992; Schneitz et al. 1993). Among the 16 PRD-specific amino acids, 14 were either identical or conserved (Bopp et al. 1986). One of the two nonconservative changes among the PRD-specific amino acids was the serine to glutamine change at position 50. This residue has been shown previously to be a major determinant of homeo domain DNA-binding specificity in PRD and BICOID (BCD; Hanes and Brent 1989; Treisman et al. 1989). The *AL* and UNC-4 homeo domains have the same amino acid at residue 50 as REPO (Fig. 9B; Miller et al. 1992; Schneitz et al. 1993). A subset of PRD-like homeo domain proteins contains an additional homology of 18 amino acids extending from the amino terminus of the homeo domain (Bopp et al. 1986). Certain PRD-like homeo domain proteins also contain two additional regions of homology: an ~130-amino-acid paired box and a conserved octapeptide (Bopp et al. 1986). The *repo* sequence did not contain significant homology to other PRD-like homeo domain proteins outside of the homeo box.

The predicted REPO protein also contains multiple sequences consisting of single amino acid repeats. The longest and most frequent was six stretches of four to nine glutamines, referred to as opa repeats, found frequently in other developmentally important *Drosophila* proteins (Wharton et al. 1985; Finkelstein et al. 1990; Nambu et al. 1990).

Examination of glial cell lineage in the embryo using *repo* as a marker

Many glial cell types have been described in *Drosophila* embryos (Fredieu and Mahowald 1989; Klämbt and Goodman 1991; Udolph et al. 1993). However, the origins of the glia in the embryo are known in only a few cases. The midline glial cells are the progeny of mesectodermal precursors (Crews et al. 1988; Klämbt et al. 1991). The longitudinal glial cells originate from lateral glioblasts (Doe et al. 1988; Jacobs et al. 1989). Both the midline and longitudinal glia derive from progenitors restricted to glial cell fate. Recently, it has been found that the so-called A and B glial cells derive from the same precursor as the aCC and pCC neurons (Udolph et al. 1993).

In situ hybridization of wild-type embryos with *repo* DNA confirmed that *repo* was expressed in glia in a bilaterally symmetrical pattern consistent with the *lacZ* expression pattern described above. Expression of *repo* first appeared during germ-band extension in stage 9 embryos (Fig. 10A,B). Expression was restricted primarily to the glioblasts (GBs) that were identified based on their location (Jacobs et al. 1989). The GBs were located in the lateral-most row of neuroblasts (NBs) and at the anterior

Figure 8. RNA blot analysis. *repo* RNA-expressed in wild-type and *repo*¹ adults. (Lanes 1,3) Wild-type RNA; (lanes 2,4) *repo*¹ RNA. Lanes 1 and 2 were probed with a *repo* 0.6-kb *Pst*I-cDNA fragment (nucleotides 1365–1985); lanes 3 and 4 were probed with a ribosomal protein cDNA, rp49. The sizes of the *repo* and rp49 RNAs are indicated at right in kb.

Figure 9. (A) Nucleotide sequence of the *repo* cDNAs and deduced amino acid sequence of the putative *repo* protein. The predicted amino acid sequence is shown under the nucleotide sequence. The homeo domain sequence is underlined. Three mRNA instability motifs (ATTTA; Shaw and Kamen 1986) and two consensus 3'-end processing signals (AATAAA) were found in the 3'-untranslated region. The 3.2-kb sequence was obtained from analyses of *pcrepo-2.6* (nucleotides 1–2552) and *pcrepo-2.2* (nucleotides 985–3210). (B) Sequence homology to members of the PRD-like homeo domain proteins. A comparison between *repo* and several other homeo box sequences is shown [Bopp et al. 1986; Frigerio et al. 1986; Finkelstein et al. 1990; Goulding et al. 1991; Krauss et al. 1991; Ton et al. 1991; Miller et al. 1992; Schneitz et al. 1993]. Amino acid identities with REPO are indicated by a dash, and the number of amino acid identities with REPO are shown to the right of the lineup. The PRD-specific amino acids are shown at the bottom [Walther and Gruss 1991]. The two PRD-specific amino acids not conserved in the REPO homeo domain are underlined.

ery hemisegment. Although the exact number was difficult to determine (Fig. 10H,I), it was in excess of the six reported previously (Jacobs et al. 1989).

