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Developmental expression and tissue distribution of the *lethal* (2) *giant larvae* protein of *Drosophila melanogaster*

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Recessive mutations in the *Drosophila* tumor gene *lethal* (2) *giant larvae* affect the growth and tissue specificity of determined cells in imaginal discs and presumptive optic centers of the brain. To analyse the function of the *l* (2) *gl* gene during development, we have raised monoclonal antibodies against the *l* (2) *gl* protein. These antibodies detect a 130-kd protein in wild-type tissue which is absent in homozygous mutant tissues. The protein is detected in increasing amounts up to mid-embryonic stages. Antibody binding to embryo sections and indirect immunofluorescence labeling indicate that the protein is localized at the cellular membranes or in the intercellular matrix of the embryonic cells. The primordia of all larval tissues are labeled in the embryo. Much less labeling is found in the neural primordia of the central nervous system, except that within the supraoesophageal ganglion the regions of the presumptive optic centers are distinctly labeled. Moreover, the axon bundles of the ventral cord are labeled in the embryo, apparently a reflection of the accumulation of cell membranes here. After embryogenesis the *l* (2) *gl* protein is found at a low level until the end of the 3rd larval instar, when it is preferentially seen in the brain and imaginal discs. The protein distribution in embryonic and larval tissues correlates with already known proliferation patterns, which could indicate that the *l* (2) *gl* protein is involved in proliferation arrest of cells.

Key words: Drosophila/recessive tumor gene/monoclonal antibodies/developmental expression

Introduction

Recessive mutations in the *Drosophila* gene *lethal* (2) *giant larvae* [*l* (2) *gl*] cause morphologically unstructured growth in developing homozygous mutant larvae (Gateff and Schneiderman, 1969). The most dramatic effects are visible in cells of imaginal discs, as well as in neuroblasts and ganglion mother cells from the presumptive optic centers of the brain hemispheres (Gateff, 1978a), although other tissues are also affected by the mutation (Gloor, 1943; Grob, 1952; Hadorn, 1955). During larval stages these tissues grow in an unstructured fashion (Gateff, 1978a). At the end of the 3rd larval instar the larvae fail to enter metamorphosis and become bloated and transparent due to hormonal malfunctions (Hadorn, 1937; Karlson and Hauser, 1952). The absence of metamorphosis in mutant larvae has been experimentally shown to be a consequence of defective ring gland function, since metamorphosis can be induced by transplantation of wild-type ring glands into giant larvae (Hadorn, 1937). It is not known whether the ring gland fails to synthesize ecdysone because of a direct effect of the mutation on ring gland cells or due to the absence of neurosecretory stimulation of gland cells from the mutant brain (Gateff, 1978a).

Mutant brain tissues grow in a dispersed fashion when subcultured in adult wild-type female hosts, invading host tissues and eventually killing the host fly (Gateff and Schneiderman, 1967). Thus the phenotypic effects of the *l* (2) *gl* mutation on the presumptive optic centers appears to be due to a neoplastic growth of the mutant cells (Gateff, 1978b). Recently a temperature-sensitive *l* (2) *gl* mutation has been recovered and analysed (Hanratty, 1984a). Subculturable neoplastic growth of imaginal discs is retained in transplanted mutant tissues after subsequent growth of the tissue at a permissive temperature (Hanratty, 1984b). This indicates that the phenotypic effects caused by the mutation are essentially irreversible and thus apparently are cell autonomous.

Using cloned fragments from the *l* (2) *gl* gene region (Mechler *et al.*, 1985) we have recently isolated cDNA fragments corresponding to transcripts from the *l* (2) *gl* gene (R.Lützelschwab *et al.*, unpublished results). The nucleotide sequence of a 5.3-kb cDNA fragment was determined and shown to contain an open reading frame of 3.5 kb length with a coding capacity of 1172 amino acids. A *Hin*dIII cDNA subfragment was subsequently cloned into a bacterial expression plasmid and the corresponding fusion protein was used as antigen for the production of antibodies against the *l* (2) *gl* protein.

Here we report the establishment of hybridoma cell lines producing monoclonal antibodies against the *l* (2) *gl* protein, the identification and stage-specific expression of the *l* (2) *gl* protein by Western blot analysis and its localization in embryonic tissue.

Results

Monoclonal antibodies against fusion proteins

A 2.2-kb *Hin*dIII fragment (Figure 1), containing 900 bp of open reading frame sequences from the 3′ end of the sequenced cDNA clone pcE7-9 (R.Lützelschwab *et al.*, unpublished results), was subcloned in proper orientation and in the correct reading frame into the bacterial expression plasmids. Cloning into the vector pATH10 (kindly provided by J.T.Körner) in-frame with the bacteria *TrpE* gene provided the recombinant plasmid pPTH6. Cloning into the vector pUR291 (Rüther and Müller-Hill, 1983) in-frame with the bacterial *lacZ* gene provided the recombinant plasmid pPGH5. The resulting fusion proteins from bacterial extracts were analysed on SDS–PAGE slab gels and compared with the respective proteins coded by the expression vectors without the *Drosophila* insert. In both constructs the addition mol. wt of the fusion protein was ~30 kd, which agrees well with the expected coding capacity of the inserted *Drosophila* cDNA sequence. The fusion protein from the plasmid pPTH6 was recovered from a preparative gel and used to immunize BALB/c mice. Spleen cells were fused with myeloma cells according to Köhler and Milstein (1976) and Campbell (1984). About 300 hybridoma cell clones grew up and were monitored for *l* (2) *gl* protein-specific antibody production. The screening procedure was considerably simplified by submerging small Western blot filter slices directly into microtiter wells containing antibody supernatant (see Materials and methods). From 50 positive

hybridoma lines, two lines 4E2XA and 4H7XD were subcloned and subsequently grown in BALB/c mice for ascites production.

