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Expression of the *Drosophila optomotor-blind* gene transcript in neuronal and glial cells of the developing nervous system

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SUMMARY

Mutations in the complex gene locus *optomotor-blind* (*omb*) can lead to defects in the development of both the optic lobes and external features of the adult fly. We describe here the expression of *omb* in the developing and adult nervous system using in situ hybridization. During embryogenesis, *omb* expression is first observed in the optic lobe anlagen. It later expands to a larger part of the developing larval brain and to the gnathal lobes. Cells in the ventral and peripheral nervous systems begin to express *omb* after completion of germ band extension. Later in embryonic development, expression declines and only persists in the antennomaxillary complex and in part of the brain hemispheres. During the larval and pupal stages, *omb* expression in the brain is confined to the developing optic lobes and contiguous regions of the central brain. At these stages, only a few cells show expression in the ventral ganglion. In the eye imaginal disc, transcript accumulation is most conspicuous in a group of presumptive glia precursor cells posterior to the morphogenetic furrow and in the optic stalk. In the adult brain, expression is prominent in several regions of the optic lobe cortex and along the border between central brain and optic lobes. In the mutation *In(1)omb^{H31}*, 40 kb of regulatory DNA, downstream from the transcription unit, are removed from the *omb* gene. *In(1)omb^{H31}* is characterized by the lack of a set of giant interneurons from the lobula plate of the adult optic lobes. We find that, already during embryogenesis, there is a drastic difference between wild type and *In(1)omb^{H31}* in the level of the *omb* transcript in the optic lobe primordia. The adult mutant phenotype may thus be caused by *omb* misexpression during embryonic development.

Key words: *Drosophila melanogaster*, neural development, gene expression

INTRODUCTION

optomotor-blind (*omb*) was originally isolated in a behavioural screen for mutants defective in the optomotor turning response (Heisenberg, 1972; Heisenberg and Götz, 1975). Histological analysis of mutant flies both homozygous and hemizygous for the first, viable allele, *In(1)omb^{H31}*, revealed the apparent absence of a set of large cells of the lobula plate, the HS and VS neurons (Heisenberg et al., 1978). More subtle defects in other regions of the optic lobes were also noted (Fischbach et al., 1989; Brunner et al., 1992). Studies in larger flies (*Musca*, *Calliphora*, *Phaenicia*) have implicated the HS and VS cells in optomotor behaviour (reviewed in Hausen and Egelhaaf, 1989). Careful analysis of the behavioural defects of *In(1)omb^{H31}* flies showed that only part of the optomotor behaviour, the large field response, is abolished in these animals; another part, the object response, still being largely intact. This excludes an essential involvement of the HS and VS neurons in the object response (Heisenberg and Wolf, 1984; Bausenwein et al., 1986).

In an effort to characterize the *omb* locus genetically and molecularly, additional mutant alleles have been investigated. These are all homozygous lethal and define the *l(1)omb* complementation group. Other, partly independent, genetic functions such as *bifid* (*bi*), *Quadroon* (*Qd*) and *lacquered* (*lac*) fail to complement several *l(1)omb* mutations and are therefore considered part of the complex gene locus (Pflugfelder et al., 1992a). *bi* and *lac* cause defects in wing venation and wing spread, respectively; *Qd* affects the pigmentation of the abdominal tergites. In mutants in which the lethal domain of *l(1)omb* is disrupted, development of the adult optic lobes is severely disturbed, leading, in the extreme case, to loss of nearly the entire optic lobe neuropil. Our previous molecular analysis has shown that *l(1)omb* is encoded by a 75 kb transcription unit in a locus of at least 120 kb, mapping at 4C on the X chromosome (Pflugfelder et al., 1990, 1992a). The 75 kb primary transcript is spliced to a 6 kb mature mRNA, designated T3. The T3 gene product has sequence homology to the murine *Brachyury* (*T*) gene (Herrmann et al., 1990) and probably functions as a transcriptional regulator (Pflugfelder et al., 1992b). T3 transcription is regulated by a 45 kb optic lobe regulatory region (OLR) in which several elements control different aspects of optic lobe development (Brunner et al., 1992). In this study, we investigate the spatiotemporal pat-

tern of *omb* expression in both wild type and the regulatory *omb* mutant *In(1)omb^{H31}*. We show that, in *In(1)omb^{H31}*, in which part of the OLR is removed from the transcription unit, T3 expression is reduced in the optic lobe primordia both at the end of embryogenesis and in the white prepupa. An attempt is made to correlate the wild-type pattern of expression with the severe phenotypes observed in loss-of-function mutants, and the altered pattern in *In(1)omb^{H31}* with the specific defects observed in these flies.

