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The homeobox gene *repo* is required for the differentiation and maintenance of glia function in the embryonic nervous system of *Drosophila melanogaster*

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SUMMARY

We describe the cloning, expression and phenotypic characterisation of *repo*, a gene from *Drosophila melanogaster* that is essential for the differentiation and maintenance of glia function. It is not, however, required for the initial determination of glial cells. In the embryo, the gene, which encodes a homeodomain protein, is expressed exclusively in all developing glia and closely related cells in both the central and peripheral nervous systems. The only observed exceptions in the CNS are the midline glia derived from the mesectoderm and two of three segmental nerve root glial cells. Using a polyclonal antibody we traced the spatial and temporal pattern of the protein expression in detail. Embryos homozygous for null alleles of the protein exhibit late developmental defects in the nervous system, including a reduction in the number of glial cells, disrupted fasciculation of axons, and the inhibition of ventral nerve cord condensation. The expression of an early glial-specific marker is unaffected in such homozygotes. By contrast, the expression of late glial-specific markers is either substantially reduced or absent. The specificity of expression is also observed in the locust *Schistocerca gregaria* and is thus evolutionarily conserved.

Key words: *repo*, glial cells, CNS, PNS, *Drosophila*

INTRODUCTION

In vertebrates and invertebrates, the nervous system consists of two principal types of cells, neurons and glia. While neurons act as transducers of information, glial cells fulfil many tasks in supporting neuronal cells including insulation, homeostasis, and providing nutrition. Recent studies also show that glial cells play an important role in the compartmentalisation and the organisation of the complex axonal scaffold within the central nervous system (for reviews see Tolbert and Oland, 1989; Goodman and Doe, 1993). However, little is known about the determination and differentiation of the glia themselves.

The insect nervous system provides a useful model system to investigate these processes because it is relatively simple. In *Drosophila* the origin of glia in the central nervous system (CNS) is known for only a few cells. Among these are the longitudinal glia (Doe et al., 1988; Jacobs et al., 1989) and the mesectodermal midline glia (Crews et al., 1988; Klämbt et al., 1991; Bossing and Technau, 1994). Both of these glial cell types derive from neural progenitors restricted to glial fate. Recently, however, the neuroblast NB1-1 has been shown to act as a common precursor for glia and neurons (Udolph et al., 1993). Thus, common as well as distinct progenitors for these two types of cells exist in *Drosophila.* Similar results have been obtained for vertebrates (reviewed by Cameron and Rakic, 1991).

In the neuroectoderm the decision to become a neural progenitor is dependent on the neurogenic and the proneural genes (e.g. reviewed by Campos-Ortega, 1993). A number of candidate genes are known that may be involved in the specification of the individual neural progenitors and their progeny (e.g. reviewed in Goodman and Doe, 1993). Finally, committed cells differentiate upon activation of a specific set of differentiation genes.

Recently several genes have been identified that are expressed in glia and may play a role in the differentiation of these cells. These include the genes of the spitz group (*sim, slit, star, rhomboid,* and *pointed*), which are mainly involved in midline development (Crews et al., 1988; Thomas et al., 1988; Rothberg et al., 1990; Nambu et al., 1990; Bier et al., 1990; Klämbt et al., 1991; Rutledge et al., 1992; Klämbt, 1993) but also other genes such as *prospero, otd,* and *ftz* (Doe et al., 1988, 1991; Finkelstein et al., 1990). However, all these genes are expressed in only a subset of glia, and in most cases, in neuronal cells as well. This suggests that they are involved in rather specific aspects of differentiation and do not by themselves confer a more general glial phenotype.

In this paper we describe the identification of a evolutionary conserved gene whose properties are consistent with such a general function. This gene encodes a paired-like homeobox protein and is specifically expressed in most of the glia in the embryonic CNS and peripheral nervous system (PNS) from an early stage. Mutant phenotypes suggest that this gene is

required for the migration and differentiation of embryonic glial cells as well as for maintenance of their function.

While this paper was in preparation, Xiong et al. (1994) reported the characterisation of the same gene, which they term *repo*. Here we extend and amend their observations on the *Drosophila* embryo by staining with antibody to the purified protein.

MATERIALS AND METHODS

Isolation and analysis of *repo* cDNA

The binding site of *ftz* USE from 2155-2215 (Harrison and Travers, 1988) was cloned as a dimer into the *Sal*I site of pBluescript (Vector Cloning Systems) and termed pSSN3. The method of Vinson et al. (1988) was used to screen a 0-16 hour embryonic λgt11 cDNA library (a gift of B. Hovemann) with the end-labelled 159 bp long *Ava*I-*Pst*I fragment of pSSN3 (=SSN3AP) containing the dimerised *ftz* USE sequence. The cDNA portion from a positive phage clone was subcloned into the *Eco*RI site of pBluescript to give the plasmid p4α3. Using p4α3 as a probe for longer cDNA fragments the plasmids p4α3/10 and p4α3c/6 were obtained by screening an 8-12 hour cDNA library (NB40, obtained from N. Brown).

The cDNAs were sequenced by the dideoxy chain termination method using Sequenase (USB). The sequence is largely identical to that of Xiong et al., 1994) except that nucleotides 194-200 in their numbering, i.e. at the 5′ end of both cDNA clones, are GTCAATT.

Genomic DNA

Genomic clones covering the region of the *repo* gene were obtained by screening a genomic library of an isogenised strain of *Drosophila melanogaster* (iso1), which is cloned into a λ EMBL3 vector (kindly provided by J. Tamkun). Using a 2.9 kb *Hin*dIII fragment from rescue clone of strain 3-2138 (see below) as a probe, we obtained three overlapping clones covering 18 kb of the genomic region. Restriction site mapping and Southern blot hybridisation were used to map the cDNA into the cloned genomic region. The intron was detected by sequencing a genomic subclone with flanking primers.

In situ hybridisation to polytene chromosomes

Hybridisation to Canton S polytene chromosomes was achieved by the method of Langer-Safer et al. (1982) except that biotinylated probes were detected using streptavidin-HRP visualization (Detek-HRP, ENZO Biochem). The probe was prepared by nick-translation of the whole p4α3 plasmid. Band location to 90 F1-2 was determined by reference to Lefevre (1976).

Fly stocks and genetics

Lines 3-3702, 3-692 and 3-2138 were obtained from a collection of lethal P-element lines (Karpen and Spradling, 1992) kindly provided by A. Spradling. P-element excisions were achieved by crossing 3-3702 and 3-692 with females carrying the *Sb P[Δ(2-3)]* chromosome.

To recognise mutant embryos, all mutants were first crossed over the ‘Blue Balancer’ chromosome *TM3, Sb, P[hb-lacZ)]*.

To analyse glial-specific expression patterns within mutant background the marker strains rA87, M84, 2-3563/*CyO* and Kr-lacZ PH3.7 (kindly provided by C. Klämbt, A. Spradling and D. Schmucker) were crossed with the excisions EX52 and EX84. Males and females of each cross carrying no balancer chromosome were mated. Embryos out of these crosses were analyzed with anti-β-galactosidase antibody staining (Cappel). Mutants were recognized by their elongated VNC.

Localisation of P-element inserts

Genomic DNA flanking the P-element insertion sites of lines 3-692 and 3-2138 were recovered using the plasmid rescue technique (Pirrotta, 1986). PCR was used to amplify the insertion site of 3-3702 by using oligonucleotides homologous to the terminal repeat of the P-element and to the cDNA. Insertion sites were determined by sequencing with primers hybridising to the terminal repeat and to the cDNA.

Preparation of *E. coli*-expressed repo protein

The cDNA of p4α3 was subcloned into the *Eco*RI site of a modified pET11a vector to obtain plasmid pDH1. After induction the expressed protein, repo, was recovered from inclusion bodies (Nagai and Thøgersen, 1987). The pellet was resuspended in SDS loading buffer and separated on an SDS acrylamide gel. The band at 70×10^3 M_r was eluted from the gel in elution buffer (50 mM Tris-HCl, pH 8, 0.1 M EDTA, 1 mM DTT, 0.1 M NaCl, 0.1% SDS, 1 mM PMSF) by overnight rotation at 4°C. The protein was renatured by dialysis at 4°C against 8 M urea in PBS (100 mM sodium orthophosphate, pH 7.1, 100 mM sodium chloride), 6 M urea in PBS, 2 M urea in PBS, and finally twice against 10 mM Tris-HCl, pH 8.0. After lyophilisation the pellet was redissolved in 0.5 ml 50 mM Tris-HCl, pH 8.0, 1mM EDTA, 1 mM PMSF, 1 mM DTT and stored at 4°C.

Gel-retardation assay

SSN3AP was labelled at the *Ava*I end by end-filling (Hattori and Sakaki, 1986). After labelling, probes were purified by size fractionation on Sephadex G-50 columns and 5% acrylamide gels. Binding of repo to SSN3AP was performed in 10 μl mixtures containing 20 mM Hepes, pH 7.9, 50 mM KCl, 2 mM $MgCl_2$, 1 mg/ml BSA, 10 ng/μl polydIC, 1% Nonidet P-40 for 20 minutes at room temperature (RT) and the complex was then run on a 5% acrylamide gel at 180 V at 4°C.

Preparation of anti-repo antibody

E. coli produced repo protein (approx. 250 μg) was injected into a rabbit 4 times at 2 week intervals. Serum isolated in the ninth week after the first injection was partially purified by chromatography on a protein A column. The bound antibody was eluted with 100 mM glycine, pH 3.0 and preabsorbed against 0-24 hour embryos. In a western blot of nuclear extracts from 0-24 hour embryos (Harrison and Travers, 1988) anti-repo detected one strong band at 70×10^3 M_r (data not shown). This size is consistent with the calculated molecular mass of 65.6×10^3 M_r for the 612 amino acid open reading frame.

Immunocytochemistry

All antibody stainings in *Drosophila* embryos against β-galactosidase were performed as described elsewhere (Schmidt-Ott and Technau, 1992). For staining with anti-repo antibody we followed exactly the same protocol except that we used a 1:200 dilution of the protein A fraction for the first antibody.