l (2) gl protein identification

In order to test specific binding of monoclonal antibodies to *Drosophila* proteins, extracts of wild-type and mutant tissues from 3rd instar larvae were separated by SDS−PAGE and transferred onto nitrocellulose filter. We have tested the following tissues from wild-type larvae: fat body, imaginal discs, brains, salivary glands, midgut. We have also tested phenotypic mutant brains from the 3rd larval instar. After incubation with monoclonal anti-*l (2) gl* antibodies, the bound antibodies were visualized by a second, phosphatase-coupled antibody and phosphatase reaction. Antibodies produced by the hybridoma cell line 4H7XD bind to a 130-kd protein from wild-type imaginal discs and brain tissues (Figure 2, lanes b and c). No binding is observed in brain tissue extracts from larvae of the homozygous mutant *l (2) glDV275* (Figure 2, lane d). In this mutant the transcription of the *l (2) gl* gene is abolished due to an 8-kb interstitial deletion removing the 5′ moiety of the gene (Lützelschwab *et al.*, 1986). The absence of binding to a 130-kd protein or any other protein in this mutant strain therefore serves as a confirmation for the specificity of the antibodies to *l (2) gl* protein.

The same results were obtained with the antibodies from the second hybridoma cell line 4E2XA, and with monospecific rabbit anti-*l (2) gl* antiserum (C.Klämbt, unpublished results).

l (2) gl protein during development

To determine the amounts of *l (2) gl* protein found in all stages between embryo and adult, we have blotted equal amounts of electrophoretically separated proteins from each stage on nitrocellulose filters and analysed the intensity of labeling in the 130-kd protein band. In this analysis the strongest label was seen in mid-embryogenesis and is localized in tissue sections to presumptive larval tissues and the presumptive optic lobes, whereas cell bodies of most of the neural tissues are much less labeled (see below). The amount of protein labeling is reduced towards the end of embryogenesis and remains at a low level during the first two larval stages (L1 and L2). It is increased again towards the end of the 3rd larval instar, where it is found preferentially in imaginal discs and brain (Figure 2, lanes b and c). Low amounts of protein label are seen in salivary glands, gut and fat body (Figure 2, lanes a, e and g). No labeling is seen in the hemolymph (Figure 2, lane f). Thus at the two stages of expression the *l (2) gl* gene product is differentially expressed in larval and neural tissues.

Protein labeling is absent during most of the pupal stage, but re-appears before eclosion at a low level and persists throughout the adult stages in both sexes (not shown). In adults most of the protein is found in the head, but also in other tissues including ovaries (not shown).

l (2) gl protein during embryogenesis

To investigate the onset and time course of *l (2) gl* protein synthesis during embryonic development, we collected staged embryos at 2-h intervals. Equal amounts of protein were separated on SDS−PAGE slab gels and transferred onto nitrocellulose filters for antibody binding. The bound antibodies were detected by a

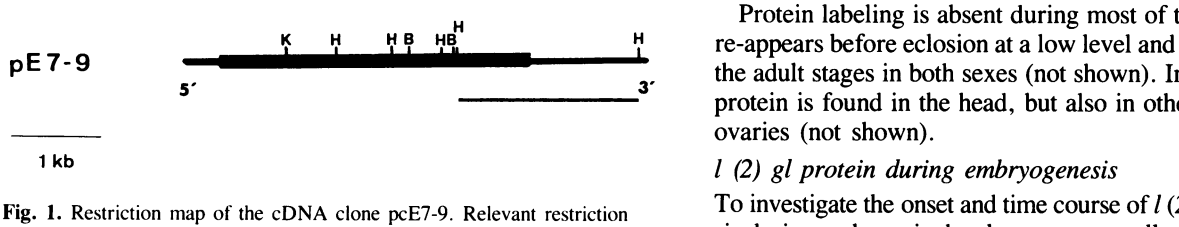

Fig. 1. Restriction map of the cDNA clone pcE7-9. Relevant restriction sites are indicated: B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I. Thick line indicates open reading frame sequences (R.Lützelschwab *et al.*, unpublished results). Lower black bar indicates the *Hin*dIII fragment used for cloning into the expression vectors.