MATERIALS AND METHODS

Drosophila stocks

In(1)omb^{H31} has been described in Heisenberg et al. (1978). *daughterless^{A60}* was kindly supplied by J. Campos-Ortega. Strain *Berlin* served as a wild-type control. Flies were raised at 23°C on standard medium (cornmeal, agar, molasses, yeast and Nipagin).

In situ hybridizations of embryo whole mounts

Embryos were fixed and hybridized according to the protocol of Tautz and Pfeifle (1989). Digoxigenin-labeled RNA probes were transcribed following the protocol of the Boehringer RNA labeling kit, using a clone containing a 5′ cDNA fragment of the *omb* T3 transcript as template (pceII-52-7, Pflugfelder et al., 1992) for both sense and antisense transcripts. Embryos were prehybridized in hybridization solution (ISH; Tautz and Pfeifle, 1989). Hybridizations were performed with 2.5-5 µg digoxigenin-labeled RNA probe in 100 µl hybridization solution supplemented with 10% sodium dextran sulfate (ISHD), at 50°C overnight. Embryos were then washed twice in washing buffer (500 mM NaCl; 10 mM Tris, pH 6.8; 10 mM $NaPO_4$; 5 mM ETDA; 50% formamide) for 30 and 60 minutes, respectively, at 50°C. After rinsing for 1 minute in TEN buffer (500 mM NaCl; 10 mM Tris, pH 6.8; 1 mM EDTA), RNA was digested for 30 minutes with 20 µg/ml RNaseA (Boehringer) in TEN at 37°C. Preparations were stringently washed for 30 minutes in 2× SSC at room temperature and, after rinsing in 0.1× SSC, for another 30 minutes in 0.1× SSC at 50°C. Subsequent signal detection again followed the protocol of Tautz and Pfeifle (1989). After dehydration and rehydration (for better signal intensities), embryos were mounted in glycerol-gelatine (Romeis, 1968; 'glycergel').

Embryos were double-stained with RNA probes and anti-HRP antibody, with hybridizations preceding antibody detection. After visualization of the RNA signal, embryos were washed three times for 5 minutes each in PBT (0.1% Tween 20 in PBS) and transferred through an ethanol series (30, 50, 70, 50, 30%), each step 5 minutes on ice and finally rinsed in PBT. After two incubations for 30 minutes each in PBTB (PBT/0.2% BSA), embryos were blocked for 30 minutes with 2% blocking serum (PBTN, Elite kit, Vectastain) and then incubated with rabbit anti-HRP (Cappel) at a dilution of 1:3000 in PBTN at 4°C overnight. After rinsing twice with PBTB, embryos were washed twice for 30 minutes each in PBTB followed by incubations first in PBTN for 30 minutes, and then in a 1:200 dilution of secondary antibody (in PBTN) for 2 hours. After washing in PBTB for 2× 5 minutes and 2× 30 minutes, embryos were incubated with ABC-complex (Vectastain, Elite kit) for 1.5 hours at room temperature and then washed 2× 30 minutes in PBTB. The final reaction with diaminobenzidine (DAB, Sigma) was done in a volume of 2 ml at a concentration of 250 µg/ml DAB in PBT solution. 4 µl of 3% H_2O_2 were added after 5 minutes preincubation. The reaction was stopped by pipetting the embryos into a vessel containing 20 ml PBT. Embryos were collected and washed two times in PBT and mounted in glycergel.

In situ hybridization of larval imaginal discs

Eye discs of late third instar larvae were prepared and then hybridized using oligo-primed digoxigenin-labeled DNA probes according to the protocol of Cubas et al. (1991), except that proteinase K digestion time was increased to 5 minutes and that discs were hybridized in ISHD. For nuclear counterstaining, eye discs after hybridization were first dehydrated and rehydrated through an ethanol series, then washed four times in PBT and finally incubated in a solution of 5 µg/ml DAPI (4′,6-diamidin-2-phenylindol, Boehringer) for 5 min. After two washes in PBT for 5 minutes each, discs were mounted in glycergel.

In situ hybridization of central nervous system whole mounts

Central nervous systems (CNSs) of white prepupae were dissected on ice in PBT and collected in 2 ml Eppendorf tubes. After washing in PBT (all following steps in 1 ml volume), whole mounts were fixed in PPTC (4% paraformaldehyde/PBS; 0.1% Tween 20; 0.1% sodium deoxycholate) for 1 hour at 4°C. The material was rinsed three times for 5 minutes in PBT and digested with proteinase K (Boehringer; 50 µg /ml in PBT) for 5-7 minutes at room temperature. The digestion was stopped with two 1 minute washes in 0.2% glycine/PBT. After washing for 5 minutes in PBT, whole mounts were refixed in PPTC for 20 minutes at room temperature and washed again four times each for 5 minutes in PBT. Preparations were prehybridized in PBT/ISH (1:1) and ISH for 10 minutes each at room temperature and for 1 hour at 50°C in ISH. Hybridization with digoxigenin-labeled RNA probes was carried out as described above. Stringent washing and signal detection followed the protocol of Tautz and Pfeifle (1989) using anti-digoxigenin antibody in a dilution of 1:2000 for 2 hours. The antibody had been preabsorbed against fixed larval tissue for 1 hour at a dilution of 1:100.