Double staining with two antibodies was performed as follows: fixed embryos were washed with BBT (10 mM Tris-HCl, pH 6.95, 55 mM NaCl, 40 mM KCl, 7 mM $MgCl_2$, 5 mM $CaCl_2$, 20 mM glucose, 50 mM sucrose, 0.1% BSA (Merck, fraction V), 0.1% Tween 20) several times. Both primary antibodies were incubated together (anti-β-galactosidase 1: 1000; anti-repo 1:100; mAb22C10 (Fujita et al., 1982) 1:50, anti-fas II (Grenningloh et al., 1991) 1:50) in BBT for 2 hours at RT. After washing several times with BBT and blocking with 2% goat serum/BBT, both secondary antibodies were incubated for 2 hours at RT in a 1:500 dilution in 2% goat serum/BBT. The anti-mouse antibody was coupled with biotin (Vector Laboratories) whereas the anti-rabbit was conjugated with alkaline phosphatase (Dianova). After several washes with PBT (PBS, 0.1% Tween 20) the embryos were incubated with AB-reagent (Vectastain Elite Kit, Vector Laboratories). Several washes with PBT were then followed by 3 washes with alkaline reaction buffer (0.1 M NaCl, 0.02 M $MgCl_2$, 0.1 M Tris-HCl, pH 9.5, 0.1% Tween 20). The alkaline phosphatase was detected with NBT and X-phosphate (Boehringer). Embryos were then intensively washed with PBT and incubated in 0.5 mg/ml diaminobenzidine (DAB, Sigma) with 0.03% H_2O_2. Stained embryos were mounted in Araldite (Sigma) for whole-

mounts or in 70% glycerol, 0.1 mM Tris-HCl, pH 7.4, 50 mM NaCl for flat preparations. Embryos were viewed under a Zeiss Axiophot microscope.

Repo staining of locust embryos (a gift of M. Akam) was performed following the protocol of Dawes et al. (1994) using a 1:250 dilution of the anti-repo antibody.

Nile Blue A staining

Nile Blue staining for cell death was performed using the method of Abrams et al. (1993).

Note on figures

In Figs 5, 7A-D, L, and 9B we combined different focal planes by using Photoshop 2.0 (Adobe).

RESULTS

Cloning of the gene

In order to identify genes that are involved in the regulation of the expression of the pair-rule gene *fushi tarazu (ftz)* we screened an expression library with a defined protein binding site within the *ftz* USE. The DNA fragment used (bp 2155-2215, numbered according to Harrison and Travers, 1988) lies in a region that is sufficient to direct *ftz* expression in stripes (Schier and Gehring, 1992). Approximately 40 bp within this fragment had been found to be protected from DNase I digestion by extracts from 5-10 hour embryos (Harrison and Travers, 1988).

Using the method of Vinson et al. (1988) we probed a 0-16 hour AEL *Drosophila* embryonic cDNA library with the USE fragment and identified one phage clone, termed 4α3. The cDNA was isolated and the protein was overexpressed in *E. coli*. The 4α3 protein was assayed by gel-retardation using a dimer, termed SSN3AP, of the *ftz* USE sequence spanning from 2155-2215 bp (Fig. 1A). The 4α3 protein retarded the SSN3AP DNA in a concentration-dependent manner (Fig. 1B, lanes 2-6). Addition of unlabelled SSN3AP competed the binding (Fig. 1B, lane 13). In order to localise the binding site three different oligonucleotides, Dah35, Dah36, Dah37, covering the region from 2155-2215m bp were added as competitors (Fig. 1A). The best competition was obtained with Dah37 (Fig. 1B, lanes 7-9) which contains the AATTA motif, a potential homeobox binding site. Dah35 and Dah36, which do not contain this motif compete SSN3AP only when added in concentrations >10× higher than Dah37. To test whether this motif is the target site for 4α3 binding we modified Dah37 by changing AATTA to GATTA (Dah 371), AGTTA (Dah372), and AAGTA (Dah374) (Fig. 1A). All three oligonucleotides only competed binding at 100× excess (Fig. 1B, lanes 10-12). Addition of a specific polyclonal antibody, raised against 4α3 protein (see below) resulted in additional retardation of the binding complex (Fig. 1B, lane15), whereas antibody alone

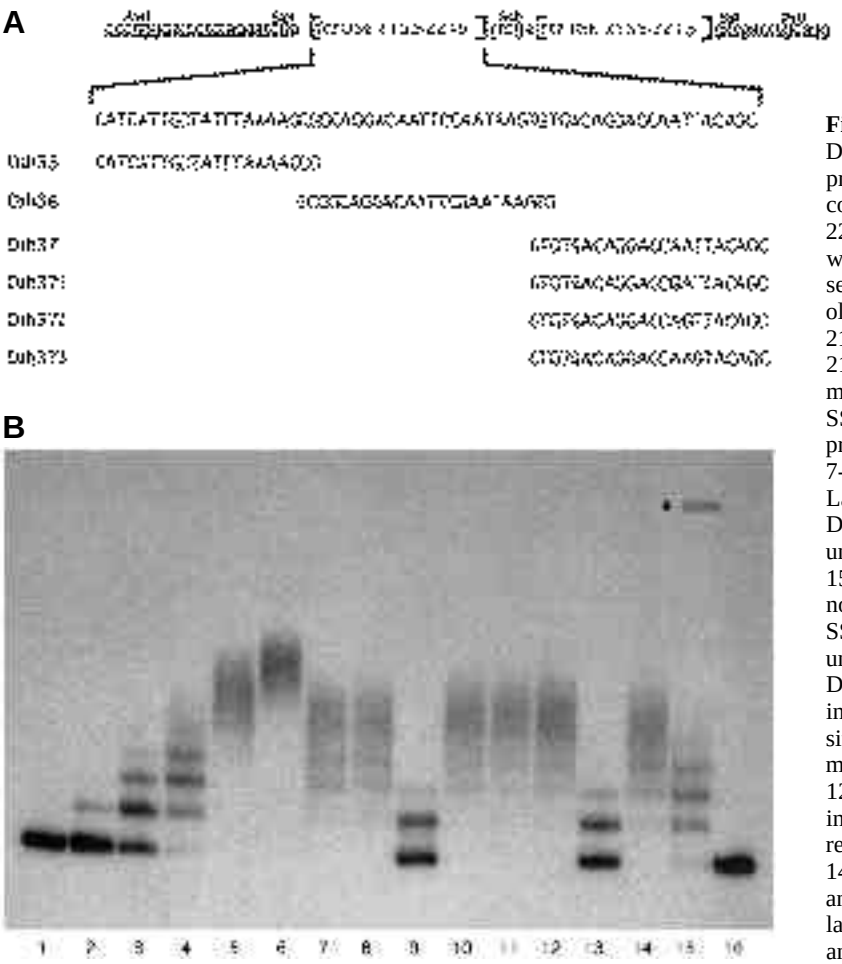


Fig. 1. DNA binding by repo protein. (A) Sequences of DNA probe and competitor DNA used in the assay. The probe was SSN3AP, a 159 bp long *Ava*I-*Pst*I fragment containing a dimer of the *ftz* USE sequence from 2155-2215 bp (Harrison and Travers, 1988). The monomers were linked by a *Sal*I site and flanked by pBluescript sequence (underlined). Dah35, Dah36 and Dah37 are oligonucleotides covering different regions; Dah35 from 2155-2175bp, Dah36 from 2173-2196 and Dah37 from 2193-2215. In Dah371, Dah372, Dah373 the AATTA motif was changed as shown. (B) Mobility shifts of SSN3AP. Lane 1: SSN3AP without addition of repo protein. Lanes 2-6: increasing addition of protein. Lanes 7-14: addition of different competitor DNA oligomers. Lane 7: Dah35; lane 8: Dah36; lane 9: Dah37; lane 10: Dah371; lane 11: Dah372; lane 12: Dah373; lane 13: unlabelled SSN3AP; lane 14: p4α3 cDNA insert. Lane 15: addition of the specific anti-repo antibody. Lane 16: no repo protein but anti-repo antibody alone. Binding to SSN3AP could be best competed either by adding unlabelled SSN3AP (lane 13) or the oligonucleotide Dah37 (lane 9). Dah37 contains a CAATTA motif shown in other homeobox proteins to be the preferred binding site. Competition could be diminished by altering the motif to CGATTA, CAGTTA or CAAGTA (lanes 10-12). The concentration of the competitors are all the same in all lanes. The mixtures for lanes 1-14 contained respectively 0, 1.4, 3.5, 7, 14, 35, 14, 14, 14, 14, 14, 14, 14, 14, 7 and 0 ng protein. 200 ng protein-A purified antibody was added to mixtures 15 and 16. The band in lane 15 marked with an asterisk (*) is a SSN3AP-repo-antibody complex.

showed no binding to SSN3AP (Fig. 1B, lane 16). We therefore conclude that 4α3 specifically binds to at least AAT in the CAATTA motif at position 2206 of the ftz USE. This sequence is homologous to binding sites of homeodomain proteins. We note, however, that 4α3 forms multiple complexes and may thus only nucleate binding at this motif.

The 4α3 cDNA codes for repo, a homeodomain protein

Sequencing of the λgt11 4α3 clone and two longer overlapping cDNA clones, 4α3/10 and 4α3c/6 (see Materials and Methods) yielded a sequence of 3033 bp, which is largely identical to the cDNA of the homeobox gene *repo* (Xiong et al., 1994). By sequencing genomic DNA using appropriate primers we found, in contrast to Xiong et al., a 416 bp intron within the coding region at position 1436. This was confirmed by the detection of an *Eco*RI site within the 3′ noncoding region of the cDNA. This site had been placed in an intron in this region by Xiong et al. (1994). However, our cDNA does not include another exon coding for the extreme 5′ noncoding region of the *repo* gene (Xiong et al., 1994).

The homeobox protein is glial-specific

To investigate the expression pattern of the gene we raised a polyclonal antibody against the *E. coli*-produced protein. Staining of whole embryos revealed that the antigen is localised in the nuclei of single cells within the developing CNS and PNS from early stage 11 onwards (Figs 2-5). From their spatial distribution and the use of glial-specific enhancer trap lines as markers we find that, with a few exceptions, the protein is expressed exclusively in glia. For the CNS this conclusion is independently supported by Xiong et al. (1994) who showed that the *lacZ*-positive cells of *repo* enhancer trap lines do not express the neuronal antigen elav (Robinow and White, 1988).