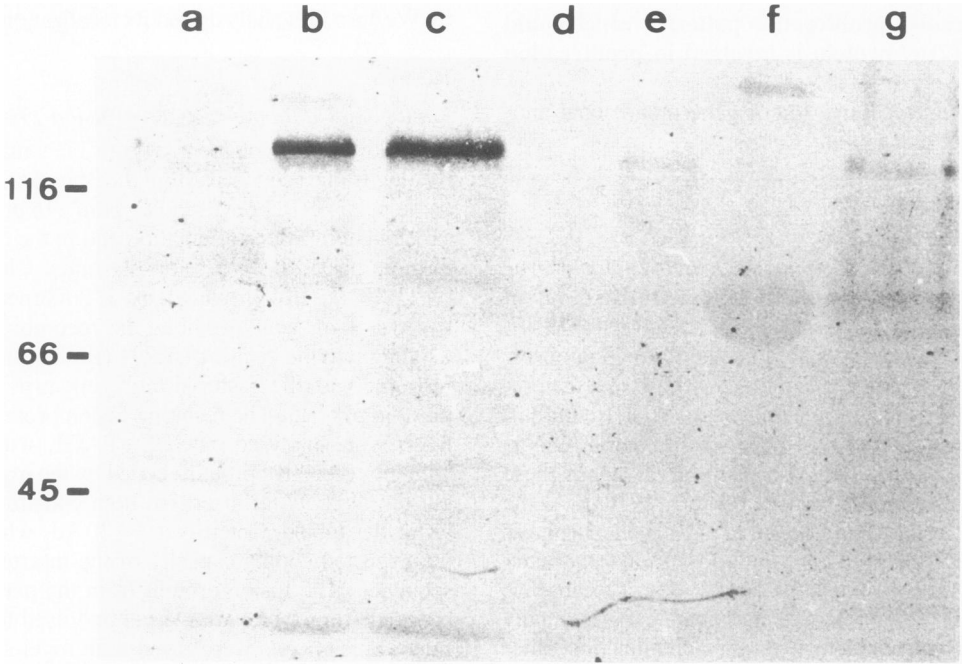

Fig. 2. Identification and tissue specificity of *l (2) gl* protein in 3rd instar wild-type and mutant larvae determined by binding of monoclonal antibody 4H7XD. Wild-type and mutant tissues from 3rd instar larvae were dissected and ~30 μg of protein was used for each lane: **(a)** wild-type (wt) salivary gland; **(b)** wt imaginal discs; **(c)** wt brain; **(d)** phenotypically mutant brain from the *l (2) glDV275* stock (Lützelschwab *et al.*, 1986); **(e)** wt gut; **(f)** wt haemolymph; **(g)** wt fat body. The protein extracts of each fraction were separated on a SDS−PAGE 10% slab gel and transferred onto nitrocellulose filter. After incubation with ascites fluid diluted 1:100 the bound antibodies were visualized with rabbit anti-mouse antibodies coupled with phosphatase. Mol. wt markers used are: ovalbumin (45 kd); bovine albumin (66 kd); β-galactosidase (116 kd).

phosphatase-coupled second antibody and phosphatase reaction. A faint band of the 130-kd protein is already visible in early stages of embryogenesis (Figure 3). Up to 4 h, no significant increase in the amount of protein labeling is detectable. After 4 h the amount of antibody binding to the 130-kd protein increases and reaches its maximum at 12–16 h. In the subsequent stages the amount of 130-kd protein decreases continuously until hatching (Figure 3, lanes 22–24) when it reaches the level seen at pre-blastoderm stages.

Tissue specificity in embryo sections

In order to investigate the distribution of the *l (2) gl* protein in embryonic tissues, we have analysed immunofluorescence antibody binding to fixed sections of embryos from various stages. At early blastoderm a faint by visible labeling is observed around the newly formed cellular membranes (not shown). At stages 10–12 differential labeling in subregions of the embryonic tissues are visible. At these stages ectodermal cells are more intensively labeled than anterior and posterior midgut primordia, which are established in the interior of the embryo (Figure 4a). Differential labeling is particularly seen within cells of the clypeolabrum of the procephalic lobe (Figure 4a). A thin layer between the ectoderm and mesoderm is also distinctly labeled (arrowheads in Figure 4a).

In stage 16 embryos the protein distribution is more complex. Although all primordia of the embryo are labeled, there are differences in the amount of label observed between different tissues at this stage. The cellular membrane of the anlagen of most of the presumptive larval tissues are strongly labeled in the embryo (Figure 4b). This is particularly seen in primordia of pharynx, gut, epidermis, salivary duct of the salivary gland, hypophysis, ring gland and proventriculus.

Much less label is seen in presumptive muscle tissue when pharyngeal muscle is compared with other pharynx structures (Figure 4b). However, within muscle tissues conspicuous fibers are labeled, which appear to be attached at the apodemata at the segmental borders and extend through the segments (Figure 5b, d). The cell bodies of neuroblasts of the supraoesophageal ganglion and the ventral cord display low amounts of *l (2) gl* protein (Figures 4a,b and 5a). Remarkable exceptions are cells of the presumptive optic centers (see below) and the neuropile of the central nervous system. The labeling of the neuropile structures is seen in horizontal sections of stage 16 embryos (Figure 5b), where the pattern of commissures and connectives of the ventral cord is easily distinguished from the less intensely labeled surrounding cell bodies of the neurones, as seen in horizontal (Figure 5b) and transversal sections (Figure 5c).

In parasagittal sections of stage 13 embryos the presumptive optic lobe cells as well as some descending axon bundles are clearly labeled within the supraoesopharyngeal ganglion (Figure 6). At this stage the optic lobe primordia consist of large neuroblasts, which are distinguished from the surrounding neuroblasts and developing neurones (Campos-Ortega and Hartenstein, 1985).