In situ hybridization of frozen tissue sections

The protocol is based on the procedure of Hafen and Levine (1986) for in situ hybridizations of frozen tissue sections with ^{35}S-labeled RNA probes. Ethanol series prior to hybridization were omitted. Sections were digested with Proteinase K (Boehringer) at 6.25 µg/ml for 10 minutes in 5× TE (TE = 10 mM Tris, 1 mM EDTA, pH 8). Following the second fixation step, sections were acetylated in 200 ml 0.1 M TEA (pH 8) containing 320 µl acetic anhydride for 10 minutes at room temperature under slight agitation. Slides were washed twice in PBS for 1 minute each and prehybridized in PBT/ISH (1:1) and ISH for 15 minutes each at room temperature.

Hybridizations were carried out overnight at 50°C in a moist chamber, using 25 µl digoxigenin-labeled RNA probe per slide, at a concentration of 2.5-5 µg/ml RNA in ISHD. Sections were covered with an 22×22 mm coverslip and sealed with rubber cement. After removing the seal, coverslips were floated off the slides by dipping the slides in PBT. The slides were then washed three times for 1 hour each in 1× SCC/50% formamide at 42°C. After rinsing for 1 minute in TEN, the preparations were digested with RNase for 10 minutes at 37°C at a concentration of 20 µg/ml RNaseA. The sections were then washed first in 2× SSC (30 minutes, room temperature) and after a 1 minute change in 0.1× SSC, for 15 minutes in 0.1× SSC at 50°C. Signals were detected according to the Digoxigenin Detection Kit (Boehringer), by first rinsing slides for 1 minute in buffer 1 and then incubating them for 30 minutes in 0.5% blocking solution. Slides were again rinsed in buffer 1 and then incubated with anti-digoxigenin antibody at a dilution of 1:500 in buffer 1 for 2 hours at room temperature.

The antibody solution was removed and the slides were washed twice in buffer 1, then for 10 minutes in buffer 3. Preparations were stained in a moist chamber using staining solution in buffer 3. Colour development (4-16 hours) was monitored under the microscope. Reactions were stopped by dipping the slides in PBT (2×5 min). The sections were mounted in glycergel.

Alternatively, CNSs were dissected from third instar larvae in PBT on ice, washed, fixed in 4 % paraformaldehyde/PBS for 20 minutes at room temperature and washed again 4 times in PBT prior to sectioning. Further treatment of sections was as described above.

Histology for [^{3}H]thymidine autoradiography

The methods involved in this procedure have been described in Hofbauer and Campos-Ortega (1990).

RESULTS

Transcription at the *omb* locus

Within the limits of the genetically defined *omb* locus, fourteen RNA signals have been identified by northern blot analysis (Pflugfelder et al., 1990, 1992a). In all experiments presented in this communication, probes derived from the cDNA clone pceII-52 were used (Pflugfelder et al., 1992a). In all experiments with RNA probes, signal authenticity was verified by hybridizations with sense probes. In experiments with DNA probes, signal specificity was tested with labeled vector DNA. These controls yielded no or low and uniformly distributed signals (data not shown).

On northern blots, pceII-52 probes exclusively detect the *omb* trancript T3 and its precursor T7. Especially during embryogenesis, T7/T3 appears to be localized in a small region of the nucleus yielding a nuclear signal, which is more prominent than the cytoplasmic signal. In the adult brain, however, T3 is predominantly found in the cytoplasm (compare Fig. 1B with Figs 5D and 6). For the sake of simplicity, the hybridization signal observed with our probe is hereafer referred to as T3. T3 is of rather low abundance, making up only the 10^5th part of total poly(A)$^+$ RNA, as judged from the yield in screens of cDNA libraries.

omb transcript levels during embryogenesis

The first *omb* transcription detectable by in situ hybridization occurs in the cephalic region of embryos in the phase of rapid germ band extension (stage 8; embryos are staged according to Campos-Ortega and Hartenstein, 1985). Dorsolaterally, *omb*-transcribing cells are located anterior to the cephalic furrow in two bilaterally symmetric domains, which laterally reach down to a mid-embryo level (Fig. 1A,B). The cells lie in a shallow groove. The transcript detected by the in situ hybridization method appears to be predominantly located in a discrete part of the expressing nuclei. One or two pigment spots can be detected per nucleus. This feature is characteristic of the *omb* transcript and was not observed with probes hybridizing to RNA from different smaller control genes. It is thus not an artefact of the hybridization or detection procedure.