Many known homeo box genes, such as *Ultrabithorax*, *Antennapedia*, *prd*, *ftz*, and *en* in *Drosophila* (Hayashi and Scott 1990; McGinnis and Krumlauf 1992) and the Hox-type homeo box genes in vertebrates (McGinnis and

REPO	KKKKTRTTFT	AYQLEELERA	FERAPYPDFV	AREELAIKLN	LSSESRVQVWF	QNRRAKWRKH	60
UNC-4	-RRR---N-S	GW-----S-	--ASH-----	M--A--MR-D	-L-----	-----R	42
AL	-QRRY-----	SF-----K-	-S-TH-----	T----M-IG	-T-A-I----	-----Q	42
PAX-3	-QRRS-----	-E-----	---TH---IY	T----QRAK	-T-A-----	S-----Q	42
PRD	-QRRC-----	S--S--D-----	---TQ---IY	T----QRT-	-T-A-I----	S---RL-Q	38
PAX[zf-a]/AN	-LQRN--S--	QE-I-A--KE	---TH-----	--R--A-ID	-P-A-I----	S-----RE	37
SEY/PAX-6	-QRRRE-----	RA--DV--AL	-GKTR--I-	M---V-L-I-	-P-----	K----C-QQ	36
OTD							
PRD specific	Q	D	RA	T	PDIY T	I	AR Q S A

Figure 10. *repo* spatial expression pattern in embryos at different stages of development. (A,B,D,E,H,I) *repo* RNA expression detected by in situ hybridization to whole-mount embryos. (C,F,G) *repo*⁴ heterozygous embryos double-labeled with anti- β -galactosidase (black) and anti-EN antibodies (gray). Embryos were oriented with anterior to the left. (A) Stage 9 embryo (lateral view), arrow points to glioblast (GB); (B) higher magnification of a ventral view in the abdominal region of a stage 9 embryo; (C) higher magnification of a ventral view of a double-labeled stage 9 embryo, EN antibody staining is indicated; (D) lateral view of a stage 11 embryo; (E) ventral view in the abdomen region of a stage 11 embryo, arrows point to the third cell present in every abdominal segment but seen in only in some segments in this view, as it is in a focal plane different from the other two cells; (F) ventral view of a stage 12 double-labeled embryo; (G) ventral view of the same embryo as in F in a different focal plane; (H) lateral view of a stage 15 embryo; (I) ventral view of a stage 15 embryo. (BR) Brain.

Krumlauf 1992), appear to convey positional information rather than regulate the specification of individual cell types. There are also many homeo box genes whose expression is tissue specific. Examples include several members of vertebrate POU gene family (Pit-1, Brn-1, Brn-2, and Brn-3) that are expressed specifically in the developing CNS (Ingraham et al. 1988; He et al. 1989). The *repo* homeo box gene, whose expression was restricted to specific glial cell lineages, is a candidate for a cell-type specification gene.

Homeo domain genes have been grouped into 10 families on the basis of the relatedness of the 60-amino-acid homeo box (Scott et al. 1989). Among these classes, the REPO homeo domain was most similar to the PRD-like group (Schneitz et al. 1993). REPO did not contain other sequences frequently conserved among PRD-like proteins, such as a PRD box (Bopp et al. 1986). Therefore, REPO appears to be a PRD-like rather than PRD-type homeo domain protein.

repo appeared to be required for glial development in the visual system and embryonic nervous system

repo is a glial-specific homeo domain protein suggesting that it might be important for glial development. Examination of the expression of glial markers in the developing *repo* visual system in the third-instar larvae indi-

cated that the glia began to develop but did not terminally differentiate. No significant difference in the number of glial cells labeled by the glial-specific *lacZ* enhancer trap line 3-109 or the *repo lacZ* was observed between wild type and *repo*. However, glial expression of OTD was eliminated. In the adult visual system, expression of neither the 3-109 *lacZ* nor the OTD protein was detected. The absence of this staining did not appear to be attributable to missing glia in young *repo* adults because the normal number of cells in *repo* homozygotes expressed the *repo lacZ*. The *repo lacZ*-positive cells did not express a neuronal marker and therefore did not appear to be transformed into a neural cell fate. In the embryo, the *repo* gene product was also expressed specifically in glia. These included most glial cell types identified previously except the midline glia. The pattern of *repo lacZ* expression was similar in *repo* and wild type; however, OTD was not detected in the *repo* expressing glia. These observations suggested that the glia in the embryo and visual system had only partially differentiated.