Discussion

The results presented in this paper concern the identification, developmental expression and tissue distribution of the protein product of the recessive tumor gene *l (2) gl*. The gene has been

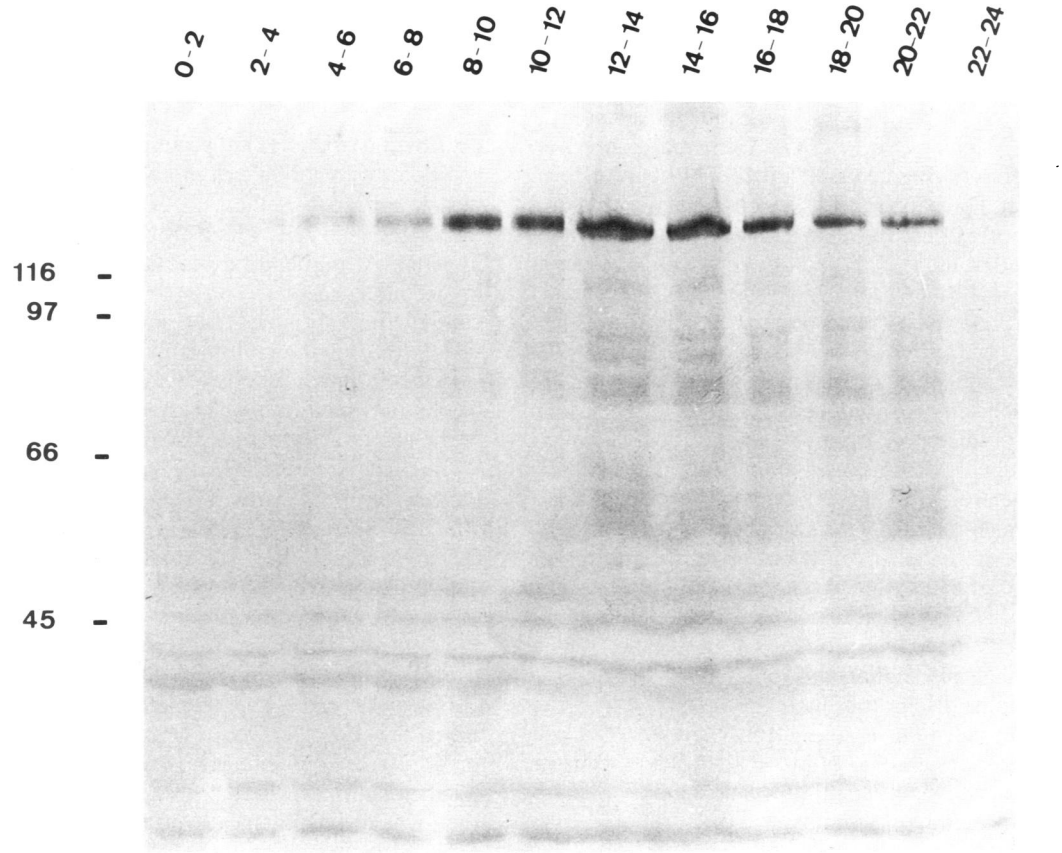

Fig. 3. Development expression of *l (2) gl* protein during embryonic stages determined by binding of monoclonal antibody 4H7XD. Staged embryos were collected at 2 h intervals. Proteins were separated on SDS–PAGE 10% gels and transferred onto nitrocellulose filters and treated as described in Figure 2. Age of embryos used in this experiment is given above the lanes. Mol. wt markers are as in Figure 2 and phosphorylase B (97 kd).

