

***loco* encodes an RGS protein required for *Drosophila* glial differentiation**

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SUMMARY

In *Drosophila*, glial cell development depends on the gene *glial cells missing* (*gcm*). *gcm* activates the expression of other transcription factors such as POINTED and REPO, which control subsequent glial differentiation. In order to better understand glial cell differentiation, we have screened for genes whose expression in glial cells depends on the activity of POINTED. Using an enhancer trap approach, we have identified *loco* as such a gene. *loco* is expressed in most lateral CNS glial cells throughout development. Embryos lacking *loco* function have a normal overall morphology, but fail to hatch. Ultrastructural analysis of homozygous mutant *loco* embryos reveals a severe glial cell differentiation defect. Mutant glial cells fail to properly ensheath longitudinal axon tracts and do not form the normal glial-glia cell

contacts, resulting in a disruption of the blood-brain barrier. Hypomorphic *loco* alleles were isolated following an EMS mutagenesis. Rare escapers eclose which show impaired locomotor capabilities. *loco* encodes the first two known *Drosophila* members of the family of Regulators of G-protein signalling (RGS) proteins, known to interact with the α subunits of G-proteins. LOCO specifically interacts with the *Drosophila* G α i-subunit. Strikingly, the interaction is not confined to the RGS domain. This interaction and the coexpression of LOCO and G α i suggests a function of G-protein signalling for glial cell development.

Key words: *loco*, RGS protein, *Drosophila*, Glial differentiation, *glial cells missing*, *pointed*

INTRODUCTION

Within the *Drosophila* nervous system, glial cells perform a variety of important functions. They are capable of controlling proliferation rates of neighboring neuroblasts (Ebens et al., 1993), they ensheath and electrically insulate axon bundles (Auld et al., 1995) and they are likely to secrete neurotrophic factors (Xiong and Montell, 1995). Furthermore, during embryonic development, neuron-glia interactions are required for the correct establishment of the axonal scaffold (Jacobs and Goodman, 1989; Klämbt and Goodman, 1991; Goodman and Doe, 1993; Hidalgo et al., 1995; Hosoya et al., 1995; Jones et al., 1995; Klämbt et al., 1996).

Based on position and gene expression, embryonic CNS glial cells can be divided into two major classes, the midline glia and the lateral glia (Klämbt et al., 1996). The lateral glial cells are a heterogeneous group of cells, characterised by the expression of a number of genes encoding transcription factors. The lineage relationships of these cells have been described (Jacobs et al., 1989; Bossing et al., 1996; Schmidt et al., 1997). Lateral glia development is initiated by the activity of the gene *glial cells missing* (*gcm*), which acts as a

master regulatory gene of glial cell development (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). *gcm* encodes a novel transcription factor with an 8-nucleotide DNA-binding site (Akiyama et al., 1996; Schreiber et al., 1997). In its absence, glial-specific gene expression is abolished and glial cells are transformed into neuronal cell types (Hosoya et al., 1995; Jones et al., 1995; Bernardoni et al., 1997). *gcm* governs glial development by activating transcription factors such as TRAMTRACK, POINTED and REPO, which in turn control glial differentiation by two different mechanisms (Hosoya et al., 1995; Jones et al., 1995; Giesen et al., 1997). On the one hand, inappropriate, neuronal differentiation appears to be suppressed in developing glial cells by the BTB-Zn-finger domain protein TRAMTRACKP69 (Giesen et al., 1997). On the other hand, glial differentiation needs to be activated by transcription factors such as the POINTED ETS domain proteins or the REPO homeodomain protein (Campbell et al., 1994; Klaes et al., 1994; Xiong et al., 1994; Halter et al., 1995).

pointed encodes two proteins, PNTP1 and PNTP2, which share a common ETS DNA-binding domain. Within the embryonic CNS, both POINTED proteins are specifically expressed in non-

overlapping sets of glial cells, with *PNTP1* being expressed in many lateral glia and *pntP2* being restricted to the midline glial cells (Klämbt, 1993; Scholz et al., 1997). In *pointed* mutant embryos, glial cells fail to differentiate and do not properly ensheath the neuropile (Klaes et al., 1994; Giesen et al., 1997). Ectopic expression and single cell transplantation experiments demonstrated that *pointedP1* is not only required but also sufficient for several aspects of glial cell differentiation (Klaes et al., 1994). *POINTEDP1* binds directly to DNA through a conserved consensus site and subsequently acts as a transcriptional activator (O'Neill et al., 1994; Albagli et al., 1996).

To understand *pointed* function during glial cell development, it is necessary to identify its glial-specific target genes. A number of approaches have been employed to identify direct targets of transcription factors (Gould et al., 1990; Wagner-Bernholz et al., 1991). Here we have utilized the enhancer trap technique to isolate a candidate target gene of *pointed*. The integration of a modified P-element in the vicinity of genomic enhancer regions often leads to a tissue- or cell-specific expression pattern of the *lacZ* reporter gene (Bellen et al., 1989; Bier et al., 1989). In many cases, *lacZ* expression precisely mimics the expression of a nearby endogenous gene. Thus genes can be isolated solely based on their expression pattern irrespective of their mutant phenotypes.

We have screened 11,000 enhancer trap insertion lines for expression in the developing CNS. About 30 lines lead to specific expression in various subsets of CNS glial cells. Among this collection, P-element insertions into the genes *gcm*, *pointed*, *tramtrack* and *gliotactin* have been identified (Klämbt, 1993; Auld et al., 1995; Jones et al., 1995; Giesen et al., 1997). Here we report on two P-element integrations at the cytological interval 94B/C. Within the CNS, β -GALACTOSIDASE expression pattern conferred by the lines 3-109 and *rC56* is restricted to lateral glial cells (Klämbt and Goodman, 1991; Winberg et al., 1992) and corresponds well to the expression pattern of an endogenous gene near the P-element integration sites named *loco*. *loco* encodes the first two known *Drosophila* members of the RGS protein family, whose members are known to regulate G-protein signalling. One LOCO isoform is specifically expressed in glial cells. During glial cell development, *loco* is required for the correct extension of cell processes. *loco*-deficient glial cells fail to ensheath axons as well as failing to perform normal glia-glia cell interaction resulting in a loss of the blood-brain barrier. In a yeast two-hybrid screen, a specific interaction of LOCO with $G\alpha i$ could be demonstrated. This interaction can also be mediated by protein domains outside the RGS domain.

MATERIALS AND METHODS

Fly work

Two P-element insertions into the *loco* gene were isolated in a large enhancer trap screen (C. K., A. Nose and C. S. G., unpublished data), *loco*³⁻¹⁰⁹ and *loco*^{C56}, both of which carry a P[*rosy*, *lacZ*] insertion at the cytological position 94B/C. Both P-element insertion lines are homozygous viable. Four X-ray-induced alleles reverting the *rosy* marker carried by the *rC56* enhancer trap insertion in the

loco gene were recovered: *Df(3R)23D1* deleting the cytological region 93F-94E; *Df(3R)5C1* deleting the cytological region 93E/F-94C/D; *Df(3R)15CE1* deleting the cytological region 93F-94C/D; *Df(3R)17D1* deleting the cytological region 93E/F-94B/C.