In the CNS of stage 16 embryos the anti-repo antibody (4α3) stains 27-29 cells per hemineuromere: 8-9 cells are associated with the longitudinal tracts (interface glia, IG; for the nomenclature of CNS glia see Ito et al., 1994); these include the 6-8 longitudinal glial cells which are the progeny of the longitudinal glioblast (Jacobs et al., 1989) (Figs 2A, 3A, 4A). 2-3 cells are associated with the nerve roots, among them 2 intersegmental nerve root glial cells (M-ISNG and L-ISNG), and in some segments 1 segmental nerve root glial cell (SNG; Klämbt and Goodman, 1991) (Figs 4G, 7C). Within the cortex we identified the medialmost cell body glia (MM-CBG: Ito et al., 1994; VUM support cells in Klämbt and Goodman, 1991) by their irregular nuclear shape and characteristic position. There are 2 MM-CBG per hemineuromere in the thorax and 1 per hemineuromere in the abdomen as previously described (Klämbt, 1993; Figs 3H, 7A). A further 2-3 repo-positive glial cells are found in the cortex by anti-repo/anti-β-galactosidase double staining in a strain which labels cell body glia (Kr-lacZ PH3,7; Fig. 9B). Finally, 10-12 repo-expressing cells per hemineuromere are associated with the CNS surface. 7-8 of these cells represent subperineurial glia (SPG) including the A- and B-SPG (Klämbt and Goodman, 1991; Udolph et al., 1993) (Fig. 7C) and 3-4 cells are channel glia (CG) associated with the dorsoventral-channel, a structure that demarcates the segmental border (Ito et al., 1994) (Fig. 4G). Since no other cells are labelled in the VNC, *repo* is glial-specific within this tissue. However, we see no expression in midline glia and two of the three segmental nerve root glial cells (Klämbt and Goodman, 1991). Thus most, but not all, glia express the gene.

Fig. 2. *repo* expression through embryonic development. Ventral views of whole–mount embryos, anterior is up. (A) Early stage 11. First expression is detected in the longitudinal glioblast (LGB). (B) Early stage 12. Approximately 13 cells per abdominal hemineuromere are labelled. (C-E) Embryos at mid stage 12 (C), stage 13 (D) and 14 (E). At stage 14, approx. 28 cells per abdominal hemineuromere are labelled. (F) Stage 16. The stained cells reach the maximum number (see text). Note the migration of the nerve associated glia (exit glia and peripheral glia) by comparing E and F. At stage 16, exit glia and peripheral glia have migrated out of the CNS (see Fig.5B-D). Within the CNS 28–29 cells per abdominal hemineuromere are repo positive. EG, exit glia; LG, longitudinal glia; LGB, longitudinal glioblast; PG, peripheral glia; MM-CBG, medialmost cell body glia (or VUM support cells).

Double staining with anti-repo and mAb22C10, which mainly stains neuronal cells of the PNS, shows that the antigen is expressed in glial cells associated with the segmental nerves, including the peripheral glia as well as the exit glia (Klämbt and Goodman, 1991). We find 9-10 cells in the abdominal and 13-14 cells in the thoracic hemisegments (Fig. 5B-D). In addition, the glial cell of the dorsal bipolar dendritic neuron expresses repo (Fig. 5A,D). Some cells that are not usually classified as glia are also repo-positive: the ligament cells of the tri- and pentascolopidial chordotonal organ and the lateral bipolar dendritic neuron (Bodmer and Jan, 1987) (Fig. 5A,D). However, we do not detect expression in cells associated with the transverse nerve (TN), previously described as dorsal root glia (DRG; Nelson and Laughon, 1993), or TN exit glia (Gorczyca et al., 1994).

Repo expression also appears to be glial-specific in the brain. Further repo-positive cells in the head are associated with the nerves and with PNS-organs such as the antenno-maxillar-complex (data not shown).

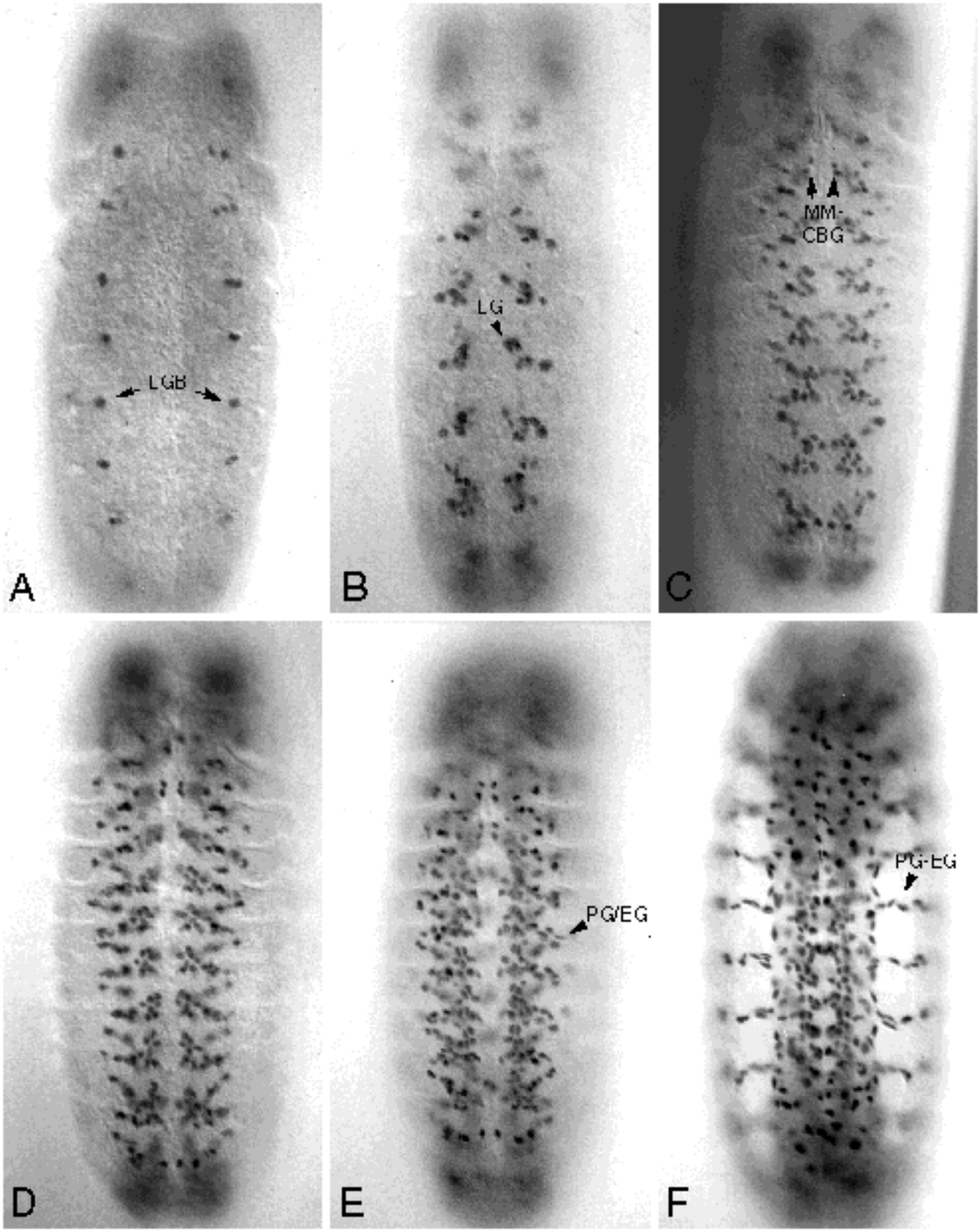
To determine whether the protein is evolutionarily conserved we stained locust embryos (*Schistocerca gregaria*) with anti-repo antibody. We observed an expression pattern comparable to that of *Drosophila* (Fig. 6) in which positively identified glia corresponded to those homologous to the *Drosophila* interface and nerve root glia (M. Bate and D. Shepherd, personal communication). Since the two organisms are separated by 300 million years of evolution, this suggests that there is a high degree of conservation of the gene within insects.