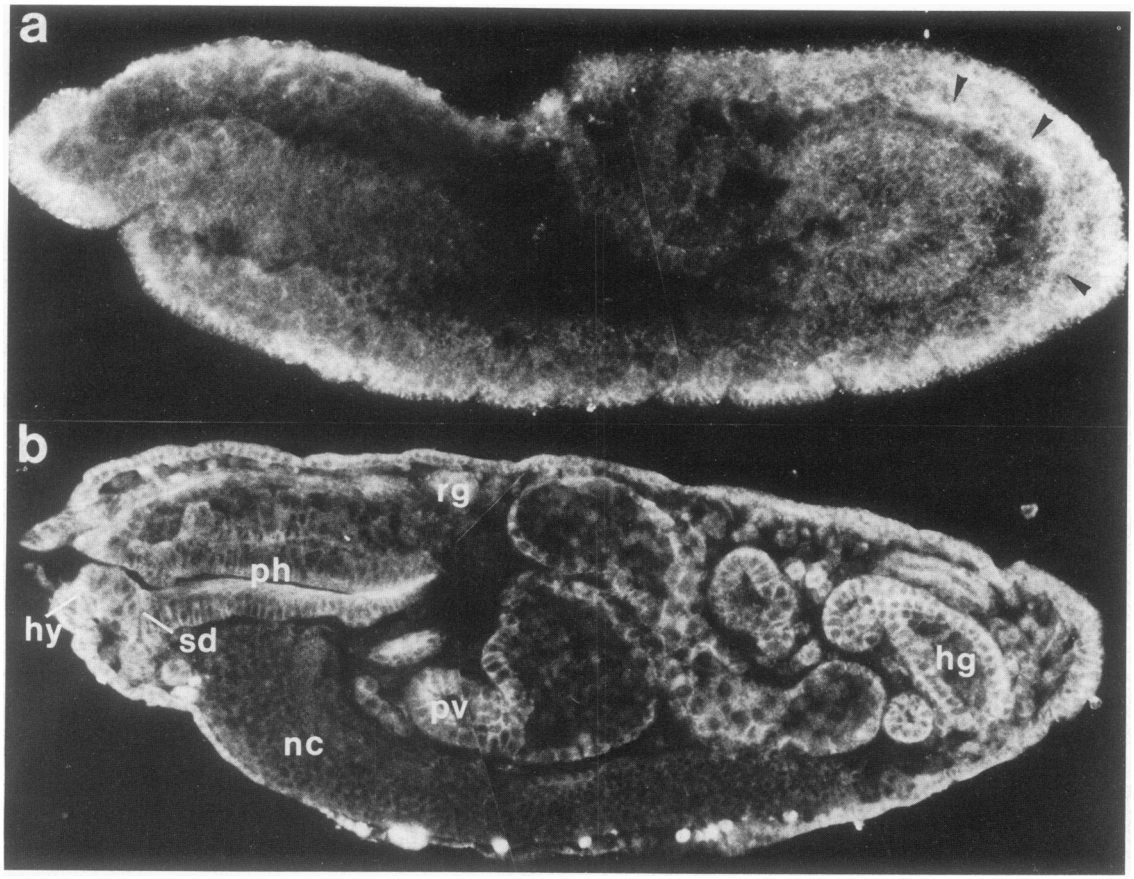

Fig. 4. Localization of *l (2) gl* protein in embryonic sections using 4E2XA ascites fluid. **(a)** Sagittal section of a stage 11 embryo. Arrowheads indicate the region between ectoderm and mesoderm. **(b)** Sagittal section of a stage 16 embryo. Abbreviations: hg, hindgut; hy, hypophysis; ph, pharynx; pv, proventriculus; rg, ring gland; sd, salivary duct; vc, central cord.

cloned recently (Mechler *et al.*, 1985). Genomic clones were used to isolate cDNA fragments (R.Lützelschwab *et al.*, unpublished results). To analyse the *l (2) gl* protein we have used monoclonal antibodies raised against a fusion protein synthesized from the 3′ end of the *l (2) gl* coding sequences. These antibodies recognize a 130-kd protein in Western blots, which is localized at the cellular membranes of embryonic tissues. These results corroborate our analysis of nucleotide sequence of a 5.3-kb cDNA fragment containing a full length 3.5-kb open reading frame (R.Lützelschwab *et al.*, unpublished results). The deduced protein has a predicted mol. wt of ~130 kd and contains a transmembrane signal peptide at the amino end of the protein, suggesting that the protein is secreted into the intercellular space.

The principal finding of the present structural and developmental analysis is a differential distribution of the *l (2) gl* protein within tissues of the embryo and of the larvae. Within presumptive neural tissues the *l (2) gl* protein is found in the presumptive optic lobes whereas much less protein is seen in other regions of the supraoesophageal ganglion and ventral cord. We have not analysed the imaginal disc primordia in detail since at this stage these cells are difficult to distinguish from the epidermal cells of the presumptive larval cells. However patches of cells are sometimes visible with significantly lower amounts of labeling (Figure 5c) which could represent imaginal disc primordia. This would indicate that at this stage cells of the imaginal discs contain lesser amounts of the *l (2) gl* protein compared with larval cells which contain high amounts of the protein.

It is interesting to consider whether the observed structural distribution of the *l (2) gl* protein in embryonic wild-type tissues correlates with normal cell proliferation and cell adhesion properties. It appears that the *l (2) gl* protein in embryonic tissues is associated with cells which are in the process to discontinue cell division. At mid-embryogenesis the *l (2) gl* protein appears to be accumulated mainly in presumptive optic lobes and in presumptive larval tissues. Larval cells are known to stop cell division towards the end of embryogenesis. In successive larval stages they grow in size by enlargement of the cells. Observations on the proliferation rates of neural tissues indicate that not all of the neural cells continue to divide in subsequent larval stages (White and Kankel, 1978). The most remarkable difference in the rate of proliferation is seen in the primordia of optic lobes which form during embryonic stage 12 and remain inactive in cell division until the 2nd larval instar (Hofbauer, 1979; White and Kankel, 1978). In contrast, we observe rather low amounts of the *l (2) gl* protein in tissues which are known to proceed with cell division. This is true for neural cell bodies except presumptive optic lobes and possibly for imaginal disc primordia.

Neuroblasts and ganglion mother cells of the presumptive optic lobes have been shown previously to be the origin of tumorous growth in *l (2) gl* mutant larvae (Gateff, 1978a). Therefore the localization of the *l (2) gl* protein in these cells coincides with the focus of phenotypically affected cells and indicates that the *l (2) gl* protein is already crucial in the embryo for the emergence and maintenance of cell specificity and cell proliferation of the optic lobe cells. Taken together with the finding that the *l (2) gl* protein appers to be located in embryonic tissues which are