Lethal *loco* alleles were recovered as transposase-induced revertants of the *loco*^{C56} allele. From 700 independent excision strains analyzed, 2 lines were lethal over the X-ray-induced deficiencies and failed to complement each other. The genomic breakpoints of *loco* ^{Δ 13} were cloned by inverse PCR: 35 cycles of 10 seconds 94°C/1 minute 54°C/4 minutes 72°C) using primers PZ-3547r (5'-GTCGCCACCAATCCCCAT-3') and PZ-3588 (5'-TGGAGCCCCGTCAGTATCG-3'). EMS-induced *loco* alleles were generated following standard mutagenesis procedures (Lewis and Bacher, 1968) on an isogenised *st e* chromosome and identified by non-complementation of *loco* ^{Δ 13} at 29°C.

All *loco* alleles were kept over a *TM3 Sb* balancer chromosome carrying a P[*elav-lacZ*] construct in order to allow the unambiguous identification of mutant embryos.

Histology

Immunohistochemistry and electron-microscopic analyses were performed as described previously (Klämbt et al., 1991; Stollewerk et al., 1996).

DNA techniques

Genomic *loco* DNA sequences were isolated by plasmid rescue and used to screen a genomic EMBL4 library, kindly provided by M. Noll, Zürich. Genomic phages were hybridised to polytene chromosomes to verify the cloning of the correct chromosomal region at 94B/C. cDNA clones were isolated from a λ gt11 library (Zinn et al., 1988). Nested deletions were constructed after DNase I treatment (Sambrook et al., 1989) and subsequently sequenced using a T7 based sequencing kit (Pharmacia) or an ABIPRISM 310 automated sequencer. The sequence of the longest cDNA clone for each transcript class (p109c1 corresponding to the glia transcript and p109c2 corresponding to the PNS transcript) was determined and confirmed by sequencing corresponding genomic clones. The clone p109c1 is 2918 bp in length. At base 207, there is an ATG in frame with a long open reading frame (ORF) coding for a hydrophilic protein of 829 amino acids, with a deduced molecular weight of approximately 97 kDa. The 3918 bp clone p109c2 corresponds to the *loco-c2* transcript and encodes a protein of 1175 amino acids. The relative molecular weight of the deduced LOCO-c2 protein is 135 kDa. Sequence analysis was performed using the Lasergene software package.

Several differences between cDNA and genomic sequences were noted. The ones resulting in amino acid substitutions are (G204→C204; R18S) (C206→G206; N19K) (A316→G316; E56G) (A435→G435; I95V) (A718→G718; N190S) (C1101→T1101; L317W) (C3675→T3675; R1175stop). Homology searches were performed using FASTA (Altschul et al., 1990).

Yeast two-hybrid screen: strains, plasmids and procedures used for the two-hybrid screen were essentially as described (Golemis and Brent, 1997). Construction of $G\alpha i$ bait: the $G\alpha i$ -coding region was fused to the *lexA*-coding region, by cloning a *Bam*HI-*Xho*I fragment containing the coding region of a $G\alpha i$ cDNA clone into the bait vector pEG202-PL1, to make the $G\alpha i$ bait plasmid pEG202GAIwt. pEG202-PL1 was based on the bait vector pEG202 (Gyuris et al., 1993; Golemis and Brent, 1997), but with some alterations to permit more flexibility in inserting bait sequences in frame with the upstream *lexA* sequence. pEG202 was cut with *Eco*RI and *Xho*I, and a fragment made from two 25-mer oligonucleotides that had been annealed to form a double-stranded linker with overhangs that could ligate to the cut pEG202, was inserted. This gave a polylinker sequence (codons shown in frame with upstream *lexA*) of CTG GAA TTC CGC GGA TCC ATG GCG GCC GCT CGA GTC GAC CTG AGC, and a vector with unique *Eco*RI, *Sac*II,

*Bam*HI, *Nco*I, *Nor*I, *Xho*I and *Sal*I sites, with all sites except *Eco*RI in a different reading frame from pEG202.

The G α i-coding region fragment was prepared as follows. (1) An *Eco*RI-*Xho*I C-terminal fragment of a G α i cDNA clone, containing DNA from the *Eco*RI site at codon 157 to a *Hinc*II site 180 bp 3' to the stop codon (Provost et al., 1988) was subcloned into these sites of pBluescript, to give pBSKS-GAiwt-RI-*Xho*I. (2) The 5' end of the gene was amplified by PCR, using a left-hand primer that included the ATG initiation codon and added a *Bam*HI site and an *Nde*I site that included the ATG, and a right-hand primer 3' of the *Eco*RI site at codon 157. (3) The *Bam*HI-*Eco*RI fragment of this PCR product was subcloned into pBSKS-GAiwt-RI-*Xho*I to give pBSKS-GAiwt-ATG-*Xho*I. (4) A *Bam*HI-*Xho*I fragment, carrying the whole of the Gi gene, was cut out of this plasmid and cloned between the *Bam*HI and *Xho*I sites of pEG202-PL1, to give pEG202GAiwt and putting G α i in frame with *lexA*.

Library screening: the *Drosophila* ovary cDNA library RFLY3 (Finley et al., 1996) in vector pJG4-5 (Gyuris et al., 1993) was obtained from Russ Finley. This library is estimated to contain 3.2 \times 10⁶ independent cDNA clones. An aliquot was used to generate over 10⁷ transformants in *E. coli* strain XL1-Blue, and these transformants were pooled and used to prepare library DNA. About 3 \times 10⁷ transformants of yeast strain EGY48 (pSH18-34) that carried pEG202GAiwt were generated using this library preparation, of which about 350 had a LEU2+ phenotype that indicated a possible interaction. PCR amplification and restriction digestion was used to identify duplicate clones and 25 unique clones were identified. One clone of each type was used to retransform yeast strain EGY48 (pSH18-34) that carried pEG202-GAiwt. 16 clones conferred a LEU2+ phenotype that suggested a bona fide interaction; 9 clones did not. Neither the interacting clones nor the LexA-Gi fusion alone activated *LEU2* expression. None of the *loco* clones showed any interaction with a number of other control LexA bait constructs, including a fusion of LexA to *Drosophila* neuronal synaptobrevin (DiAntonio et al., 1993).

As the ovary cDNA library had been oligo(dT)-primed (Finley et al., 1996), DNA sequencing of the 3' end of each clone, using a primer that annealed to downstream pEG202-PL1 vector sequences, was used to sort 14 of the 16 clones into 6 different genes. The 5' boundary of the *loco* fragment in each clone was determined by one or more sequencing reactions using a primer that annealed to upstream pEG202-PL1 vector sequences, followed by alignment with the existing *loco* sequence.

In situ hybridisation

DNA probes were labelled by random priming. Non-radioactive in situ hybridisation experiments using digoxigenin-labelled probes were performed as described (Tautz and Pfeifle, 1989). The stained embryos were embedded in GMM (Ashburner, 1989) and photographed on a Zeiss Axiophot.

RESULTS

Enhancer trap lines with a *pointed*-dependent glial-cell-specific β -GALACTOSIDASE expression

The ETS transcription factors encoded by *pointed* control CNS glial cell differentiation presumably through activation of target genes. In order to identify possible glia-specific target genes, we made use of the enhancer trap technique. Two enhancer trap lines, *3-109* (KlÄmbt and Goodman, 1991) and *rC56*, were selected based on their specific β -GALACTOSIDASE expression in the lateral CNS glia (Fig. 1). Both lines show identical β -GALACTOSIDASE expression patterns and carry a P-element insertion at the cytological position 94B/C. In embryos carrying the *rC56* enhancer trap insertion, first β -GALACTOSIDASE expression can be detected in early stage 12 in cells which, based on their position, appear to be the progeny of the lateral glioblast (Fig. 1A).