Glial cell fate as revealed by anti-repo antibody as a glial-specific marker

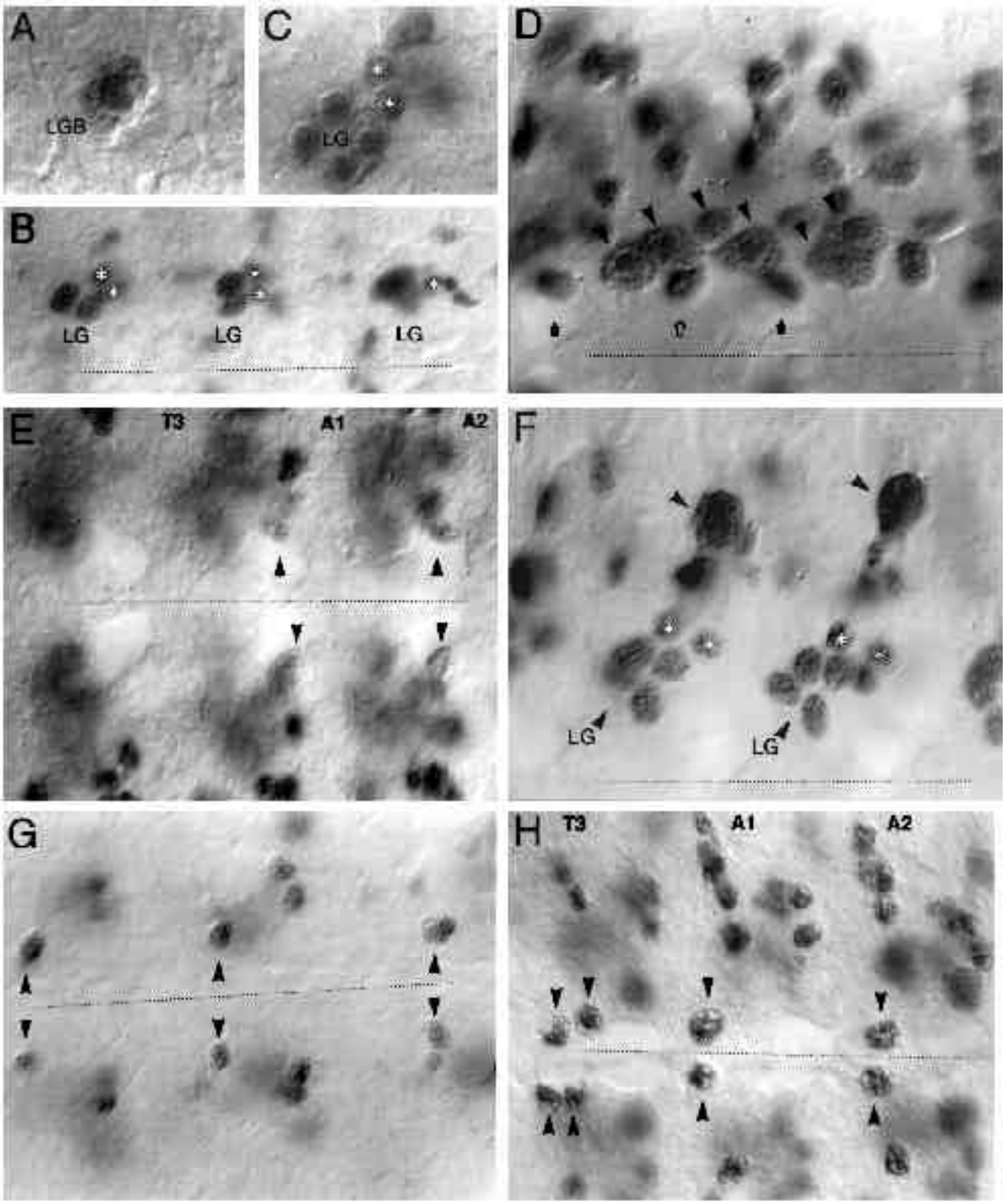
Since *repo* is expressed from early stages of glial development, we traced the embryonic development of certain of the labelled glial cells to elucidate their origin (Figs 3, 4). In some cells, division could be detected after the onset of repo expression, taking the appearance in the cytoplasm of the otherwise strictly nuclear repo antigen as an indication of cell division. Confirmation of division was obtained by comparison of the number of relevant cells between the neuromeres within the same embryo.

Neuropile-associated glia

The longitudinal glioblast (LGB) (Jacobs et al., 1989) is first



detected at the beginning of stage 11 (Figs 2A, 3A, 4A). The LGB then divides symmetrically and both cells migrate towards the midline as previously described (Jacobs et al., 1989; Figs 2B, 3B, 4B). At late stage 11 two other cells with slightly smaller nuclei start expressing repo (Figs 3B, 4C). They are located laterally to the two inward migrating longi-



tudinal glial cells (LG). These two cells are unlikely to be progenies of the LGB because there is no indication of cell division among the LG before their appearance. In early stage 12 the LGB progenies undergo a second round of division. The resulting 4 cells are then arrayed in a characteristic diamond pattern (Figs 3C,F, 4D). By this time they already occupy a position where the longitudinal tracts will form. During stage 13 and 14 the group of longitudinal glia stretches in an anterior-posterior direction (Fig. 4F). Thereafter the two additional cells intermingle with the longitudinal glia and can no longer be distinguished. At stage 14 a wave of cell divisions occurs within this population (Fig. 3D) resulting in dorsal longitudinal rows

Fig. 3. Proliferation and migration of identified glial cells during early wild-type gliogenesis. Whole–mount embryos, stained with anti-repo antibody; ventral view, anterior is left. Dotted line indicates the midline. (A-C) Proliferation of the longitudinal glioblast (LGB). Early stage 11 (A), late stage 11 (B) and early stage 12 (C). Two waves of mitoses can be detected, giving rise to four longitudinal glial cells (LG) that derive from the LGB. In B, C and F, two additional cells, which presumably are not part of the LGB–lineage, are labelled with stars (see text). (D) Abdominal hemineuromeres at stage 14. Dividing cells (arrowheads) can easily be identified by their size and the existence of cytoplasmic staining. A–SPG (solid arrow) and B–SPG (hollow arrow) lie at their characteristic position beside the dorsoventral channel. (E) Segment specificity of the presumptive A- or B–SPG. Mid stage 12, showing segments T3-A2. While present in abdominal segments the progenitor (arrowheads) is missing in thoracic segments. (F) An unknown anterodorsal progenitor (arrowheads) is in process of dividing at early stage 12. In addition, the characteristic rhomboidal arrangement of the longitudinal glia (LG) is shown. (G,H) Migration of MM-CBG (VUM support cells). (G) MM-CBG (arrowheads) at stage 11 shortly after the onset of repo expression. (H)By mid stage 12 they have reached a position close to the VUMs (see also Fig. 7A). Note that there are two MM-CBG in thoracic hemineuromeres.

of 9-10 cells per hemineuromere at the interface between the longitudinal tracts and the cortex (Figs 4G, 7C). At stage 17 one of these cells lies at the intersegmental nerve root representing the M-ISNG (Ito et al., 1994; segment boundary cell in Jacobs and Goodman, 1989; ISG1 in Klämbt and Goodman, 1991; Figs 4G, 7C).

MM-CBG

The MM-CBG (Ito et al., 1994; VUM support cells in Klämbt and Goodman, 1991) first stain during stage 11 after the first division of the LGB (Fig. 4B). They appear in a lateral position within the posterior compartment of each neuromere. During stage 11/early stage 12, these cells migrate along the ventral surface of the developing nerve cord towards the midline, reaching their final position lateral to the VUM neurons (compare Fig. 3G and H).

A- and B-SPG

The A- and B-SPG (Klämbt and Goodman, 1991; Ito et al., 1994) are abdominal-specific progeny of the neuroblast NB1-1 (Udolph et al., 1993). In the abdominal segments, at the beginning of stage 12, one cell per hemineuromere in a medial dorsoventral position near the dorsoventral channel starts expressing repo (Figs 3E, 4C). In the middle of stage 12 another cell appears in the vicinity (Fig. 4D). During further development both cells reach the dorsal surface of the nerve cord. One of the cells is then shifted anteriorly into the next neuromere assuming the characteristic position and nuclear shape of the B-SPG whereas the other cell remains in the position of the A-SPG (Figs 4D-F, 7C).

Unknown glial progenitors

We also identified two glial progenitors which have not been described before. They appear at stage 12 in a lateral position of the CNS (Fig. 4C). Both of these cells lie near the position of the future segmental nerve, one anterodorsal and one posteroventral to it. The anterior cell divides once resulting in two progeny at early stage 12, one of which divides a second time at the end of this stage (Figs 3F, 4C-E). For the posterior cell only one division was detectable at the end of stage 12 (Fig. 4D-E). We were not able to follow the fate of these cells further.

Peripheral glia and exit glia

The majority of the peripheral glia and exit glia can be first recognised when they begin to migrate out of the VNC at stage 15 (Figs 2E,F, 5B,C). At stage 17 they consist of 4 exit and of 4-5 peripheral glial cells per abdominal hemisegment that line up along the segmental nerve between the VNC and the lateral pentascolopidial chordotonal organ (Fig. 5D).

One cell, PG3 (Klämbt and Goodman, 1991), expresses repo while already at a lateral position at the end of stage 12 (Fig. 5A). It remains in position during further embryonic development and is found associated with the dorsal part of the segmental nerve at stage 17 (Fig. 5D).

The *repo²* and *repo³* alleles do not express the repo antigen

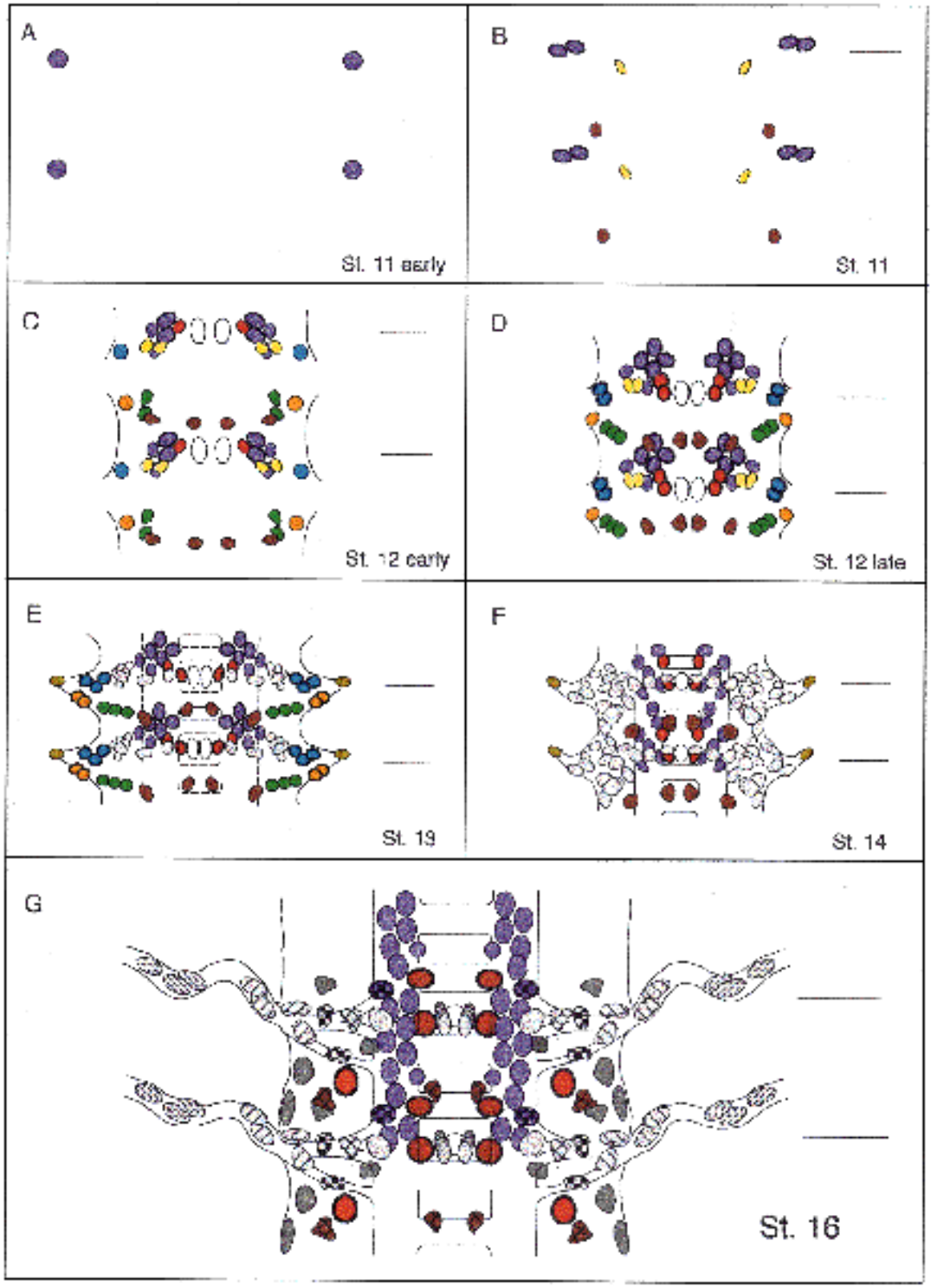
We identified three enhancer trap lines 3-692, 3-3702 and 3-2138 among a collection of lethal P-element insertions (Karpen and Spradling, 1992) as alleles of *repo*. These lines have also been used by Xiong et al. (1994) and were named *repo²*, *repo³* and *repo⁴* respectively. The *lacZ*-expression in heterozygous embryos of all three lines corresponds to the staining pattern of the anti-repo antibody except for some additional segmental lateral patches in the epidermis (data not shown). All three insertions are embryonic lethals and map at 90 F1-2. This corresponds to the region to which the *repo* cDNA hybridises. We cloned the flanking regions of all three lines and mapped the insertions at the sequence level (see Materials and Methods). In contrast to Xiong et al. (1994) we found that in the alleles *repo²* and *repo³*, the P-element is inserted at exactly the same site, and in the same orientation, within the 5′ untranslated region (UTR) of the gene between bp 202-203 of the published sequence (Xiong et al., 1994). The P-element in *repo⁴* is found 100 bp upstream of the 5′ end of our cloned cDNA sequence which starts at a position corresponding to bp 194 of the published sequence of Xiong et al. and is in the opposite orientation to the other two insertions (Fig. 8).