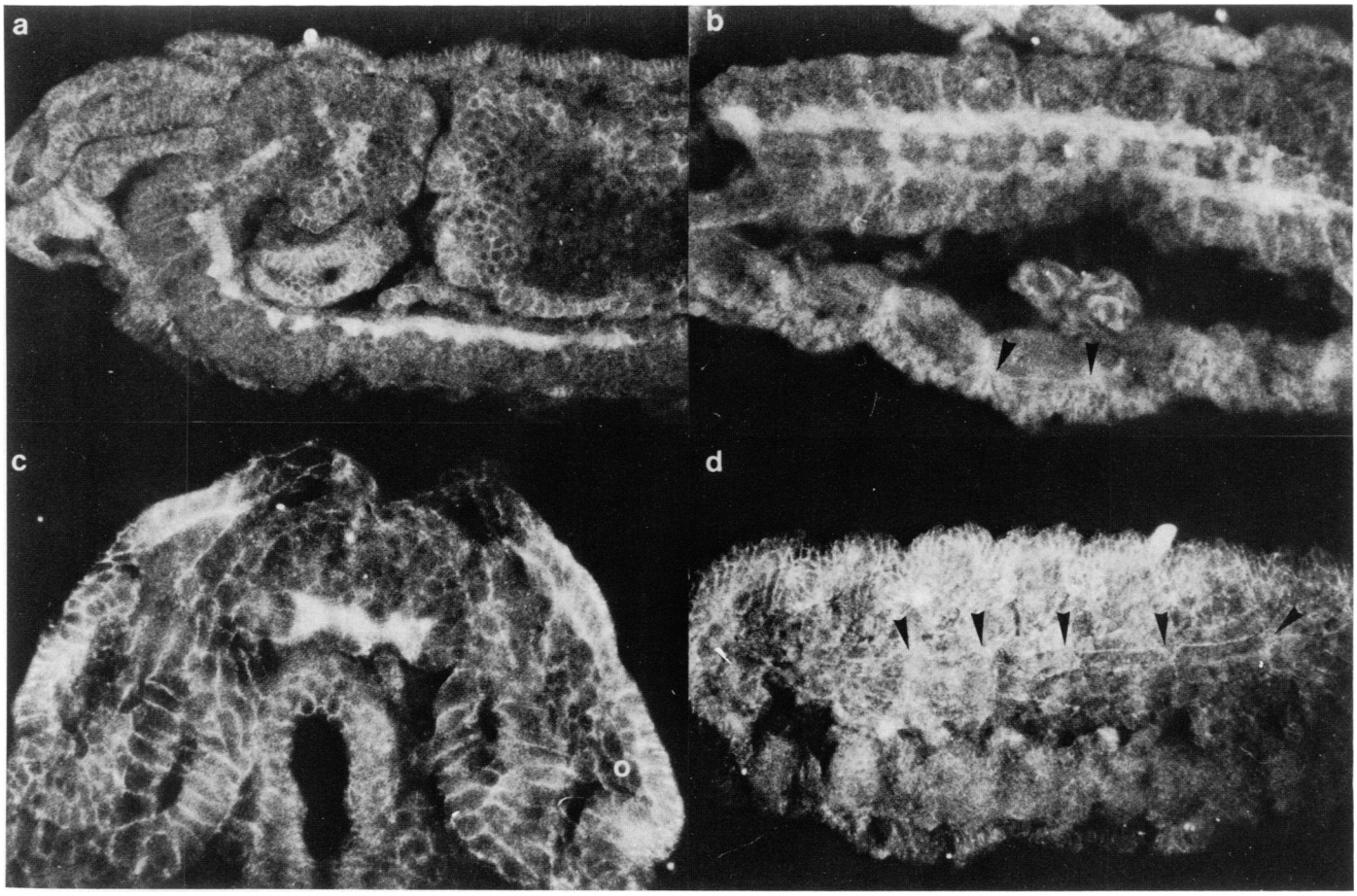

Fig. 5. Differential distribution of *l (2) gl* protein in tissues of stage 14 embryos using 4E2XA ascites fluid. **(a)** Parasagittal section of a stage 16 embryo. Immunofluorescence labeling is seen in the neuropile structure extending into the supraoesophageal ganglion and in cell membranes of the supraoesophageal ganglion corresponding to the presumptive optic lobes. In addition cells of the midgut, epidermis, proventriculus and the salivary gland primordia are also labeled. **(b)** Horizontal section of stage 16 embryo cut at the ventral cord. The neuropile and segmental fascicles are labeled. Note also label in the apodemata and conspicuous fibers between the segmental muscles (arrowtips). **(c)** Transversal section through an anterior region of a stage 16 embryo. Differences are seen in labeled cell membranes between the neural cord and other tissues. **(d)** Grazing section of the embryo seen in **b**. The embryo is cut ventrally between epidermis and neural cord. Conspicuous fibers are seen between segmental borders and indicated with arrowtips.

known to slow down with cell proliferation at the second half of embryogenesis, it is possible that the protein is involved in this process by preventing cells from cell division.

If we assume a function of the *l (2) gl* protein in slowing down cell division in embryogenesis it is interesting to analyse the protein distribution at the end of the 3rd larval instar, when the gene is expressed again. Although we have not yet analysed protein distribution in tissue sections, Western analysis of dissected tissues indicate that the protein is localized preferentially in the larval brain and in imaginal discs (Figure 2). Thus a shift in tissue-specific expression of the *l (2) gl* gene is observed between embryogenesis and 3rd larval instar.

It is known that during larval stages imaginal disc cells and at least part of the brain cells divide until the end of the 3rd larval instar. At the onset of metamorphosis these cells stop dividing and undergo differentiation. It is at this stage that the *l (2) gl* protein is expressed in large amounts and appears to be located preferentially in the larval brain and in imaginal discs. From this observation the *l (2) gl* protein at this stage could exert its function on brain cells and imaginal disc cells by preventing cell division in tissues where it is expressed. The phenotypic effect of the *l (2) gl* mutation could be explained by continued cell proliferation of optic lobe cells during larval stages due to the absence of the protein.

In this context it is not obvious why larval tissues are not phenotypically affected by the absence of protein in the mutant, whereas cells in the presumptive optic centers and imaginal disc cells show aberrant proliferation rates and unstructured growth in the homozygous mutant larvae. One of several possible explanations for this could be the existence of a redundant protein similar to the *l (2) gl* protein which would function exclusively in the presumptive larval tissues during embryogenesis. However, experimental evidence is necessary to test the proposed function of the *l (2) gl* gene during development.