The number of T3-expressing procephalic cells increases upon further germ band elongation. Mediodorsally, a narrow strip of cells connecting the two dorsolateral domains begins to transcribe *omb* (Fig. 1C). Upon completion of germ band extension (stage 11), T3 is expressed in the lateral germ band in a segmentally repeated pattern (Fig. 1D). Staining intensity decreases from anterior to posterior, being, at this stage, barely detectable in abdominal segments 8 or 9. At the same time, expression in the gnathal segments becomes apparent. No expression in the ventral nerve chord is yet detectable. With further development, the number of cells expressing T3 in the procephalic region increases. These cells are now localized on each side of the embryo in two discernible but contiguous regions, the ventrolateral one corresponding to the antennal segment (Cohen and Jürgens, 1991). With the onset of germ band retraction (stage 12), small clusters of cells flanking the ventral midline begin to express T3. Transiently, also cells of mesodermal origin appear stained in a ventrolateral position. A dorsal view of embryos at this stage shows that the dorsal domain of T3 expression is indented by a groove which helps to identify this region as the optic lobe anlage (Campos-Ortega and Hartenstein, 1985; Fig. 1E).

Upon further germ band retraction, expression within the segments increases. The pattern of the lateral signal becomes less uniform, a mediolateral cluster of cells showing the highest T3 level within this expression domain. The lateral staining probably reflects expression of T3 in neurons of the peripheral nervous system (PNS) but also in some additional cells (see below). The overall T3 pattern is very similar to the PNS structure as it is visualized by anti-HRP staining (Campos-Ortega and Hartenstein, 1985; Hartenstein, 1988), mab21A4 staining (Ghysen et al., 1986) or *lacZ* expression in the enhancer trap line A37 (Ghysen and O´Cane, 1989; Fig. 1F). An abdominal segment contains 40-42 PNS neurons (Ghysen et al., 1986). There are, however, at stage 13, more than 50 T3-expressing cells in the lateral zone of abdominal segments, suggesting that T3 expression is not limited to ganglion cells. Preparations, double-stained for both T3 and the general neuronal antigen detectable with anti-HRP, directly reveal T3 expression in PNS neurons (not shown). In embryos homozygous for *daughterless* (*da*), lateral T3 expression is reduced to a straight line of presumably epidermal cells (Fig. 1L). Zygotic *da* expression is required for PNS development (Caudy et al., 1988). This again indicates that, laterally, T3 is also expressed in non-neuronal cells.

From stage 10 to 13, an increasing number of cells in the ventral nervous system express T3 (Fig. 1G). The most strongly expressing cells are located just outside of the neuropil region anterior to the posterior commissure. With head involution and the formation of the antennomaxillary complex, the staining disappears from the region of the former gnathal protuberances and the antennal segment. Cells in the antennomaxillary complex continue to express T3. In the second half of embryonic development, T3 expression in most of the peripheral and ventral nervous system gradually declines, starting ventrally in the PNS (Fig. 1H,I). Shortly before hatching, T3 expression is limited to the antennomaxillary complex and to a caudolateral region within the larval brain, posterior to the horizontal commissure which connects both hemispheres (Fig. 1J,K).