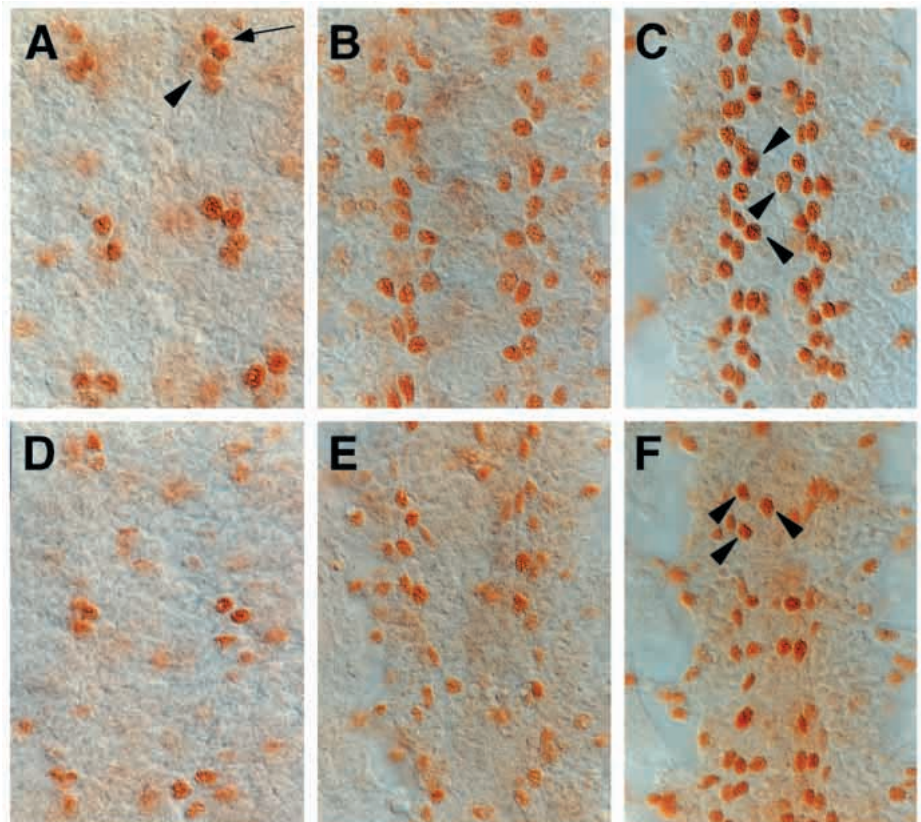


Fig. 1. *rC56*-directed β -GALACTOSIDASE expression in wild-type and mutant *pointed* embryos. Frontal views of dissected embryonic CNS preparations. β -GALACTOSIDASE expression is detected using anti- β -GALACTOSIDASE antibodies and subsequent HRP-immunohistochemistry. Anterior is up. (A-C) β -GALACTOSIDASE expression associated with the *rC56* enhancer trap insertion. (D-F) β -GALACTOSIDASE expression in homozygous *rC56 pointed*^{8B74} mutants. (A) Stage 13 embryo, the lateral glioblast has divided twice. Note that the two anterior pairs of glial cells (arrow) show a slightly higher level of β -GALACTOSIDASE expression compared to the posterior pairs of glial cells (arrowhead). (B) Stage 14 embryo. (C) Stage 16 embryo. In wild-type embryos, glial cells have divided to form 8 longitudinal glial cells covering the longitudinal connectives. The A and B glial cells are indicated (arrowheads). (D) In a stage 12 *pointed* mutant embryo, *rC56*-directed β -GALACTOSIDASE expression is reduced in the progeny of the longitudinal glioblast. (E) In a stage 14 embryo, a reduction in the level of β -GALACTOSIDASE expression can be observed in *pointed* mutant embryos. This is most evident in neuromeres T3 to A5. (F) In stage 16 embryos, β -GALACTOSIDASE expression in the longitudinal glial cells is reduced or absent, while the expression in the A and B glial cells appears unchanged (arrowheads). The position of these cells is altered due to the *pointed* mutation. Note that this phenotype is not as pronounced in the abdominal-most neuromeres.

Interestingly, at this early stage these cells appeared to be already different. The anterior pair of progeny expresses elevated levels of β -GALACTOSIDASE. As CNS development continues, these cells migrate medially and divide (Jacobs et al., 1989; Halter et al., 1995). By the end of embryogenesis, most glial cells except the midline glia express β -GALACTOSIDASE. In addition, β -GALACTOSIDASE expression can be detected in the dorsal leading edge cells in the lateral ectoderm (data not shown). To test whether CNS glia expression depends on the function of *pointed*, we assayed β -GALACTOSIDASE expression directed by the *rC56* enhancer in a *pointed*^{8B74} mutant background (Fig. 1) or in *rC56 pnt*^{8B74}/*pnt* ^{Δ 88} heterozygous embryos (data not shown). First expression directed by the *rC56* element at early stage 12 in the progeny of the longitudinal glioblast appears to be slightly weaker in the mutant. During later stages of CNS development, a reduction in the expression level as well as in the number of cells expressing the *rC56* reporter gene can be detected. In stage 16 mutant embryos, the activity of the *rC56* enhancer is most prominently reduced or absent in the longitudinal glial cells, whereas A and B glial cells and the VUM glial cells appear relatively unaffected (Fig. 1F, see also Fig. 3G). The influence of *pointed* on the *rC56* enhancer is less pronounced in the posteriormost 2-3 neuromeres. Furthermore, the *rC56* reporter can be ectopically activated by ectopic expression of *pointed*P1 (Klaes et al., 1994). This suggests that the *lacZ* gene located in the *rC56* P-element is under at least partial control of a *pointed*-dependent genomic enhancer element, which encouraged us to look for a corresponding gene in the vicinity of the P-element insertion.

loco is expressed in the CNS glia and the developing PNS

35 kb of genomic DNA sequences flanking the *3-109* P-element insertion site were isolated. The location of the P-elements *rC56* and *3-109* is indicated in Fig. 2. The mapping of exons by restriction analysis and genomic sequencing revealed two different variants differing in their 5' ends (c1 and c2) of a gene that we named *loco* (locomotion defects, see below) (Fig. 2, see below). In situ hybridisation experiments with transcript-specific digoxigenin-labelled cDNA probes showed that both *loco* RNA classes are expressed in the embryo (Fig. 3). *loco*-c1 transcription is very weak and is detected only after prolonged incubation (6-12 hours) in the staining solution. Using a 200 bp *loco*-c1-specific probe, expression can be first detected in late stage 12 embryos (Fig. 3D). In stage 16 embryos, *loco*-c1 RNA is found in the leading edge cells (Fig. 3F, arrowhead), in the tracheal cells (Fig. 3D,F, asterisk) and in the lateral glial cells within the CNS (Fig. 3E, arrow). Except for *loco* expression in tracheal cells, this corresponds well with the β -GALACTOSIDASE expression pattern observed for the two P-element insertions in the *loco* gene. *loco*-c2 transcripts are found only in scattered cells in the lateral ectoderm. Based on their position, these cells might correspond to PNS progenitor cells (Fig. 3A-C). No expression can be detected in the CNS.