Materials and methods

DNA

A 2.2-kb *Hin*dIII DNA fragment of the cDNA clone pcE7-9 (R.Lützelschwab *et al.*, unpublished results) containing an open reading frame sequence of 900 bp which codes for the carboxy-terminal 30 kd of the *l (2) gl* protein was cloned into two bacterial expression vectors according to standard procedures (Maniatis *et al.*, 1982). The pUR290-292 plasmids (Rüther and Müller-Hill, 1983) allow cloning of DNA fragments in-frame to the 3′ end of the β-galactosidase gene. The bacterial gene of the vector codes for a 116-kd protein. pPGH5 is a fusion of the 2.2-kb *Hin*dIII fragment and the β-galactosidase gene in pUR291. The other vectors used in this work: pATH1, pATH10 and pATH11, were a generous gift of Dr T.J.Körner (New York). They enable cloning in-frame to the 3′ end

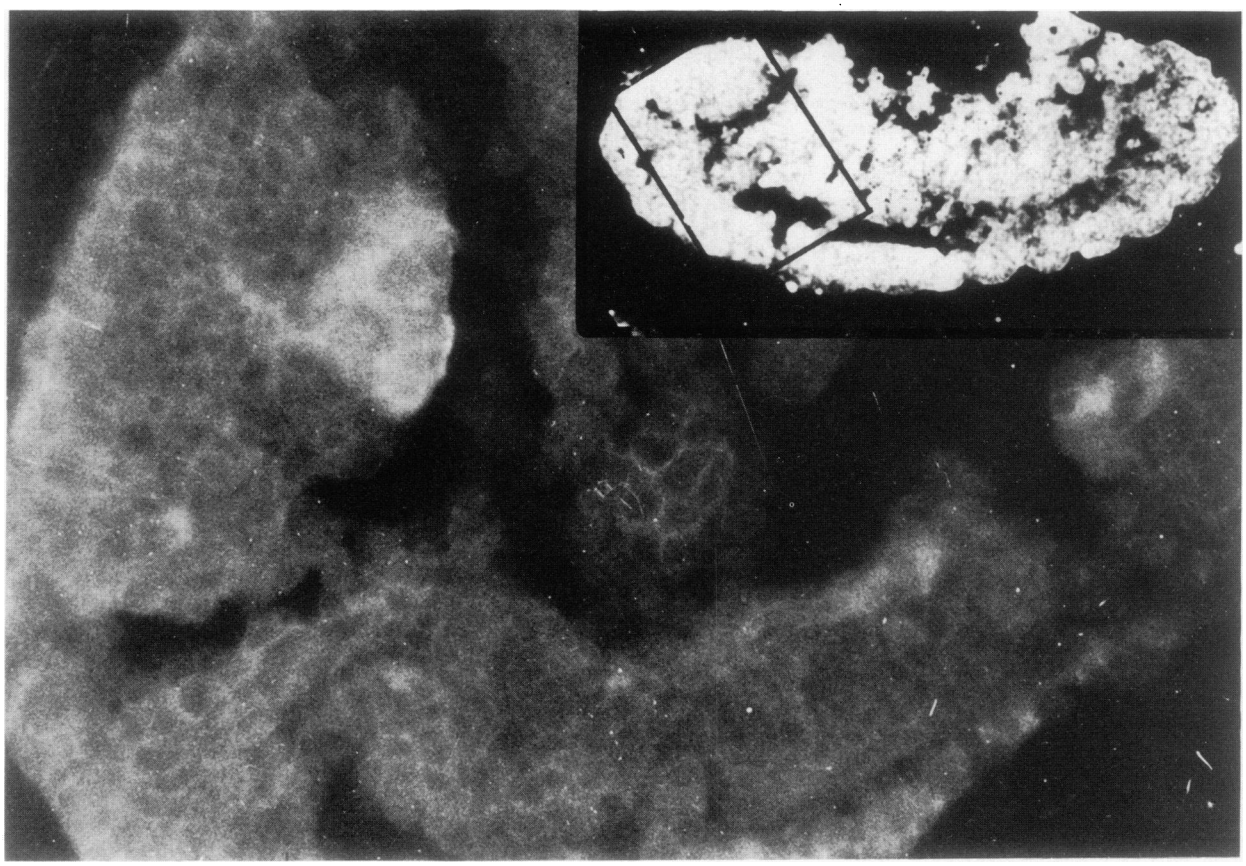

Fig. 6. Labeling of neural tissue in a sagittal section of stage 13 embryo using 4E2XA ascites fluid. The *l (2) gl* protein is labeled in the presumptive optic centers seen as a subregion of the supraoesophageal ganglion of the embryo. Insert: dark field micrograph of the same embryo at a lower magnification with the enlarged part framed.

of the *TrpE* gene. The *TrpE* gene in this vector codes for a 37-kd protein. The pPTH6 is a fusion of the 2.2-kb *Hin*dIII fragment and the *TrpE* gene in pATH10.

Expression of fusion proteins

pPGh5 was transformed into BMH 71-18: (*lac pro*) *thi supE/F lacI^{Q}Z^{-}* M15 *pro*$^{+}$. The bacteria were grown to midlog in M9/glycerin and induced for 4 h with IPTG. 5 ml were pelleted, re-suspended in 0.5 ml sample buffer and boiled for 6 min. The probe was separated on a preparative SDS−PAGE 10% gel (Laemmli, 1970). pPTH6 was transformed into C600:F^{-}, *thi-1, thr-1, leuB6, lacY1, tonA21, supE44,* λ^{-}. The bacteria were grown to midlog in M9 and induced for 4 h with β-indole-acrylic acid. 5 ml were pelleted, re-suspended in 0.5 ml sample buffer and boiled for 6 min. The probe was loaded on a preparative SDS−PAGE 10% gel (1 mm thick). After separation of the proteins the gel was stained with aqueous Coomassie solution and the protein band containing the fusion protein was eluted (White and Wilcox, 1984). This procedure yielded ~100 μg of fusion protein per gel.