repo ERG phenotype appears to be attributable to defects in glia in the visual system

The failure of many glial cells in the adult visual system to differentiate fully had a major effect on the waveform

of the ERG. In wild-type flies, the ERG measures principally the light response of the retinal cells and is corneal negative. The response of the second-order cells in the lamina to light is corneal positive (Shaw 1975; Stephenson and Pak 1980). However, the contribution of the laminal response to the wild-type ERG is relatively minor, presumably attributable to electrical insulation between the retina and lamina. The corneal-positive ERG in *repo* flies did not appear to be the result of a defect in the regulation of photoreceptor cell ion channels, or any other disruption in the photoreceptor cells, because intracellular recordings of *repo* photoreceptor cells were indistinguishable from wild type. Additionally, the *repo* gene was not expressed in the retina, but in the subretinal glia and laminal glia.

A possible explanation for the *repo* corneal-positive ERG is that there may have been defects in electrical insulation and K^+ buffering. The satellite glia and subretinal cells are the glia most likely to be important in electrically insulating the lamina and retina because they separate these two layers (Saint Marie and Carlson 1983). In the absence of the normal insulation, the ERG would presumably measure the summation of both the corneal-negative retinal potential and the laminal potential, which is of opposite polarity. However, this alone would not fully explain the corneal-positive ERG because the amplitude of the laminal corneal-positive response is not larger than the retinal corneal-negative response (Stephenson and Pak 1980). Glial cells in vertebrates have been shown to buffer K^+ by producing an inward K^+ current (for review, see Barres 1991). The amplitude of the laminal corneal-positive response in *repo* could be larger than in wild type because of the failure of laminal glia to buffer the K^+ released by the laminal neurons (for review, see Nicholls et al. 1992). Consistent with this proposal, we have found that the amplitude of the laminal response in *repo* was more than twice as large as that in wild-type flies (W.-C. Xiong and C. Montell, unpubl.). Thus, it is possible that the reversed-polarity ERG observed in *repo* might have resulted from a combined deficiency in K^+ buffering and electrical insulation.

repo embryonic phenotype appears to be attributable to defects in glia

Because *repo* was expressed specifically in glia and there was an apparent disruption of glial differentiation, the embryonic lethality in the strongest *repo* alleles appeared to be caused by defects in glia. The thinning in the longitudinal connectives in some *repo/Df(3R)P14* embryos suggests that condensation of these axon tracts may be affected by a failure in terminal differentiation of the longitudinal glia. The formation of these axon tracts was probably not affected in *repo* embryos as breaks were not detected between any segments. However, these results do not exclude a role for the longitudinal glia during formation of the longitudinal axon tracts as suggested previously (Jacobs and Goodman 1989) because

the *repo* longitudinal glia appeared to be normal during the early stages of development when the pioneer axon tracts form (Bastiani and Goodman 1986; Jacobs and Goodman 1989).

The late embryonic lethality in the strong *repo* alleles may not have been attributable exclusively to defects in the longitudinal connectives, as the morphology of these axon tracts appeared normal in >50% of the embryos. It is possible that the lethality was attributable to a combination of defects. Previous studies indicate that glia provide essential nutrients to neurons and provide ionic homeostasis (for review, see Barres 1991). In the absence of the ionic homeostasis, there appear to be defects in neuronal function. The *repo* mutation might have resulted in defects in these glial functions, thereby causing embryonic lethality.

Potential targets for the *repo* putative transcription factor

The apparent defect in glial cell differentiation and the finding that *repo* encodes a glial-specific homeo domain protein suggest that *repo* controls glial development at the transcriptional level. Potential targets for *repo* in the embryonic nervous system are other genes that are expressed in longitudinal glia. These include *ftz*, *otd*, and *pros* (Doe et al. 1988, 1991; Finkelstein et al. 1990; Vaessin et al. 1991; Wieschaus et al. 1992). These genes could be potential targets for *repo* in the longitudinal glial cells but not in all of the cells in which they are expressed, as *repo* is glial-specific. Consistent with the proposal that *otd* might be a target for *repo* in the embryonic nervous system, expression of OTD initiated subsequent to *repo* (Xiong and Montell, unpubl.) and, in the *repo* mutant, was absent in those glia in which *repo* was normally expressed.