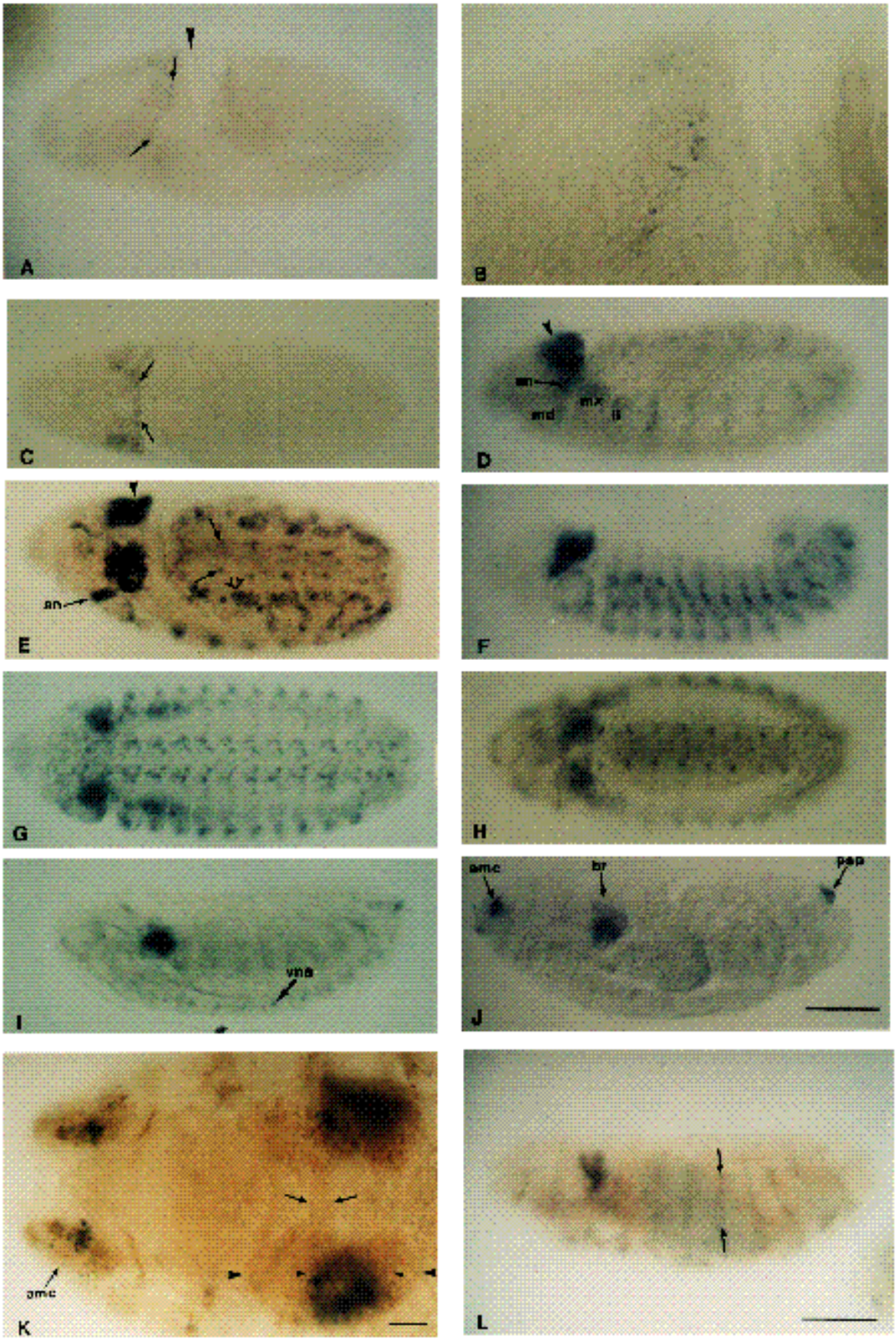

Fig. 1

Fig. 1. T3 expression during embryogenesis. Staging is according to Campos-Ortega and Hartenstein (1985). (A,B) First appearance of T3 signal at stage 8 in a row of cells in the procephalon. (A) Slightly dorsolateral view. Arrows indicate dorsolateral extent of T3 expression; arrowhead points to cephalic furrow. (B) 2.5-fold magnification of A to visualise the discrete localization of the hybridization signal. (C) Stage 10 embryo, dorsolateral view. The procephalic *omb* transcription domains begin to enlarge. Cells connecting the two symmetrical domains dorsally lie between arrows. (D) Stage 11 embryo, lateral view. Expression in the antennal segment (an) and in the gnathal lobes (md, mandibular; mx, maxillary; ll, labial) is indicated; arrowhead points to furrow in the dorsolateral expression domain of the procephalon, the presumptive location of the optic lobe anlage. A segmentally repeated pattern of T3 transcription on the dorsolateral side of the extended germ band also appears at this stage. (E) Stage 12 embryo, dorsolateral view. Onset of germ band retraction. Arrows point (in one abdominal segment only) to the first cells to express T3 in the ventral nervous system in a bilaterally symmetrical and segmentally repetitive pattern. Expression in the antennal segment and around the procephalic groove is indicated as in D. Open arrow points to mesodermal cells transiently expressing T3. (F) Late stage 12 embryo, lateral view. Compared to D, the number of cells expressing T3 laterally in the germ band has increased, also by extending ventrally. (G) Ventral view of stage 13 embryo. A regular pattern of T3-transcribing cells is evident in the ventral nervous system. At this focus, only expression in the lateral cluster of the PNS can clearly be recognised (cf. Fig 2A). (H) Ventral view of stage 14 embryo after the onset of head involution. Expression in the ventral and peripheral nervous system has begun to decline. (I) Lateral view of late stage 14 embryo. Arrow points to T3-expressing cell in ventral nervous system (vns). (J) Lateral view of embryo at the end of embryogenesis (stage 17). T3 transcript has disappeared from the segmented nervous system. T3 remains in the brain (br) and in the antennomaxillary complex (amc). The signal in the posterior spiracles (psp) is a staining artefact. The bar corresponds to 100 μm. (K) *omb* transcript in brain and antennomaxillary complex (amc) of late (stage 17) embryo. Dorsal view. The horizontal commissure connecting the hemispheres is indicated by two arrows. The rostrocaudal extent of the brain and the T3 expression domain is indicated by big and small arrowheads, respectively. T3 expression is limited to the caudolateral part of the brain. The bar corresponds to 20 μm. (L) Lateral expression (between arrows) remaining in homozygous *daughterless*^{A60} embryos is in a single stripe of epithelial cells. The mutant embryo presented shows da phenotype at high expressivity, only remnants of the ventral nerve chord still being visible. The bar corresponds to 100 μm. In all panels, anterior is to the left. In lateral views, dorsal is up. With the exception of B and K, all panels are at the same magnification.