loco encodes two RGS domain proteins

The sequences of the different cDNA clones were determined and compared to genomic sequences to deduce the exon-intron structure. We observed a number of differences between genomic and cDNA sequences (see Materials and Methods). The glia-specific exon of *loco*-c1 encodes only the initiator methionine, such that the remaining cDNA sequences are shared with the *loco*-c2 transcript. A second, in-frame ATG is

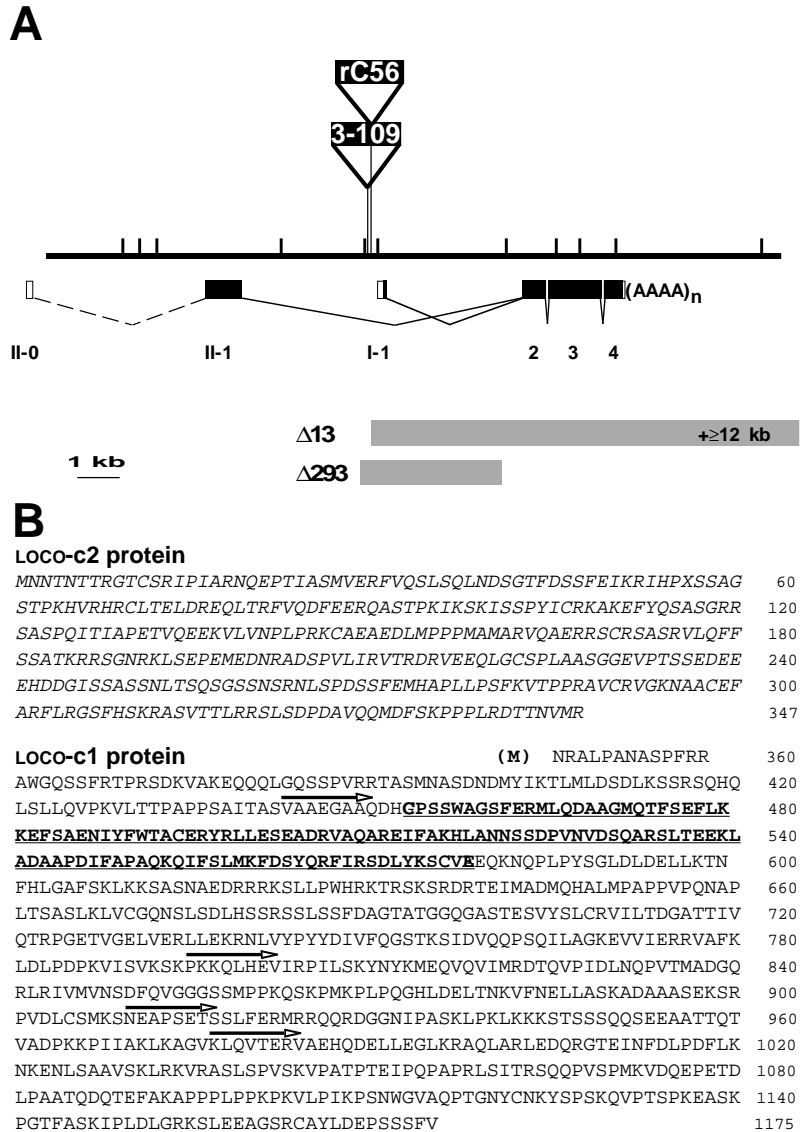


Fig. 2. Genomic organization of the *loco* gene. (A) Schematic drawing of the *loco* gene. Transcription is from left to right. Exon (bars), intron (lines) and coding regions (black shading) are indicated. Exon II-0 maps outside of the cloned region. The extent of the chromosomal deletions associated with *loco* ^{Δ 13} and *loco* ^{Δ 293} is indicated. The insertion sites of the two P-element insertions, *3-109*, and *rC56*, are separated by 1 bp and represented by triangles. In both cases, the *lacZ* gene is 5'. Coding regions are indicated by black shading. (B) Deduced amino acid sequences of the LOCO proteins, numbering refers to the LOCO-c2 protein (Accession numbers: AF130745 (*loco*-c1); AF130744 (*loco*-c2)). The RGS domain is in bold and underlined. LOCO-c2 contains an additional N-terminal domain of 346 amino acids shown in italics. The methionine shown in brackets is encoded by the glial cell-specific exon I-1. Horizontal arrows at V443, P795, N910 and K977 indicate the start points of the *loco* fusions (clones designated C25, D1, D7 and H2 respectively) recovered in the yeast two-hybrid screen.

Table 1. Isolation of EMS induced *loco* alleles

	A1	F1	L1	M1	T1
A1	–	larval - pupal	larval	viable*	larval - pupal
F1		–	larval	pupal‡	larval - pupal
L1			–	pupal§	larval - pupal
M1				–	pupal§
T1					–

*Flies eclose but appear paralysed.

‡Pupal lethal at 25°C; At 18°C escapers* eclose.

§Pupal lethal at 25°C; At 18°C rare adult escapers* appear.

Complementation data were obtained at 25°C. The lethal phase is indicated. At 29°C, all *loco* mutations are prepupal lethal in trans to *loco*^{Δ13}.

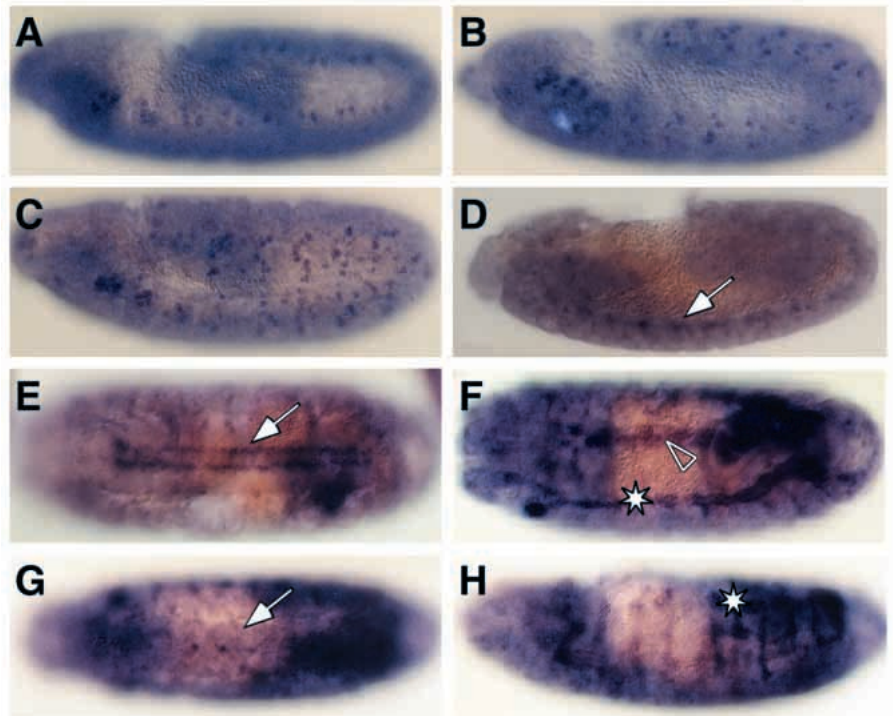
found 46 codons 3' of the c1 initiator. The introns between exons 2/3 and 3/4 are 67 and 72 bp in size.