Analysis of the homozygous mutant embryos of all three lines revealed no detectable expression of repo protein in *repo²* and *repo³*. Since anti-repo is a strong polyclonal antibody, this suggests that the P-element insertion causes a null mutation for the protein product in these two lines.

Remobilisation of the P-element in both lines gave 34% and 48% rescue from lethality, respectively, demonstrating that the phenotype, which is identical in both cases, must be due solely to the P-element insertion.

Structural defects in the nervous system

We inspected the neuropile of the *repo²* mutant for possible defects by double staining with mAb22C10 or anti-fas II and anti-β-galactosidase. Up to and including stage 13, comparison of the axonal tracts of mutant and WT embryos stained with mAb22C10 or anti-fas II revealed no obvious defects in the mutant (data not shown). However, at late stage 14 disruptions in the axonal scaffold become apparent (Fig. 7F). The projections of the VUM neurons are particularly affected: in a variable number of segments they fasciculate with the projection of the aCC neuron at rather abnormal positions (Fig. 7E-



G). The PNS phenotype is much weaker. In early stages, outgrowth and fasciculation of fibers seemed normal. At later stages, fibers occasionally are partially defasciculated (compare Fig. 7K and L).

Additionally, in mutant embryos at stage 17, the VNC remains extended to varying degrees whereas in WT it condenses significantly during this stage (compare Fig. 7H and J). Moreover, the surface of the VNC in all mutant embryos has a very rough and loosely packed appearance in comparison to WT (compare Fig. 7C and D).

Fig. 4. Scheme of glial development in the abdominal neuromeres using the anti-repo antibody as a glial-specific marker. Ventral views, anterior is up. The colour code labels cells that start *repo* expression at a common position. Cells within the groups that are likely to be clonally related have a thickened outline. At later stages, cells that were no longer identifiable regarding their origin are drawn in grey. (A) early stage 11. The longitudinal glioblast (LGB, purple) appears first. (B) Mid stage 11. The LGB has divided (purple) and the MM-CBG start *repo* expression (brown). A further cell is labelled near the anterior border of the segment (yellow). (C) Early stage 12. The LGB-progeny (LG) reach their dorsomedial position. Two additional cells (purple) appear close to the LG presumably being of different origin. The MM-CBG migrate ventrally. Near the forming dorsoventral channel (open circles) at the midline the presumptive A- or B-SPG can be identified (red). Laterally two glial progenitors, one anterodorsal (blue) and one posteroventral (orange), start repo expression. (D) Late stage 12. The LG and the anterodorsal cell (blue) have divided. A second cell near the dorsoventral channel appears which represents A- or B-SPG (red). A group of cells (green) line up in a characteristic row near the ventrolateral surface of the developing nerve cord. (E) Stage 13. One of the progeny of the anterodorsal progenitor (blue) and the posteroventral progenitor (orange) have divided. An additional even more lateral cell starts repo expression (light green). The LG begin to stretch in an anterior-posterior direction. (F) Stage 14. Most of the cells can no longer be unequivocally assigned to their origin. The LG become aligned in the anterior-posterior direction and the two additional cells have intermingled. This group of cells is about to divide. (G) Stage 16. Glial cell types can be identified by their positions (see Ito et al., 1994). Interface glia (purple, without pattern); nerve root glia (checkered; M-ISNG is purple in addition); subperineurial and dorsoventral channel glia (dorsal, dark grey; ventral, light grey; one of the ventral SPGs and the A- and B-SPG, which derive from NB 1-1 (Udolph et al., 1993) are shown in red); cell body glia (cross-hatched); exit glia (hatched); peripheral glia (broken hatched).

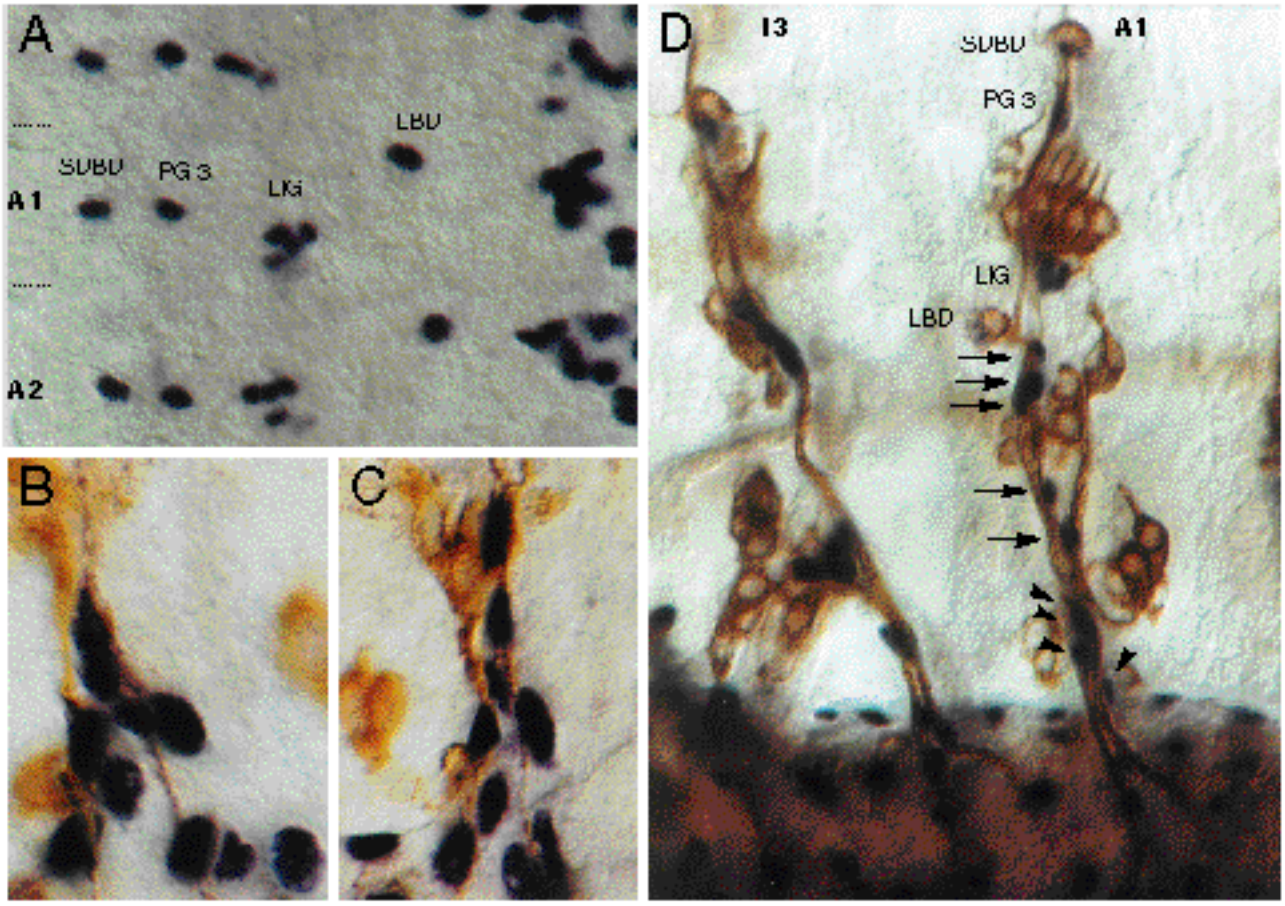


Fig. 5. Development of glial cells in the PNS (flat preps). (A) Repo-expressing cells at early stage 13; anterior is up, dorsal is left. (B-C) Late stage 14 and stage 15 embryos, double stained with anti-repo (black) and mAb22C10 (brown); anterior is left, dorsal is up. Peripheral and exit glial cells begin to migrate out, following the peripheral nerve. (D) Segments T3 and A1 at stage 17, double stained with anti-repo antibody and mAb22C10; anterior is left, dorsal is up. Arrows point to peripheral glial cells and arrowheads to exit glial cells, most of which derive from the CNS. LBD, lateral bipolar dendritic cell; LIG, ligament cells of the pentascolopidial chordotonal organ; PG 3, peripheral glia 3; SDBD, support cell of the dorsal bipolar dendritic cell.