T3 expression in the optic lobes of late third instar larvae and white prepupae

Northern blot analysis showed that, after a decline in the first two larval instars, T3 expression increases again in late third instar larvae, remaining high in the prepupa and during the first half of pupal development (Pflugfelder et al., 1992a and unpublished data). The most prominent CNS expression around pupariation is found in the developing optic lobes (Fig. 2A,B). There is virtually no T3 expression in the central part of the brain hemispheres and in the abdominal ganglia. Whole-mount preparations (Fig. 2A,B) demonstrate that *omb* is transcribed in the developing lamina. The zone of labeling follows the crescent-shaped outline of the lamina and at the end of the crescent extends dorsocaudally, embracing the central cell plug, which originates from the inner anlage (White and Kankel, 1978; Hofbauer and Campos-Ortega, 1990). In the dorsocaudal hemisphere, this labeling zone fuses with a T3-expressing domain, which is oriented mediolaterally and which apparently is associated with the suture in the outer anlage. Along this suture, the outer anlage opens up at this stage of development to give space to the cortex of the lobula plate (Hofbauer and Campos-Ortega, 1990). Medially, the labeling zone reaches the border between the developing optic lobes and the central part of the hemispheres. There the labeling is continuous with an expression zone, which includes most of the border line between the optic lobes and the central hemisphere.

Hybridizations of sections through the larval brain show that the labeling in the lamina actually derives from a population of cells in the developing lamina cell body area and from an area near the developing lamina neuropil (Fig. 3A). The labeling at the border between the optic lobes and the central brain appears to be rather patchy, including cells on both sides of the border line and showing an intermingling of labeled and unlabeled cells. In addition, scattered labeled cells are observed at the surface of the developing medulla neuropil.

The whole mounts and sections do not allow a clear identification of the cell types stained (Figs 2, 3). The density and the location of the labeled cells suggest that, at the border between central brain and optic lobes as well as in the dorsocaudal hemisphere and in the lamina, T3 expression is in ganglion cells. However, expression in other cell types cannot be excluded. The labeled cells close to the developing neuropil zones of lamina and medulla probably are glia.

T3 expression in the eye imaginal disc

Eye development proceeds in a posterior-anterior direction, ommatidial assembly occurring in a regular fashion behind the morphogenetic furrow. Axons from differentiating retinula cells run underneath the disc posteriorly toward the optic stalk, through which they enter the developing optic lobes in the lateral part of the brain hemispheres (Ready, 1989). Apically, the disc is covered by a thin peripodial membrane. In the region of the eye disc that gives rise to the retina, T3 expression is found on the basal side of the disc (Figs 3A,B, 4). There is also expression in a few cells of the optic stalk (Fig. 4A). The existence of cells outside of the disc epithelium and posterior to the morphogenetic furrow is most evident from sections through the disc. Pulse-labeling experiments with tritiated thymidine show that these cells still replicate, when cell divisions in the overlying disc epithelium can no longer be observed (Fig. 3C). Expression in this area is confined to cells that lie several ommatidial rows posterior to the morphogenetic furrow. Compared with the regular pattern of expression of many genes that are involved in ommatidial spacing and assembly (Banerjee and Zipursky, 1990), the pattern of T3 expression is highly irregular. The ratio of T3-expressing cells to the number of ommatidia is less than one in the expression domain. As observed in embryos, most of the T3 hybridization signal in eye disc cells appears highly