Monoclonal antibodies

The fusion protein was dissolved in PBS, BALB/c mice were immunized i.p. with 25 μg of fusion protein in 150 μl of PBS and 150 μl of Freund's incomplete adjuvant. Four, 8 and 12 weeks after the first injection the mice were boosted with the same amount of antigen in Freund's incomplete adjuvant. Four days after the last immunization the animals showing a good response were sacrificed and the spleen was recovered. The spleen cells were fused with XAg63 myeloma cells (kindly provided by T.Hecht) and plated out on a spleen cell feeder layer in 384 0.2-l wells in HAT/RPMI 1640/20% fetal calf serum according to Köhler and Milstein (1976) and Campbell (1984). About 300 hybridoma clones grew up and were screened on micro-protein blots. For the preparation of micro-nitrocellulose filters the soluble proteins of the bacteria expressing the *l (2) gl*−β-galactosidase fusion protein were applied on a preparative SDS−PAGE slab gel and run into the separation gel for ~1 cm. The gel was electroblotted onto a nitrocellulose filter strip and cut into slices of ~0.5−1 mm. These blot slices containing a narrow but distinct protein pattern were incubated in microtiter plates overnight at 4°C with 100 μl of cell culture supernatant. To prevent the filter slices from floating away a mesh was spanned over the microtiter plate. In this way the filters could be washed by placing the mitrotiter plate in a larger tank of blocking buffer. Because there is no immunological cross-reaction between the β-galactosidase and *TrpE* gene product, all antibodies labeling the *l (2) gl*−β-galactosidase fusion protein are probably produced by hybridoma cell lines specific for the *l (2) gl* part of the *trp* fusion protein. With this procedure ~50 positive hybridoma clones were identified. The antibodies were shown to be specific to the *l (2) gl* portion of the fusion proteins. In Western blots containing soluble proteins from bacteria harboring the plasmids pPGH5 and pUR291, binding is observed to the 145-kd fusion protein but not to the 116-kd β-galactosidase protein.

Ascites

Six positive hybridoma cell lines were subcloned twice by limited dilution. Two clones 4E2XA and 4H7XD were used to generate ascitic fluid. 0.5 ml Pristane (Sigma) was injected i.p. in BALB/c mice. Two to four days after injection, 10^{6}−10^{7} hybridoma cells were injected i.p. Three to four weeks later the mice were sacrificed and the 1−3 ml ascites fluid was recovered. The proper dilution factor was determined in protein blot experiments or with immunofluorescence experiments.

Protein probes

For collecting staged eggs, flies were kept at 20°C on agar plates complemented with yeast. Embryos or larval tissues were homogenized in PBS and boiled for 3 min. The protein concentration was measured according to Bradford (1976). Sample buffer was added and the homogenate was boiled for 6 min. Aliquots containing 30 μg of protein were electrophoretically separated according to Laemmli (1970).

Protein blots

Electroblotting of SDS−PAGE separated proteins was performed according to Towbin *et al.* (1979). Immediately after the transfer the nitrocellulose filter was incubated for 1 h at room temperature in BB (20 mM Tris−HCl pH 7.4; 0.5 M NaCl; 0.05% NP-40), 3% BSA, incubated with ascites fluid diluted 1:100 in BB, 3% BSA at 4°C overnight. The filter was washed four times for 15 min with BB and then incubated for another 2 h with the second antibody [anti-mouse IgG alkaline phosphatase conjugate (Sigma)] diluted 1:1000 in BB, 3% BSA. After another wash for 1 h in BB, the filter was stained for phosphatase activity with 0.05% 5-bromo-4-chloro-3-indolyl phosphate, 0.03% Nitro Blue Tetrazolium (Sigma) in 100 mM Tris−HCl pH 8.8, 100 mM NaCl, 5 mM $MgCl_2$.

Immunofluorescence

The embryos grown at 20°C were collected at different times of embryonic development and dechorionated with 3% Na-hypochlorite. Stages of individual embryos (Campos-Ortega and Hartenstein, 1985) were ascertained under the stereo microscope. Sections were prepared according to Mitchison and Sedat (1983) and Dequin *et al.* (1984). Embryos were washed with PBS and fixed for 5 min in a 5 ml PBS/5 ml 4% paraformaldehyde/*n*-heptane mixture by gentle agitation. The fixed embryos were transferred into a −70°C pre-cooled 5 ml methanol/5 ml *n*-heptane mixture and vigorously shaken for 10 min. After rapidly warming the mixture to room temperature the devitellinated embryos sank to the bottom and were recovered. The embryos were washed three times with methanol and twice with PBS without detergent. The embryos were mounted in Tissue Tek O.T.C. (Miles Scientific). Sections of 8 μm were cut on a Cryotome WK 115 and positioned on gelatin-coated slides. The preparations were incubated for 2 h with ascites diluted 1:50 in PBS, 3% BSA, washed three times for 10 min in PBS and incubated for 1 h with the second FITC-conjugated anti-mouse IgG antibody (Sigma) diluted 1:100 in PBS, 3% BSA. After washing for 30 min the sections were embedded in 90% glycerin PBS, 4% *n*-propylgallate and inspected under u.v. light with a standard Zeiss microscope using a fluorescein filter set (450−490). Photographs were taken using Ilford HP5 1600 ASA and forced development with Ilford Microphen developer.

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