The deduced protein sequences of both transcripts were compared with those in the EMBL database using the FASTA program (Altschul et al., 1990). A conserved domain of 125 amino acids, encoded by exon 2, identifies LOCO as a member of the RGS (Regulators of G-Protein signalling) protein family (Figs 2, 4, 5). To date about 16 different members of the family have been described. *loco* represents the first *Drosophila* RGS family member. Within the RGS domain, LOCO is most similar (>40% homology) to rat RGS12 and rat RGS14 (Snow et al., 1997). Interestingly, homology to rRGS12 and rRGS14 extends beyond the RGS domain to the C terminus of the deduced LOCO sequences. Three additional regions of homology were designated as B (48 of 160 aa are identical in LOCO and rRGS12 which corresponds to 30% identity), C (14/59 identical, 26% identity), and D (20/51 identical, 39% identity) (see Figs 4, 5). No homologies were found in the LOCO-c2-specific domain.

Generation of mutations in the *loco* gene

To determine the function of *loco* during glia development, we first generated chromosomal deficiencies removing the *loco* gene (see Materials and Methods). Subsequently, we mobilized the P[*rosy, lacZ*] insertion *rC56* and generated 700 independent P-element excision lines. Among these lines, two non-complementing lethal mutations, *loco*^{Δ13} and *loco*^{Δ293}, were recovered. These mutations also failed to complement the X-ray-induced deficiencies, indicating that the lethal hit was in the vicinity of the P-element insertion. The extent of the genomic deletions associated with the two alleles was determined by Southern blot analyses and is indicated in Fig. 2. In *loco*^{Δ13}, the proximal deletion breakpoint lies within the P-element leaving the *lacZ* gene intact. However, the relative level of β-GALACTOSIDASE expression in different glial cells appears to be altered and, in *loco*^{Δ13} embryos, the longitudinal glial cells express only low levels of β-GALACTOSIDASE (data not shown). This indicates that glia-specific enhancer elements reside upstream of the *rC56* enhancer trap insertion whereas a

Fig. 3. Expression of *loco* transcripts during embryogenesis. Whole-mount in situ hybridizations using digoxigenin-labelled exon-specific probes to assay the expression of the two transcripts generated from the *loco* gene. Anterior is to the left. (A-C) *loco*-c2-specific probe, (D-H) *loco*-c1-specific probe. (A) Lateral view of an early stage 11 embryo. *loco*-c2 expression can be detected in 2-3 cells per hemisegment in the lateral body wall. (B) Lateral view of a late stage 11 embryo. Additional cells express the *loco* gene. (C) Lateral view of a stage 12 embryo. *loco*-positive cells appear to divide and form a regular pattern reminiscent of PNS progenitor cells. (D-H) Using a 200 bp long probe, *loco*-c1 expression can be detected from stage 12 onwards. (D) In a lateral view of a stage 12 embryo, *loco*-c1 expression is found only in the longitudinal glial cells in the CNS (arrow). (E) Ventral view of a stage 16 embryo, expression of *loco*-c1 in two longitudinal rows of cells in the CNS can be seen (arrow). These cells correspond to the longitudinal glial cells covering the connectives. (F) In a dorsolateral view of a stage 16 embryo, expression of *loco*-c1 is detected in the main tracheal trunk (asterisk) and the dorsal leading edge cells of the epidermis (arrowhead). (G) In a ventral view of a stage 16 *pointed*^{Δ88} mutant embryo, CNS expression of *loco*-c1 is undetectable except for cells close to the midline, which could correspond to the VUM glial cells (arrow). (H) The same *pointed* mutant embryo as shown in G. The tracheal cells show the *pointed*-specific differentiation defect. Note that in contrast to the CNS glial cells they still express the *loco* gene (asterisk).



transcriptional activator acting specifically in the longitudinal glial cells must reside 3' of the *rC56* insertion. The breakpoint in *loco*^{Δ13} was cloned and is at least 7 kb downstream of the *loco* gene. A small inversion as well as a deletion of about 2 kb of genomic sequence is associated with the *loco*^{Δ293} allele (Fig. 2). Here, putative promotor sequences as well as the first exon are deleted. Both mutations are homozygous embryonic lethal.

Additional *loco* alleles were obtained following EMS mutagenesis (see Materials and Methods). From 3.088 chromosomes analysed, we obtained five mutations, defining a single complementation group (see Table 1 for details). They are lethal in *trans* to both *loco* excision mutations at 29°C. Based on the complementation analyses, they were placed into the following allelic series: *loco*^{L1}>*loco*^{F1}>*loco*^{T1}≈*loco*^{A1}>*loco*^{M1}.

At 25°C, *loco*^{M1} but none of the other EMS-induced alleles produces adult escapers when in *trans* to *loco*^{Δ13} or *loco*^{Δ293}. About 10% of the expected numbers of transheterozygous adults appear. They often fail to eclose from the opened puparium. Eclosed flies show a paralytic phenotype and drop into the food and die. If such flies are rescued from the food, they show a severe impairment of spontaneous locomotor activity and display a 'shaking' phenotype. Response to mechanical stimulation (e.g. after stimulation of thoracic bristles) is weak in *loco*^{M1}/*loco*^{Δ293} and undetectable in *loco*^{M1}/*loco*^{Δ13}, indicating that ^{Δ13} is a stronger allele than ^{Δ293}. Similar phenotypes, albeit with lower expressivity, are seen in flies heterozygous for *loco*^{M1} and other EMS-induced alleles at lower temperatures (see Table 1 for details). All adult escapers die after a maximum of 2 days.

Loss of *loco* function leads to a glial cell differentiation phenotype

The strongest *loco* allele is represented by the embryonic lethal mutation *loco*^{Δ13}. No abnormal CNS axon pattern phenotype was detected using the mAb BP102, which labels the overall axon pattern. mAb 1D4 recognizes the FASCICLIN II protein, which is expressed on a subset of longitudinal fascicles (Fig. 6A). In mutant *loco*^{Δ13} but not in mutant *loco*^{Δ293} embryos, a slight defasciculation of axons can be found. In addition, we observed an occasional crossing of fasciclin II-positive axons within the longitudinal connective (Fig. 6B).

To analyse the different glial cells in mutant *loco* embryos, we used anti-REPO antibodies (Halter et al., 1995) which label most lateral glial cells. No gross defects were detected in the number and position of these cells. This indicates that birth and migration of the lateral glial cells do not depend on *loco* function. To analyse terminal differentiation of glial cells, we used the *M84* enhancer trap marker (Klämbt and Goodman, 1991). In wild-type stage 16/17 embryos, a regular pattern of evenly spaced glial cells can be detected (Fig. 6). In *loco*^{Δ293} as well as in *loco*^{L1} mutant embryos defects in the positioning of some of the *M84*-positive cells were observed. In particular, β-GALACTOSIDASE expression is reduced specifically in the A and B glial cells compared to more laterally positioned glial cells (Fig. 6C,D, arrows).