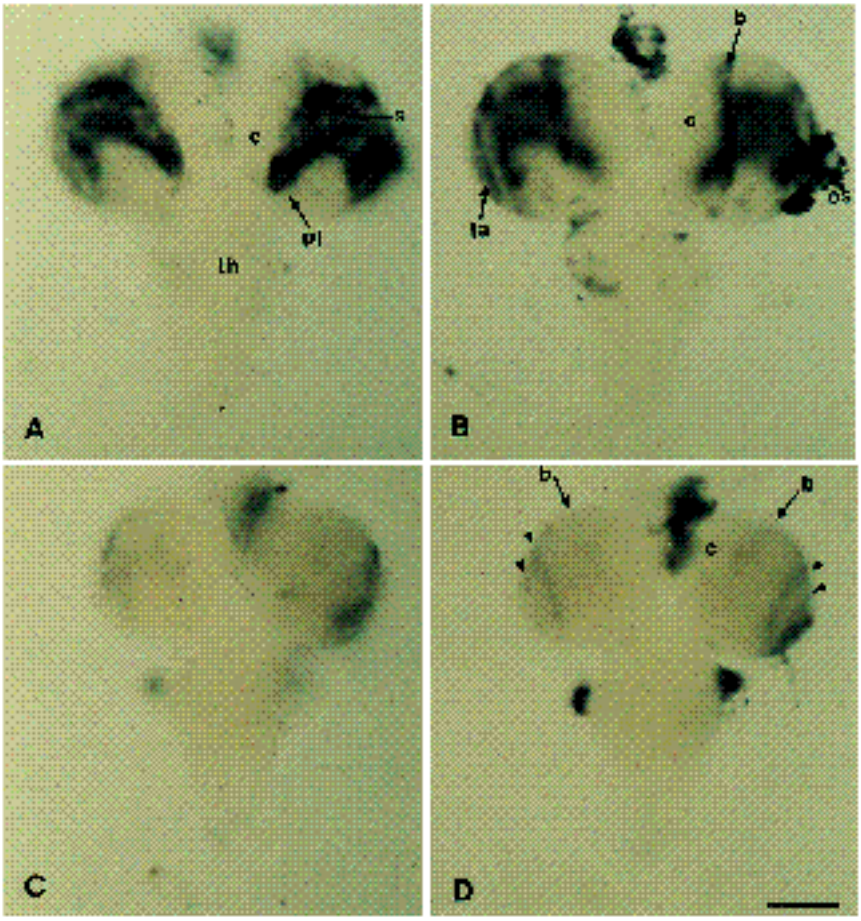

Fig. 2. T3 expression in the central nervous system of wild type (A,B) and *In(1)omb*^{H31} (C,D) white prepupae. In A and C, the focus is on the dorsal, in B and D, on the ventral hemisphere of the same preparations, respectively. (A) There is prominent staining along the suture (s) of the outer optic anlage in the dorsocaudal part of the hemisphere. Medially this staining area is continuous with the staining of the proliferation zone of the posterior shank of the inner anlage (pi). Laterally, the zone of labeling widens and is continuous with the staining of the lamina crescent (la). (B) In addition, cells outlining the border between optic lobes and central brain are stained in the anterior and ventral hemisphere (b). Only few stained cells are observed in the thoracic ganglia (th), virtually none are found in the central brain (c) and in the abdominal ganglia. The heavily stained fragments adhering to the optic stalk (os) and to the central brain are preparation artefacts. (B, D) In *In(1)omb*^{H31} mutant flies T3 expression at the same stage of development is drastically reduced compared to wild type. Remnants of the wild-type expression pattern can still be recognized. Arrows and arrowheads in D point to residual signals. Fragments adhering to the central brain and the thoracic ganglion are preparation artefacts. Bar in D corresponds to 100 μm.

localized. At high magnification, and most clearly in counterstained preparations, it can be seen that the signal is localized in the nucleus (Fig. 4C). There is only one hybridization spot per nucleus.

Hybridization signal is also found along the dorsal and ventral margins of the eye disc (Fig. 4A). These parts of the disc will secrete parts of the head capsule that surround the compound eye (Ouweneel, 1970).

T3 expression in the adult brain

In situ hybridization of cryostat sections of adult fly brains results in the staining of several areas in the cell body rind of the protocerebrum and the optic lobes as well as along the contact zone between the optic lobes and the central brain (Fig. 5). There is little staining of neuropil or retina. Compared with the neuropil, the entire cortex appears somewhat stained. In situ hybridizations with ^{35}S-labeled sense and anti-sense probes (not shown) suggest a general low level expression of T3.

There are labeled cells in the cell body rind of the central brain, the lateral horn of the protocerebrum, and in an area just anterior to the anterior optic foci (= optic tubercles of Strausfeld, 1976; Fig. 5A). A few cell bodies with strong labeling are localized in the caudal cell body rind at a level slightly dorsal to the oesophagus.

Many cell bodies near the boundary between optic lobes and central brain show strong T3 expression although not all cells are stained (Fig. 5B,C). Judged from their location, ganglion cells associated with the optic lobes as well as cells projecting into the central brain are labeled. As was observed in the prepupal stage, in some areas labeled and unlabeled cells are intermingled.

Another cell body area with distinct labeling is located caudally, between medulla and lobula plate, bordering laterally on the most posterior region of the medulla cortex and medially on the lobula plate cortex (Fig. 5C). The cells in this area are generated as a distinct population during larval development. They project toward the medulla and connect either medulla and lamina (C2 and C3 cells) or medulla and lobula complex (T2, T3 and Y cells; Fischbach and Dittrich, 1989). Here too, not all cells are stained. Labeled and unlabeled cells are interspersed.