The *otd* gene is also a potential target for *repo* in glial cells in the visual system, as *otd* was not expressed in *repo* subretinal cells. The 3-109 *lacZ* marker is not a likely candidate for transcriptional regulation by REPO, as 3-109 was expressed in *repo* larvae. Several other genes have been identified that are expressed either specifically in glia (*anachronism*) or in both neurons and glial cells in the visual system (*cut*, *tramtrack* and *optomotor-blind*) (Blochliger et al. 1993; Ebens et al. 1993; Poeck et al. 1993; Xiong and Montell 1993). It is possible that *repo* regulates the expression of one or more of these genes.

Materials and methods

Electrophysiology

ERG recordings were performed by inserting glass electrodes, filled with Ringer's solution, in small drops of electrode cream placed on the surface of the compound eye and thorax as described previously (Porter et al. 1992). Intracellular recordings were performed by placing a low-resistance electrode, containing Ringer's solution, into a small hole in the compound eye and a reference electrode on the thorax as described previously

(Barash et al. 1988). Recordings were performed on flies <7 days posteclosion.

Fly stocks and genetics

The *repo*¹ mutant was identified among ~7000 homozygous viable P-element lines screened by performing ERGs. In A.S. Spradling's laboratory (Karpen and Spradling 1993), 6000 lines were generated, 400 homozygous P-element insertion lines were kindly provided by M.C. Mullins, and an additional 400 lines were a gift of M.E. Fortini (both from University of California, Berkeley). The *repo*¹ mutant was found among the Mullins lines, which contained a single modified P-element consisting of a *rosy*⁺ + (*ry*⁺) gene and a copy of the chloramphenicol acetyltransferase (CAT) reporter gene (Mullins et al. 1989). The *repo*², *repo*³, and *repo*⁴ alleles were identified among the collection of homozygous lethal P-element insertion lines from the Spradling laboratory (stocks 3702, 692, and 2138 respectively; Karpen and Spradling 1992). The *repo*⁴ allele was also identified in a screen of the P-element lethal lines for mutations affecting neural development (N. Patel, unpubl.). To demonstrate that the P element induced the *repo* mutation, the P element from *repo*¹ and *repo*⁴ was mobilized by crossing *repo* flies with a stock, referred to as *jumpstarter* or $\Delta 2-3$, which provided the P-element transposase required in *trans* for transposition. Among 59 *ry*⁻ lines that were established from *repo*¹, 26 reverted to wild type, 5 were recessive lethals, and 28 were semilethal or homozygous viable lines that exhibited a *repo* ERG phenotype. One of these lines that exhibited a *repo* ERG phenotype, *repo*^{1ae44}, was used in the analysis of 3-109 expression in the developing and adult visual system, as the *ry*⁻ background facilitated genetically introducing the 3-109 *ry*⁺ element into a *repo* background. Among 40 *ry*⁻ lines that were established from *repo*⁴, 22 reverted to wild type and 18 were recessive lethals. *repo*^{4e19} and *repo*^{4e25} were two of the lethal lines obtained by mobilization of the *repo*⁴ P element. The deletion in *repo*^{4e19} was mapped by digesting the genomic DNA with *NarI* and probing the DNA blot with a *PstI*-*NarI* fragment cDNA fragment (nucleotides 1366-1905). The wild-type *NarI* genomic fragment, which hybridizes to the cDNA probe, encodes REPO amino acids 23-465. The breakpoints of *Df(3R)P14* are 90C2-D1 and 91A2-3 (Detwiler and MacIntyre 1978).