Loss of repo affects glia migration and development

The *repo*^{2} mutant, in which no repo protein is detectable, still expresses β-galactosidase. Consequently, we could use this strain to follow the development of the glia in the mutant background. In *repo*^{2} homozygous mutant embryos the longitudinal glioblast starts to express β-galactosidase at its normal

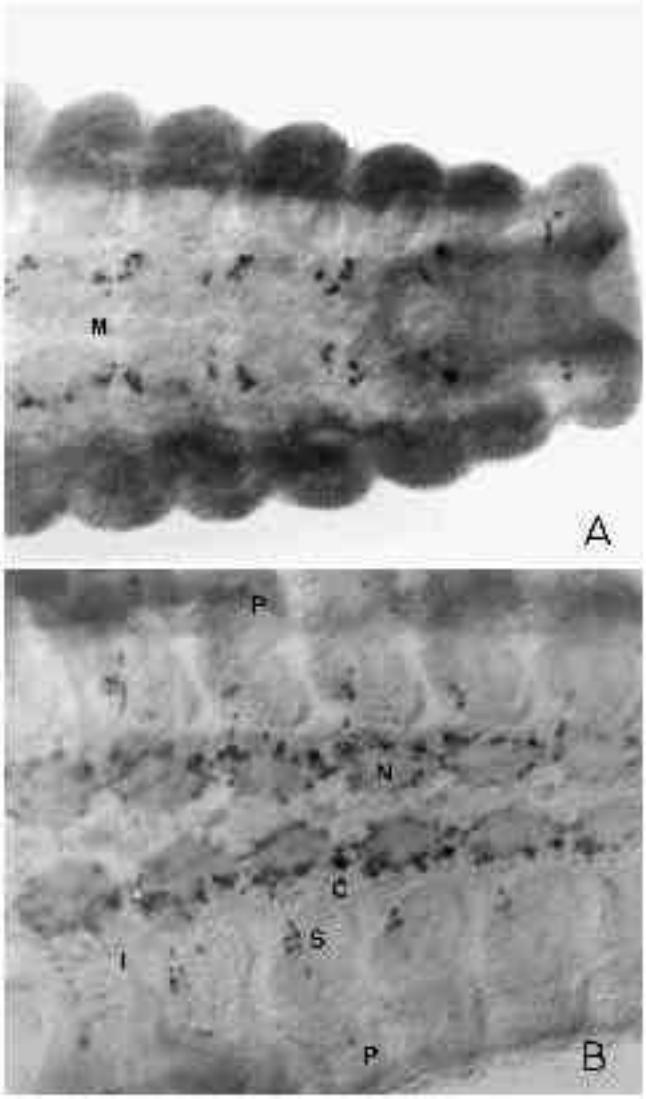


Fig. 6. Anti-repo antibody recognises a homologous protein in the nervous system of *Schistocerca gregaria.* Dorsal view of a stage 40% (A) and stage 45% (B) embryo, anterior is left. (A) No staining is visible in the ventral midline. (B) One cell is stained in a very lateral position (P), whereas, like in *Drosophila*, most of the other identified labelled cells are around the neuropile (interface glia). C, glia of the connectives (longitudinal glia); I, intersegmental nerve glia; N, neuropile; M, median neuroblast; S, segmental nerve glia; P, peripheral glia.

position. As in WT embryos the glioblast divides and migrates towards the midline. At the end of stage 12 the mutant phenotype becomes visible: the MM-CBG, which derive laterally do not reach their final position close to the VUM neurons but stop migrating halfway (compare Fig. 7A and B). Also, from this stage on, the migration of the interface glia and other glia is disturbed and the cells show a rather irregular distribution at the end of embryogenesis (Fig. 7D).

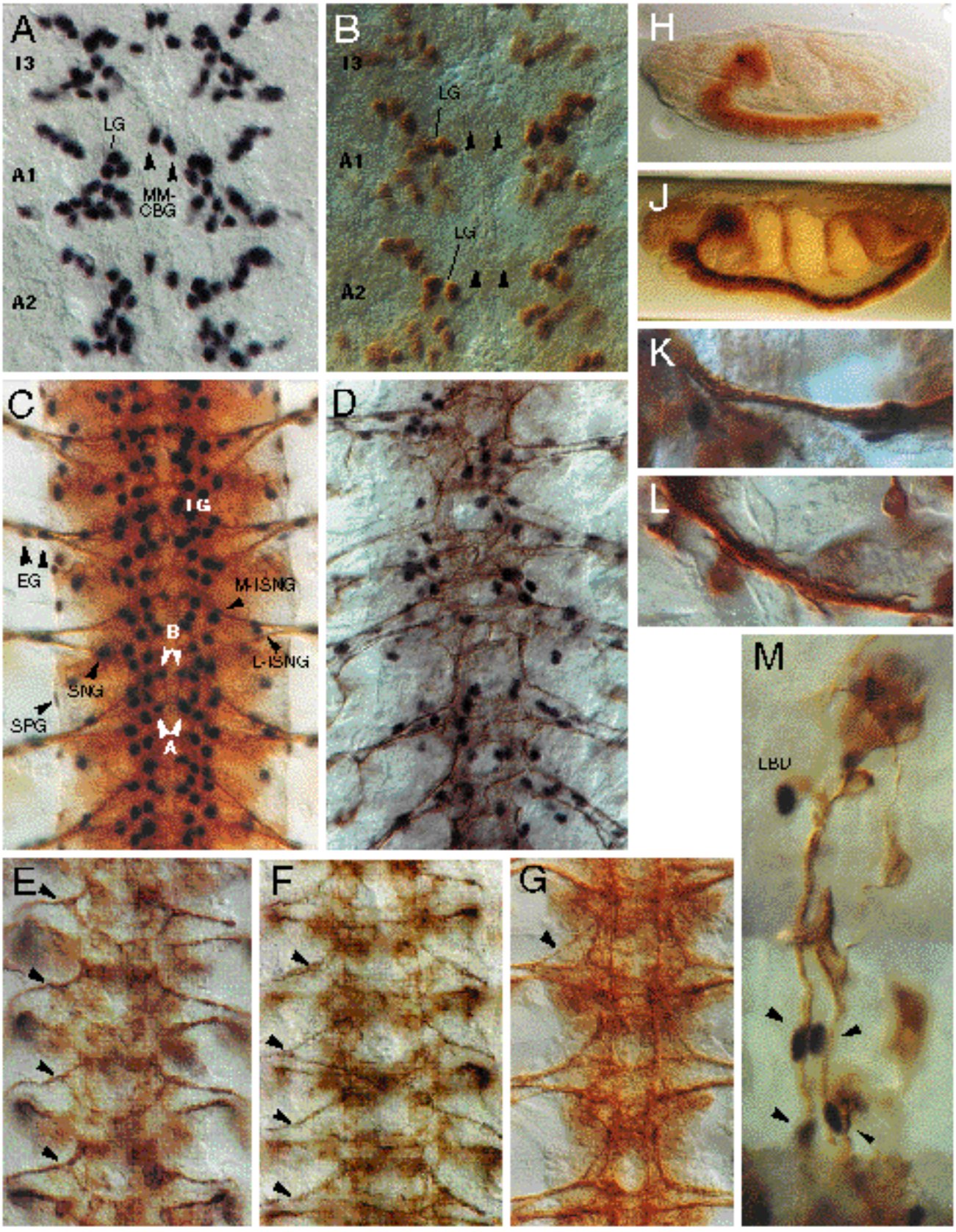
At stage 12 there is no significant difference in the number of glial cells in the developing VNC compared to those in WT. However, at stage 16, the total number of labelled cells within the CNS is reduced to an average of 32 cells per abdominal

Fig. 7. Different aspects of the *repo* phenotype (B,D,F,G,J,L,M) in comparison to WT (A,C,E,H,K). (A,B) Segments T3 to A2 at mid stage 12, dorsal view, anterior is up; (A) WT stained with anti-repo antibody, (B) *repo*^{2} stained with anti-β-galactosidase. In *repo*^{2} the overall pattern seems normal; however, the MM-CBG is not migrating to its normal medial position (arrowheads). (C,D) Abdominal segments, at stage 17; flat preps, dorsal view, anterior is up. (C) WT stained with anti-repo (black) and mAb22C10 (brown), (D) *repo*^{2} stained with anti-β-galactosidase and mAb22C10. In the mutant, the number of glial cells is reduced to approx. 55% and glial cells show an irregular distribution. In addition the surface of the VNC is irregular. (E,F,G) Abdominal segments at stage 14 (E,F) and stage 16 (G); flat preps. WT (E) and mutant embryos (F,G) stained with mAb22C10. At stage 14, the two roots of the intersegmental nerve often fasciculate at abnormal positions in the mutant (compare arrowheads in E and F). This is still the case at later stages (arrowhead in G). (H,J) Lateral views of whole-mount embryos at stage 17 stained with BP102. Mutant embryos (J) do not retract their VNC properly. (K,L) Abdominal peripheral nerves in the WT (K) and mutant (L) at stage 17; flat prep. In the mutant, fibers are less tightly fasciculated. (M) Abdominal peripheral nerve of mutant embryo at stage 17, double stained with anti-β-galactosidase and mAb22C10; anterior is left. Numbers of exit glia and/or peripheral glia are reduced at the ventral part of the nerve (arrowheads; compare with Fig.5D). A,A–SPG; B, B–SPG; SPG, subperineurial glia; EG, exit glia; LBD, lateral bipolar dendritic cell; IG, interface glia including longitudinal glia; LG, cells derived from the longitudinal glioblast; L-ISNG, lateral intersegmental nerve root glia; M-ISNG, medial intersegmental nerve root glia or segment boundary cell; SNG, segmental nerve root glia; MM-CBG, medial-most cell body glia (or VUM support cells).

segment (*n*=70 segments) as compared to an average of 58 cells in WT (*n*=21 segments) (Fig. 7C,D). These mean values were determined by counting the cells of 3-4 consecutive abdominal segments in 21 and 7 different embryos respectively. In the PNS of mutant stage 16 embryos, there are only 4-6 glial cells associated with the abdominal segmental nerves compared with 9-10 in WT (compare Figs 5D and 7M). This is in contrast to the findings of Xiong et al. (1994) who observed no difference in glial cell numbers in the same mutant line.