To obtain a higher level resolution, we performed an electron

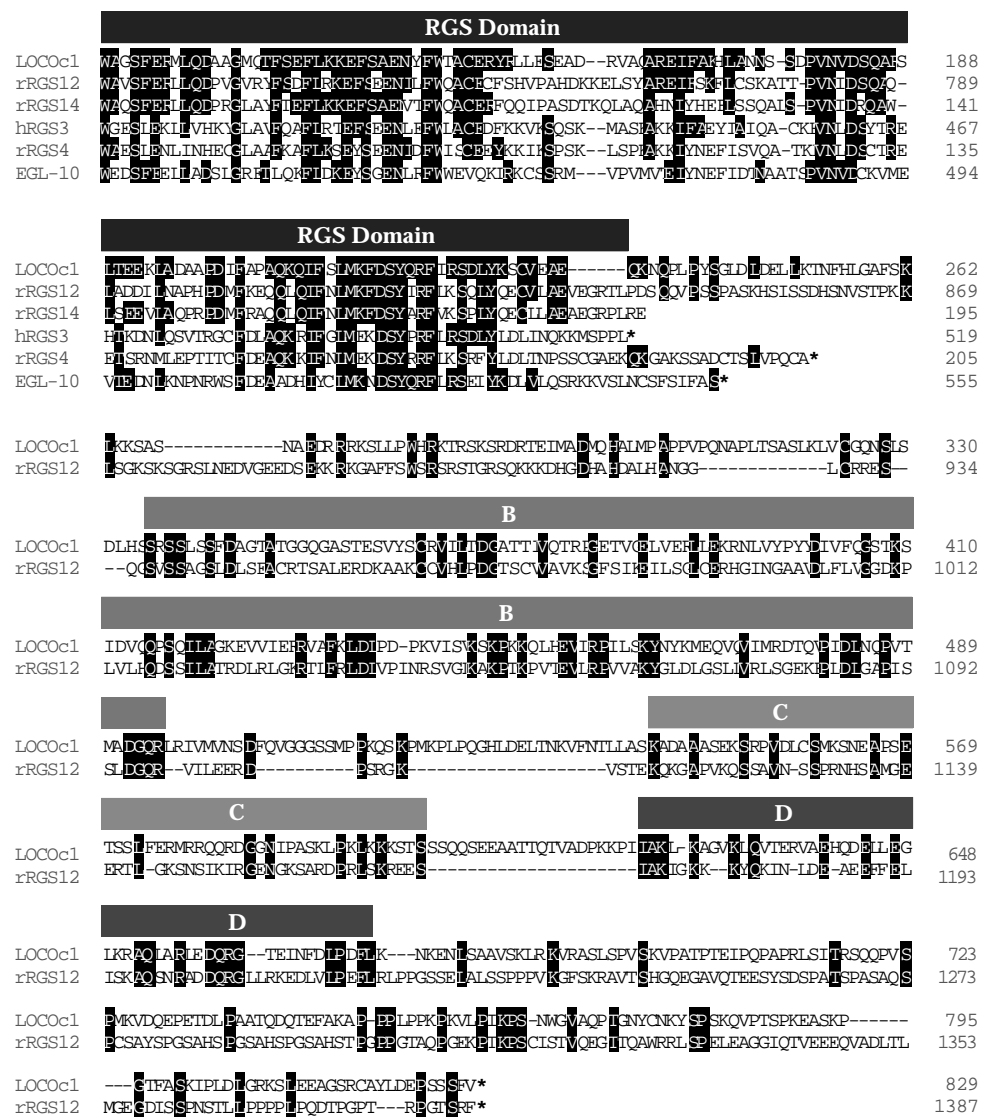


Fig. 4. *loco* encodes two members of the RGS protein family. Amino acid sequence comparison of the RGS domains of LOCO and rat RGS12, rat RGS14, human RGS3, rat RGS4 and *C. elegans* EGL-10 shows conservation of the RGS domain. Residues identical to LOCO are shaded in black. Sequence conservation between LOCO, RGS12 and RGS14 extends beyond the RGS domain. Only RGS12 is shown. Three additional regions of higher homology (B-D; see also Fig. 5) can be identified. Alignments were performed using the Clustal algorithm with standard parameters.

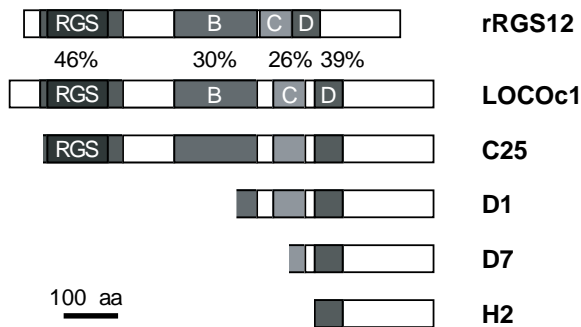


Fig. 5. Clones recovered in the yeast two-hybrid screen. The schematic drawing shows the array of conserved sequences in LOCoc1 and rRGS12. The percentage of sequence identity is indicated. Four N-terminal deletion variants of the LOCO protein were recovered in the two-hybrid experiment. All proteins bind to G α i and contain region D.

microscopic analysis of homozygous *loco* ^{Δ 293}, *loco* ^{Δ 13} embryos as well as of *loco* ^{Δ 13}/*Df(3R)15E1* embryos (Fig. 7). In wild-type embryos, the longitudinal glial cells cover the longitudinal axon tracts and extend processes into the connectives. Furthermore the entire cortex is covered by a thin perineurial glia sheath (Fig. 7A,C arrowheads). In *loco* mutant embryos, the differentiation of both glial cell types appears to be affected. In *loco* ^{Δ 13}/*Df(3R)15E1* mutant embryos, the perineurial sheath is virtually absent (Fig. 7 compare A,C to B,F). This is most clearly seen at the CNS midline. In wild-type embryos, glial cells form a continuous barrier preventing the contact of midline cells with the hemolymph (Fig. 7A,B arrowheads; see G,H for enlargements).

A similar phenotype can be seen for the longitudinal glial cells. The nuclei are found at relatively normal positions, but no glial cell processes can be detected within the connectives. In addition, the intimate glial-glial cell contact, that is observed in wild-type embryos is severely disrupted in *loco* mutant embryos. Often we find axons on the dorsal surface of longitudinal glial cells, apparently in direct contact with the hemolymph (Fig. 7D-F, arrowhead).

In summary, the *loco* mutant phenotype can be described as a late glial cell differentiation defect, where the formation of glial cell processes enwrapping neuronal cell bodies and axons does not occur.

***loco* interacts with G α i**

RGS domains directly interact with G-protein alpha subunits, displaying a remarkable degree of specificity (De Vries et al., 1995; Berman et al., 1996; Drucey et al., 1996; Hunt et al., 1996; Watson et al., 1996). If LOCO indeed functions as a regulator of G-protein signalling, we would anticipate the presence of a G-protein in the lateral glial cells. We therefore analysed the expression of G α s, G α i and G α o RNAs in the embryonic nerve cord and found that the G α i subunit appears to be specifically expressed in the glial cells (Wolfgang et al., 1991; S. G. et al., unpublished data).