Immunolocalizations, histochemical localizations, and in situ hybridizations

Antibody staining was performed as described previously (Patel et al. 1989). The fixation was done either in 4% formaldehyde for 15 min (whole-mount embryos) or in 2% paraformaldehyde for 45 min (8 μ M frozen sections of adult fly heads, whole-mount eye discs, larval brain, and CNS). Rat polyclonal anti-OTD (1:50), rabbit polyclonal anti- β -galactosidase (1:5000), and the following mouse monoclonal antibodies were used: anti- β -galactosidase (1:1000; Promega); 22C10 (1:10); BP102 (1:10); anti-ELAV (1:5); and anti-EN (1:10). HRP-conjugated secondary antibodies were developed with 0.5 mg/ml of diaminobenzidine (DAB) and 0.03% hydrogen peroxide. Immunofluorescent localizations were performed with FITC- and RITC-conjugated secondary antibodies and were viewed by confocal microscopy. Identical anti- β -galactosidase staining patterns were obtained with *repo*², *repo*³, and *repo*⁴ heterozygotes (which contained P[*lacZ*] enhancer trap P elements) except that the embryonic expression initiated in *repo*⁴ during stage 9 and in *repo*² and *repo*³ during stage 13. *repo*¹ flies were not examined for β -galactosidase staining because the P element that was used to generate this allele did not contain a *lacZ* reporter gene (Mullins

et al. 1989). Histochemical staining of eye discs and sections of adult heads for *lacZ* activity were done as described (Winberg et al. 1992). Localization of RNA in whole-mount embryos was performed using single-strand polymerase chain reaction (PCR)-generated, digoxigenin-labeled probes prepared (Patel and Goodman 1992) from the *repo* cDNA.

Isolation and analyses of repo genomic and cDNA clones

The initial *repo* genomic DNA clone was isolated from a *repo*¹ DNA library probed with the 5'-P-element end. The library was constructed by digesting *repo*¹ genomic DNA with *EcoRI*, fractionating DNA in the 3- to 5-kb range from an agarose gel, and cloning into λ gt11. The *repo*¹ genomic DNA was used to screen a wild-type genomic DNA library inserted into bacteriophage λ , Charon 4A (Maniatis et al. 1978). One of the genomic clones, λ repo-1, contained a 14.5-kb insert (0-14.5 map units, Fig. 7) and was used to construct most of the physical map. The DNA sequences flanking the P-element insertion sites of *repo*², *repo*³, and *repo*⁴ were recovered by the plasmid rescue technique (Pirrotta 1986). The genomic DNA isolated from *repo*² and *repo*³ was used to extend the physical map to the *XbaI* site at ~17 map units (Fig. 7).

Two overlapping cDNAs, *pcrepo*-2.6 (nucleotides 1-2552) and *pcrepo*-2.2 (nucleotides 985-3210), were isolated from an embryonic cDNA library probed with a ³²P-labeled 3.5-kb genomic region as described (Montell and Rubin 1989). The cDNAs were sequenced by the dideoxy chain termination method using Sequenase (U.S. Biochemical). The template DNAs used for the DNA sequencing were generated by subcloning random cDNA fragments, generated as described (Montell and Rubin 1989), into the *SmaI* site of M13mp10.

Mapping P-element insertion sites

The position and orientation of the P-element insertion in *repo*¹ was determined by probing a Southern blot containing DNA prepared from wild-type and mutant flies with P-element sequences as well as flanking *repo* genomic DNA. The sites of the P-element insertions in the three lethal alleles, *repo*², *repo*³, and *repo*⁴, were determined by isolating the flanking genomic DNA, by the plasmid rescue technique, and sequencing genomic DNA from the insertion lines, using a primer that hybridized to the 5' end of the P element.

Mapping repo-transcribed region and RNA blots

The approximate genomic region encoding the 3.5-kb *repo* mRNA was initially determined by digesting the cloned *repo* genomic DNA with various restriction enzymes, transferring to GeneScreen Plus, (NEN) and probing with ³²P-labeled cDNAs prepared from adult RNA. The transcribed region was mapped in greater detail by probing blots containing the cloned genomic DNA with the cDNAs *pcrepo*-2.6 and *pcrepo*-2.2. To perform RNA blots, total RNA was prepared as described (Montell and Rubin 1989), transferred to GeneScreen Plus and probed with a 0.6-kb *PstI* fragment of the *repo* cDNA (nucleotides 1365-1985) labeled with ³²P.

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The sequence data described in this paper have been submitted to the EMBL data library under accession number X78218.

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