In order to confirm these data with independent glial-specific enhancer trap lines as markers we first screened lethal excisions of the *repo*^{3} insertion for absence of both repo and β-galactosidase expression. We selected two excision lines, EX52 and EX84, which both showed no expression of either antigen. Furthermore, their phenotypes with respect to the neuropile and to the VNC retraction were similar to the putative protein null mutantssuggesting that no other genes are affected in these mutants.

The enhancer trap line rA87 (Klämbt and Goodman, 1991) was crossed into the mutant background of the two excision mutants. rA87 labels the same glial cells as repo in a comparable time course (Fig. 9A). Analysis of the anti-β-galactosidase staining in the excision mutants showed the same irregular distribution of the labelled cells as in *repo*^{2} and *repo*^{3} (Fig. 9E). Counting the cells within the VNC of EX84 at stage 16 revealed nearly the same reduction in cell number as in the original enhancer trap lines (33 cells per segment; *n*=21 segments; 7 embryos). This reduction in cell numbers does not seem to be due to elevated cell death, as no increased numbers of apoptotic cells were found on applying Nile Blue A (Abrams et al., 1993) to the mutants between stage 13 and 16. This



suggests that the reduced number of glial cells is due to a failure of glial cell division in the mutant.

Three additional glial-specific marker strains were crossed to EX52 and EX84: M84, which labels the subperineurial glia (Klämbt and Goodman, 1991; Udolph et al., 1993), 2-3563, which is expressed in a subset of three nerve associated glial

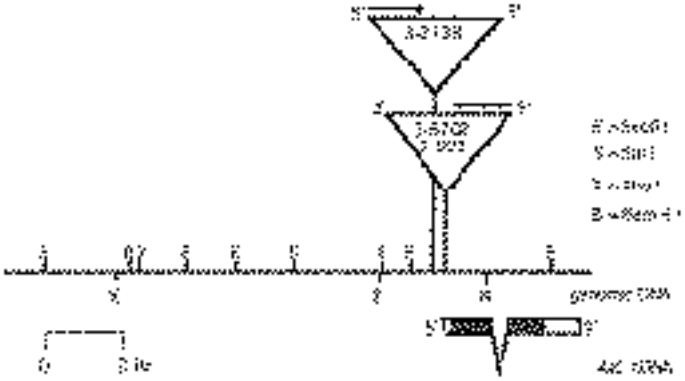


Fig. 8. Genomic organisation of the *repo* locus. The thick horizontal line represents the genomic region. The organisation of the primary transcript is shown below. The translated region is shaded and the intron shown. The P-element insertions are indicated by the triangles and the direction of *lacZ* transcription indicated by horizontal arrows.

Fig. 9. Expression of glial *lacZ* markers in WT (A,B,C,D) and in mutant background (excision mutant EX84; E,F,G,H). (A,E) Abdominal segments at stage 16 (flat preps), dorsal view; anterior is up. The enhancer trap line rA87 labels the same glial cells as anti-repo antibody. In mutant background, stained cells show similar irregular distribution and reduction of number as in *repo*^{2}. (B,F) Abdominal segments at stage 17 (flat preps); dorsal view, anterior is up. Kr–lacZ PH3.7 labels MM-CBG (arrows) and other cell body glia (arrowheads). No detectable β-galactosidase expression is found in mutant background (F). (C,G) Whole–mounts at stage 17; ventral view, anterior is left. 2-3563 stains a subset of 3 nerve-associated glial cells (exit/peripheral glia) (arrowheads) and one additional unidentified cell in each thoracic hemineuromere (arrows in C). In the mutant background marker expression in the exit glia is not detectable while it is still present in the single CNS cells (arrows in G). (D,H) Whole mounts at stage 17; lateral view, anterior is left. M84 stains subperineurial glial cells and cells of the lymph glands (D). In mutant background (H) anti-β-galactosidase expression is significantly reduced in the glial cells but clearly detectable in the lymph glands (see arrows). EG, exit glia; IG, interface glia; SPG, subperineurial glia.

cells (exit/peripheral glia), and Kr-lacZ PH3.7, which labels cell body glia (Fig. 9B-D). In contrast to rA87 these lines start expression of β-galactosidase rather late (M84: stage 14; P3563: late stage 12; Kr-lacZ PH3.7: early stage 13). The expression of all three reporter genes was no longer detectable in most mutant embryos (Fig. 9F-H), although in a few cases we still saw residual expression. Thus, the expression of these rather late glial-specific markers is severely reduced. This indicates that the expression of these markers depends on the normal function of the *repo* gene and that glial differentiation is affected in the mutant.

DISCUSSION

Is the *repo* expression glial-specific?

An increasing number of glial cells have been identified in the embryonic CNS and PNS of *Drosophila* (Fredieu and Mahowald, 1989; Klämbt and Goodman, 1991; Goodman and Doe, 1993; Nelson and Laughon, 1993; Ito et al., 1994). Based on these data and by comparing the *repo* expression pattern with glial-specific markers we were able to identify most of the *repo*-expressing cells in the CNS and PNS as glial cells. For the CNS a similar conclusion was reached by Xiong et al. (1994) who showed that the *repo*-expressing cells do not express elav, a neuron specific marker (Robinow and White, 1988). We find that all known CNS glial cells, with the exception of the midline glia and two of the previously described segmental nerve root glial cells, express *repo.*

In the PNS all known peripheral glia including the support cell of the dorsal bipolar dendritic neuron are *repo* positive. The only exceptions are the glia that are associated with the transverse nerve (Nelson and Laughon, 1993; Groczyca et al., 1994). In addition, some ligament cells and the lateral bipolar dendritic neuron (lbd) express *repo.* The lbd is also positive for the neuron-specific antibody against HRP (Bodmer and Jan, 1987). However, as no specific innervation has been identified, the function of this cell is still unknown. Our finding that the lbd expresses an otherwise strictly non-neuronal gene might argue against its neuronal identity.

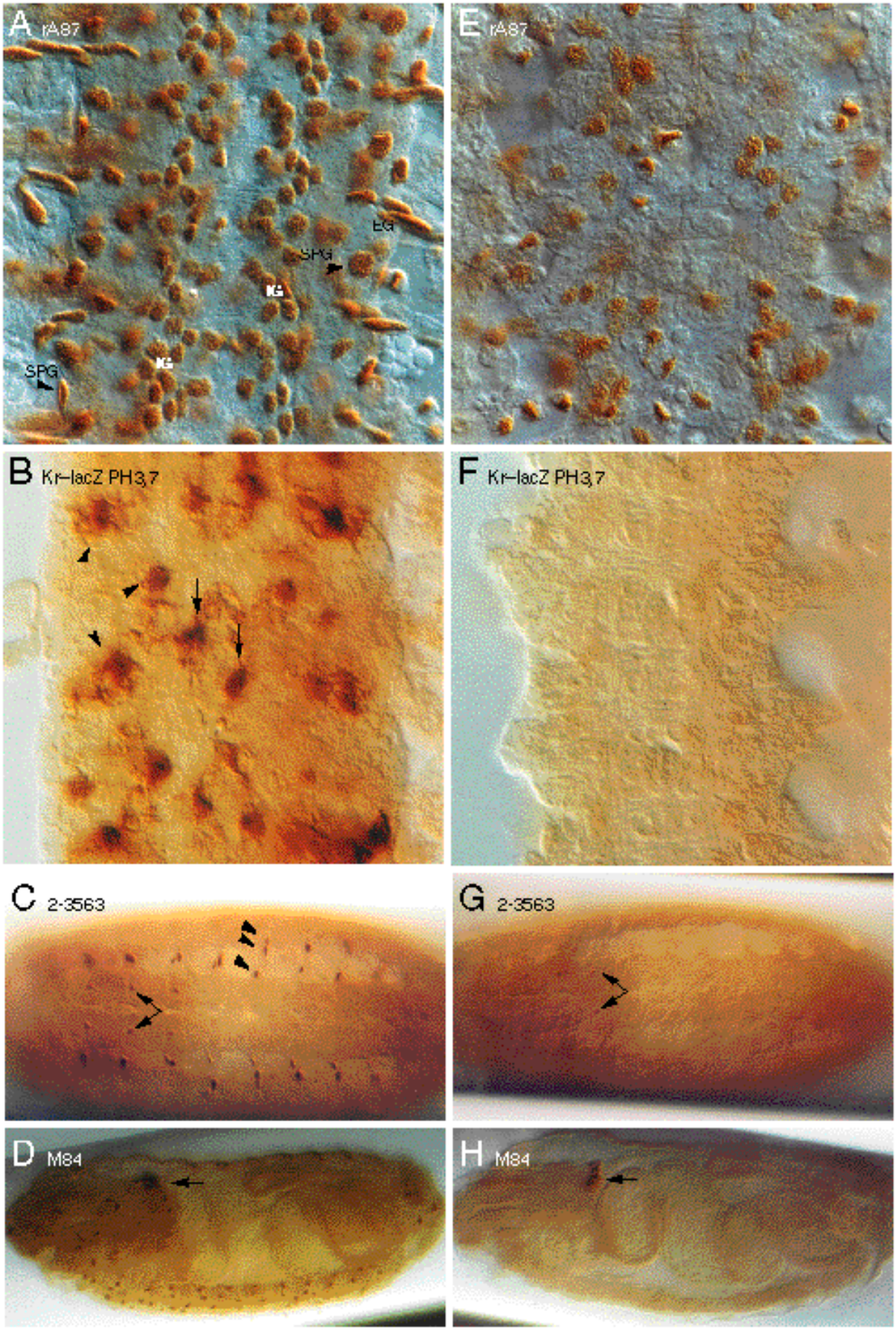
Gliogenesis as revealed by the repo expression pattern

Our data suggest that not all the glial cells associated with the longitudinal tracts derive from the longitudinal glioblast (LGB). At stage 13 two cells of unknown origin intermingle with the progeny of the LGB. At stage 17 one of the resulting 9-10 cells lies at the intersegmental nerve root (M-ISNG), whereas the others remain associated with the longitudinal tracts.