Further evidence for interaction of LOCO and G α i was found in a yeast two-hybrid screen (Gyuris et al., 1993). A cDNA clone of *Drosophila* G α i (Provost et al., 1988) was used as 'bait' for interacting proteins. The G α i gene was fused in frame

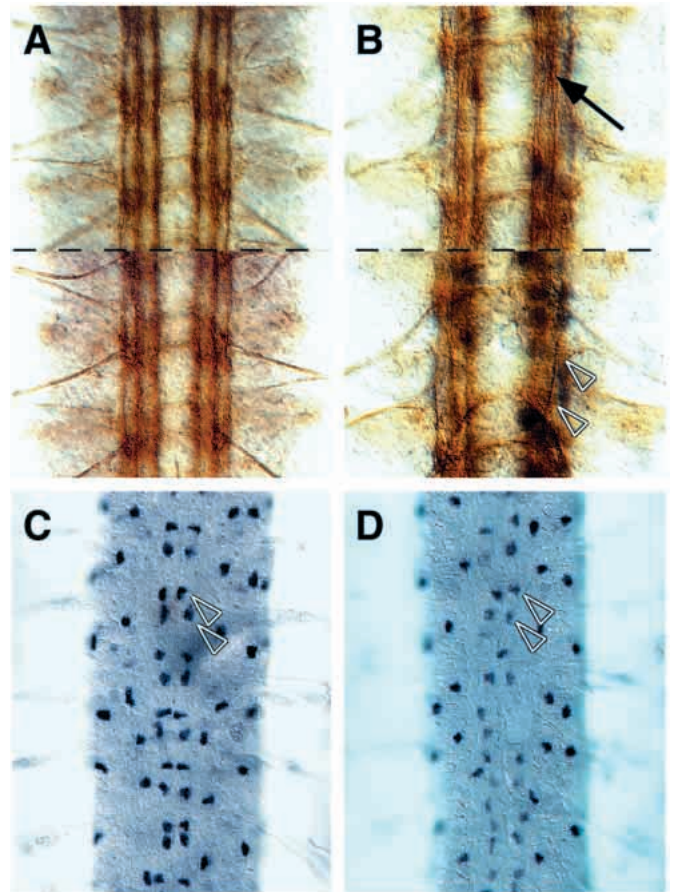


Fig. 6. CNS phenotype of homozygous mutant *loco* embryos. Frontal views of dissected embryonic nerve cord preparations. (A,B) Antibody staining with mAb 1D4, which recognizes the fasciclin II protein. The dashed line separates two focal planes. (C,D) Antibody staining with anti- β -GALACTOSIDASE antibodies. Anterior is up. (A) In a wild-type stage 16 embryo, three distinct fascicles are labelled. Note that axons never switch fascicles. (B) In homozygous mutant *loco* ^{Δ 13} embryos, a moderate defasciculation of axons can be observed. In addition axons occasionally switch between fascicles (arrowheads). (C) In stage 16 *M84* embryos, expression of β -GALACTOSIDASE can be detected in a small subset of glial cells. The medial glial cells are the A and B glia (arrowheads). The level of β -GALACTOSIDASE expression is similar in all cells. (D) In mutant *M84/+; loco*^{*L1*}/*loco*^{*L1*} stage 16 embryos, β -GALACTOSIDASE expression in the A- and B-glial cells is lower than in the laterally positioned glial cells. The position of the glial nuclei appears to be more variable.

at its N terminus to a gene encoding a LexA DNA-binding domain. Yeast that expressed this fusion was transformed with a library carrying *Drosophila* cDNAs fused to a gene for a transcriptional activation domain (Finley et al., 1996). Clones that encoded putative G α i-interacting proteins were identified by the ability of the transformed yeast colonies to express a *LEU2* gene that contained LexA-dependent regulatory elements and the interaction was confirmed by reintroducing the putative positive clones into yeast that carried the LexA-G α i fusion.

Six non-overlapping sets of interacting clones were identified. Four non-identical *loco* clones were recovered, with

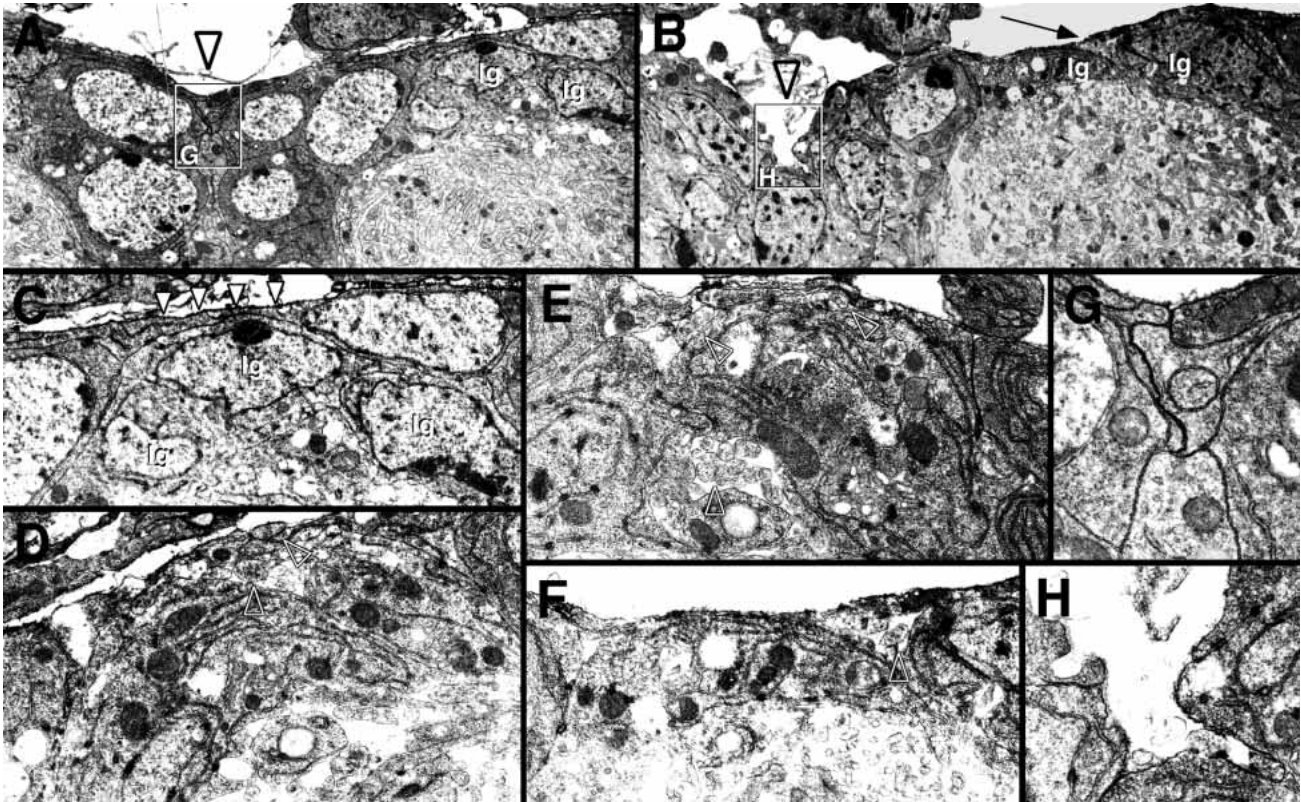


Fig. 7. Electron-microscopic analysis of the mutant *loco*^{Δ13}/*Df(3R)15E1* phenotype. The figure shows cross sections of wild-type (A,C,G) and of *loco*^{Δ13}/*Df(3R)15E1* (B,D-F, H) stage 16 embryos. lg, longitudinal glial cell. (A) In wild-type embryos, the neuropile and the entire cortex are covered by a sheath of glial cells. Note that the glial sheath surrounding the ventral nerve cord spans the midline (arrowhead). (C) In a larger magnification, longitudinal glial cells are seen in close contact to each other, dorsally to the neuropile. Note that axons never contact the hemolymph. Dorsal to the longitudinal glial cells, a thin sheath of perineurial glial cells can be seen (arrowheads). (B) In mutant *loco* embryos, the dorsal surface of the ventral nerve cord appears more irregular compared to wild-type embryos. In addition, the neuropile is not as dense in its appearance and axons are not surrounded by glial cell processes. (D-F) Three high-power microphotographs of mutant *loco* neuromeres, taken at the same position in mutant as the microphotograph in C in wild type. Glial differentiation appears impaired. Glial-glia cell contact is not established and axons are exposed to the hemolymph (arrowheads). (G,H) Higher magnification of the areas outlined in A and B. (G) At the dorsal midline of wild-type embryos, glial cells form a tight junction. (H) In mutant *loco* embryos, glial cells fail to contact each other at the midline leaving midline cells in direct contact to the hemolymph.