The MM-CBG, which in the embryo lie close to the midline-derived VUM neurons, were also assumed to originate from the midline (VUM support cells, Klämbt et al., 1991; Goodman and Doe, 1993). However, by analysing the lineages of the midline cells, this assumption could not be confirmed (Bossing and Technau, 1994). With the anti-repo antibody we now document that these glial cells originate from a more lateral site of the neuroectoderm. They appear at stage 11 and migrate ventrally towards the midline during stage 11/12.

We have identified two further progenitors with mitotic activity. Judged from their lateral position these cells may represent progenitors of the exit and/or the peripheral glia. For many other cells we did not observe any divisions after the onset of repo expression. These data suggest, that *repo* is expressed soon after a cell is determined for a glial fate and that this can occur at different stages during lineage development.

With respect to glial development in the embryonic PNS, we see an interesting parallel with the glial cells in the wing disc (Giangrande et al., 1993). In the embryo, one peripheral glial cell (PG 3) (Klämbt and Goodman, 1991; Nelson and Laughon, 1993) originates from the dorsolateral ectoderm and remains in this position. Most of the other peripheral glia and the exit glia originate within the VNC, and leave the CNS to migrate dorsally along the already existing nerves towards their final positions. In the wing disc a similar behaviour was observed: some of these glial cells stay at their site of origin whereas others, although not originating from the CNS, migrate along the nerve towards their final destination (Giangrande, 1994). Thus, in both systems the nerve associated glia consists of two populations: stationary and migrating peripheral glial cells.


A rA87
EG
SPG
MG
MG
SPG
E rA87
B Kr–lacZ PH3,7
F Kr–lacZ PH3,7
C 2-3563
G 2-3563
D M84
H M84

repo is not required for the formation of longitudinal tracts

Based on the spatial relationship between longitudinal glia and pioneering axons in locust and *Drosophila* it has been proposed that glia provide a matrix for longitudinal axon extension (Jacobs and Goodman, 1989). This is supported by the observation that in mutants with malformation of the longitudinal tracts, the longitudinal glia also show perturbations in migration and differentiation (Jacobs, 1993). Moreover, mutations in *pointed*, which is exclusively expressed in glial cells including the midline glia and longitudinal glia, cause fused commissures and disruption of the longitudinal connectives (Klämbt, 1993). In spite of the fact that the glial differentiation is heavily perturbed in mutants for the repo homoeodomain protein (see below), the longitudinal glia still migrate to the correct position and the longitudinal tracts are formed. We conclude that expression of *repo* in the longitudinal glia is not required for the formation of the longitudinal tracts.

repo function is necessary for proper fasciculation of nerve roots and segmental nerves

In contrast to the normal formation of the connectives, the structure of the intersegmental nerve roots is affected by the absence of *repo* function: all mutant embryos at stage 16 show a number of axons with abnormal fasciculation patterns in the nerve roots of the CNS. Examination of earlier stages revealed no obvious defects in the axonal projections up to stage 14 when many mutant embryos show fasciculation of the two intersegmental nerve roots at abnormal positions. Since there is a high degree of variability concerning this phenotype in the CNS we were not able to determine whether this is due to inadequate navigation or recognition of axons or to secondary defasciculation.

Similarly in the PNS, axons of some nerves are not properly fasciculated in the mutant (Fig. 7K,L). Previous ultrastructural studies on glial cell morphology showed that nerve-associated glia associated with nerves form a tight sheath around the axon bundles (Murray et al., 1984). In late embryos lacking the repo expression the number of these cells is significantly reduced (see below). The remaining cells are irregularly distributed although many of them remain associated with the axons. Therefore, the most likely explanation for this phenotype in the PNS is that these cells are no longer able to ensheath the nerves correctly, leading to partial defasciculation of axonal tracts.

The repo homeobox protein is an essential factor for glial development

The mechanisms that lead to glial-specific differentiation are still unknown. Glial-specific transcription factors are likely to be involved in this process. One recent example of such a transcription factor is the ETS homologue *pointed*, which is implicated in some aspects of glial-specific differentiation (Klämbt, 1993; Klaes et al., 1994).

We have described here another putative glial-specific transcription factor. Interestingly, because of the glial-specificity of certain artificial promoter constructs using homeodomain binding sites of *ftz* and *en* in front of *lacZ* (Nelson and Laughon, 1993; Vincent et al., 1990), the existence of at least one glial-specific homeodomain protein was expected. The *repo* gene codes for such a protein and it is very likely that it is at least partially responsible for the expression patterns of these reporter genes.

Table 1. Alignment of an octapeptide homology present distal to the homeodomain sequence in repo and other regulatory proteins

Protein	Amino acid sequence	Reference
repo	YGAYVHES	Xiong et al., 1994
Chx10	YGAMVRHS	Liu et al., 1994
ftz	YPAYSHSH	Laughon and Scott, 1984
eve	YGQYRYTP	Macdonald et al., 1986
al	YNPYLPGG	Schneitz et al., 1993

Chx10 is from mouse, the others from *Drosophila melanogaster*. The first tyrosine is at the following position in each protein: repo, 438; Chx10, 222; ftz, 363; eve, 214; al, 153.

Homeodomain proteins have been subdivided into classes based on sequence similarity (Scott et al., 1989). The repo homeodomain shows the highest homology to those of the paired class proteins especially to al, unc-4, pax-3 and chx-10 (Schneitz et al., 1993; Miller et al., 1992; Goulding et al., 1991; Liu et al., 1994). However, of the 16 conserved residues defining the prd class, repo differs in 9 and consequently Xiong et al. (1994) have suggested that repo may represent a distinct subclass of the paired group. We note that distal to the homeodomain, repo contains a sequence of eight amino acids which shows strong homology to similarly placed sequences in certain other homeodomain proteins including ftz (Table 1).

Examination of the sequences of the recognition helices reveals that repo differs in one residue (51) from prd, glutamine replacing serine. This position is an important determinant of DNA-binding specificity. In ftz, residue 51 is also glutamine, which would be consistent with the recognition of the CAATTA sequence by both ftz and repo. It has been shown for ftz, even-skipped (eve) and engrailed (en) that a glutamine-51 preferentially binds to a CAATTA motif (Desplan et al., 1988; Hoey and Levine, 1988; Percival-Smith et al., 1990). Changing serine-51 to glutamine-51 in the prd homeobox showed preferred binding of this mutated protein to the CAATTA motif (Wilson et al., 1993). Our observation that repo binds to CAATTA complements these observations and is consistent with the glial-specific expression of a reporter gene driven by *ftz* or *en* recognition elements (Vincent et al., 1990). This implies that in this situation repo acts formally as a transcriptional activator. The equivalence in recognition properties between ftz and repo does not however necessarily indicate that repo binds to the *ftz* USE in vivo. In the developing CNS *tramtrack*, a repressor of *ftz* (Brown et al., 1991), is expressed in most, and possibly all, glia (Harrison, Fairall and Travers, unpublished data) but it is unclear at present whether this expression is wholly contemporaneous with that of *repo*.

The expression of *repo* starts very early in glial development, probably when the cells have assumed a glial identity, and continues during postembryonic development (Xiong et al., 1994, our unpublished observations). This suggests that *repo* may play a major role in the differentiation and maintenance of most of the embryonically derived glia.

Analysis of *repo*^{2}, EX52 and EX84, which represent

putative null alleles for repo protein expression, reveals mainly late developmental defects within the embryonic nervous system. The first glial progenitors, like the longitudinal glioblast, originate normally and show initially normal migration behaviour. In addition the glial-specific enhancer trap line rA87, which labels the same set of glia as the anti-repo antibody from early stages on, still shows β-galactosidase expression in the mutant background. This suggests that the cells still acquire a glial identity. The typical tight association of some cells with the fiber tracts of the late mutant embryo supports this conclusion.

Nevertheless, the differentiation of the presumptive glia seems to be severely affected: at the end of embryogenesis we found a rather irregular distribution of glial cells. Moreover, three independent *lacZ*-lines, which label different subsets of glial cells at later stages, show reduced or no expression within the mutant background of the P-element excision lines EX52 and EX84. This is supported by similar results of Xiong et al. (1994). Using the weak hypomorphic allele $repo^{1}$ they detected a reduced *lacZ* expression of the glia-specific enhancer trap line 3-109 within the optic lobe. Moreover they could show that the glia-specific homeobox gene *otd* is downstream of *repo*.

The defects in axon fasciculation and the inhibition of the VNC condensation are most probably due to defects in glial differentiation. A similar failure of VNC condensation is observed in *prospero* mutants. In this case the belt glia (one or two of the subperineurial glial cells) are assumed to be responsible for the phenotype (Doe et al., 1991).

In addition to these differentiation defects and in contrast to the findings of Xiong et al. (1994), we observed an approx. 45% reduction in cell number in the mutant embryos. We could not detect any elevated cell death in mutant embryos compared to WT. Thus, it seems that a smaller number of glial cells is born in the mutant. Since *repo* codes for a putative transcription factor this reduction in cell number is probably a cell autonomous effect although we cannot at present rule out non autonomous mechanisms. Taken together, these results suggest that *repo* plays a major role in the glial differentiation process.

We thank Kevin Moses for confirming the *repo* locus on the chromosome, Rachel Dawes for staining the locust embryos, Bea Christen for assistance in generating excision lines EX52 and EX84, Michael Bate and David Shepherd for confirming the glial identity of anti-repo labelled cells in the locust, Christian Klämbt for the enhancer trap lines M84 and rA87 and the mAb 22C10 antibody, Corey Goodman for the fas II antibody, Dietmar Schmucker and Herbert Jäckle for the line Kr-lacZ PH 3.7, and Allan Spradling for the lethal enhancer trap lines 3-692, 3-2138, 3-3702 and 2-3563.

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Note added in proof

While this paper was under review similar functions of the *repo* gene were described (Campbell, G., Göring, H., Lin, T., Spana, E., Andersson, S., Doe, C. Q. and Tomlinson, A. (1994) *Development* **120**, 2957-2966).