C-terminal fragments of various lengths fused to the *lexA* gene (Figs 2, 5). The longest fragment began at residue 443 of the predicted LOCO c2 protein and included the RGS domain; the shortest encoded only 199 amino acid residues that extended C-terminal from residue 977 of the predicted LOCO c2 protein and included the final 43 amino acids of the conserved region D closest to the C terminus (Figs 2, 5). None of the *loco* clones showed any interaction with several control LexA fusions (see Materials and Methods). Thus LOCO appears to be an RGS domain protein specific for G α i.

DISCUSSION

In the present paper, we describe the *Drosophila* gene *loco* and show for the first time that RGS domain proteins are required for correct glial differentiation. *loco* is expressed in lateral glial cells throughout development and appears to be a target gene of *pointed*. *loco* is required for the proper formation of the blood-brain barrier and the ensheathment of neuronal cell bodies and axons. It is coexpressed and able to specifically

interact with G α i suggesting a role for G-protein signalling in glial development.

Analysis of glial cell development in *Drosophila*

Although glial cells are an important component in any complex nervous system, not much is known about the molecular mechanisms underlying glial development. In *Drosophila*, a number of gene functions and mechanisms required during glial development are emerging. Following lineage specification (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996), terminal differentiation of glial cells is mediated by transcription factors encoded by *repo* and *pointed* (Klaes et al., 1994; Halter et al., 1995).

loco is required for glial differentiation

The identification of genes activated by *pointed* in glial cells should provide new insights in the molecular mechanisms underlying glial differentiation. *loco*, identified by an enhancer trap approach, might represent such a *pointed* target gene. Analysis of the *loco* promoter region revealed the presence of GCM- and ETS-binding sites suggesting that *loco* might be a

direct target of *gcm* as well (S. G., unpublished data). *loco* promoter-*lacZ* fusion constructs revealed a small promoter fragment that is capable of directing *lacZ* expression in almost all *loco*-expressing glial cells. This promoter fragment is indeed dependent on *pointed* function and ectopic *pointed* expression as well as ectopic *gcm* expression result in a corresponding ectopic *lacZ* expression. Sequence analysis and in vitro mutagenesis revealed both GCM- and POINTED-binding sites within this element (S. G. and C. K., unpublished data). These data, as well as the phenotypes observed in *loco* and *pointed* mutant embryos (Klaes et al., 1994), suggest that *loco* is indeed a target of *pointed*. However, it is important to emphasise that *loco* expression in the tracheal system does not appear to depend on *pointed* function (Fig. 3).

In *loco*-deficient embryos, glial development initially proceeds normally, however, glial-glial cell-cell interactions appear to be defective. Axons within the longitudinal connectives are not completely enwrapped and are occasionally found even at the dorsal surface of the CNS (Fig. 7D-F, arrowheads). A similar phenotype can be observed following a fasII antibody staining (Fig. 6B). In addition, the blood-brain barrier is not established. Due to the high potassium concentration in the hemolymph, this is likely to result in a disruption of axonal conductance. The adult, paralytic phenotype of the weak EMS-induced *loco* alleles might be a consequence of such a defect as well.

Similar phenotypes were found in *neurexin* or *gliotactin* mutants (Auld et al., 1995; Baumgartner et al., 1996). Here too, the formation of the blood-brain barrier is defective and the animals are paralysed. GLIOTACTIN is a transmembrane protein expressed by a subset of glial cells, NEUREXIN is a transmembrane protein that is required for the formation of septate junctions (Baumgartner et al., 1996). In contrast to the above mentioned proteins, LOCO is likely to be localized within the cell. What causes the mutant phenotype found in *loco* mutant embryos?

***loco* encodes a *Drosophila* RGS protein**

LOCO is the first known *Drosophila* member of the recently described family of Regulators of G-protein Signalling (RGS) (Koelle, 1997; Arshavsky and Pugh, 1998) with highest homology to RGS12. RGS proteins were first described in yeast and *C. elegans* (De Vries et al., 1995; Druey et al., 1996; Koelle and Horvitz, 1996). RGS proteins stimulate the GTPase activity of different G α subunits as much as 100-fold (Watson et al., 1996), accelerating the transition from the GTP-bound active to the inactive GDP-bound form and thereby terminating trimeric G-protein signalling. Different RGS proteins vary in their specificities for the G α i and G α o subunits. To date, no RGS protein binding to G α s has been identified. The RGS domain itself is sufficient for both binding G α and GTPase activation (De Vries et al., 1995). Here, we have shown that LOCO physically interacts with G α i. Strikingly, the interaction with G α i is not confined to the RGS domain but can also be mediated by C-terminal sequences, possibly by a stretch of 51 amino acids that is conserved between LOCO and RGS12 (see Fig. 4). It is interesting to note that rat RGS12 also interacts with G α i (Snow et al., 1998).

Several G-proteins have been identified in *Drosophila* (Wolfgang et al., 1990; Parks and Wieschaus, 1991; Quan et al., 1993; Wolfgang and Forte, 1995). Beside their role in

phototransduction and learning (Scott et al., 1995; Connolly et al., 1996; Zuker, 1996), only few functions have been associated to date to G-proteins (Parks and Wieschaus, 1991). Interestingly G α i RNA (but not G α s and G α o) is expressed in dorsal CNS cells at a position typical of glial cells (Wolfgang et al., 1991), S. G., unpublished data). This, as well as the interaction data presented, suggests that *loco* function is required to regulate G α i signalling in glial cells.

G-protein signalling in glial cell differentiation

Taken together, our data argue for an important role of G-protein-mediated signalling in terminal glial cell differentiation. G-protein signalling is thought to be triggered by binding of a ligand to a seven transmembrane domain receptor. To date, no such receptor has been reported to be expressed in the *Drosophila* glial cells. Recently cross talk between receptor-tyrosine-kinases and G-proteins has been described (Weiss et al., 1997). Interestingly, the CNS expression of *heartless*, the *Drosophila* FGF-receptor2 gene, is restricted to glial cells (Beiman et al., 1996; Gisselbrecht et al., 1996; Shishido et al., 1997). *heartless* mutant embryos show a defect in lateral glial development. Based on immunostaining using anti-HEARTLESS antibodies, mutant glial cells appear rounded and are incapable of increasing their surface area (Shishido et al., 1997). This is reminiscent of the phenotype of mutant *loco* embryos described here. It is thus tempting to speculate that HEARTLESS and G-protein signalling involving LOCO act in concert to trigger glial cell shape changes in response to extracellular signals.

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