A further, distinctly labeled group of cells lies embedded in the medulla cell body layer just anterior to the center of the outer chiasm (Fig. 5D). The identity of these cells is unknown.

Apart from the cell populations described above, there are labeled cells scattered throughout the cell body layer of lamina and medulla (Fig. 5D). In the medulla, these cells are located mostly near the fibers of the outer chiasm or near the surface of the medulla neuropil. Similarly, in the lamina, stained cells are found associated with the proximal as well as distal surface of the neuropil and close to the fenestrated layer. Judging from their location, these cells are glia and may correspond to the marginal, epithelial and fenestrated layer glia cells of Saint Marie and Carlson (1983).

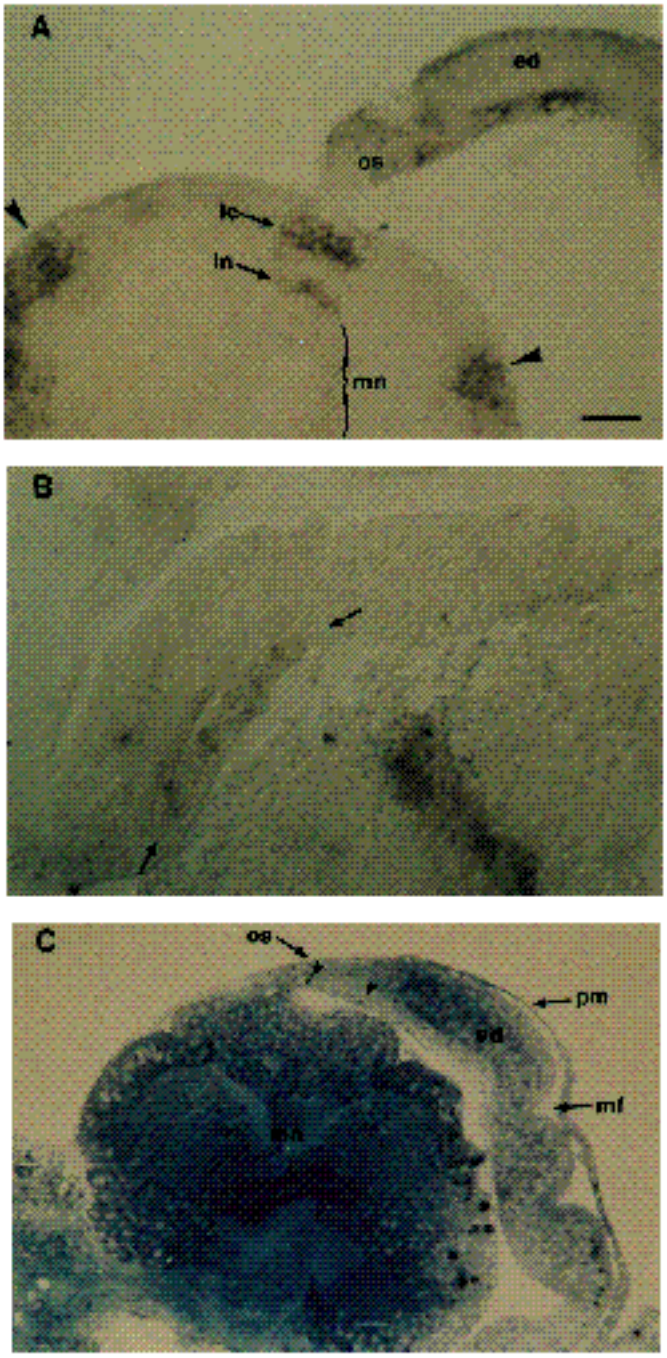

Fig. 3. T3 expression and cell proliferation in the third larval and white prepupal optic system. (A, B) In situ hybridization to 10 μm cryostat tissue sections. (A) Approximately horizontal section through a third instar larval hemisphere at the level of the optic stalk. Anterior is right, lateral is up. T3 expression is seen underneath the posterior end of the eye imaginal disc (ed) and in the optic stalk (os). In the brain, there is labeling both in the lamina cell body layer (lc) and in presumptive glia cells of the lamina neuropil (ln). Weaker expression can be seen in cells (mn) along the medulla neuropil. At the periphery of the hemisphere, there is expression in the outer anlage (arrowheads). (B) Section through a white prepupal brain. Arrows point to a strip of T3-expressing cells underneath the posterior eye imaginal disc. (C) Methylene blue/toluidine blue-counterstained autoradiograph of 2 μm section of [^{3}H]thymidine pulse-labeled third instar larva. In the eye imaginal disc, proliferation can be observed in the peripodial membrane (pm), in cells underneath the eye disc and in the optic stalk (arrowheads). Preparations A and B were photographed at the same magnification. The magnification in C is 1.6-fold less. The bar in A corresponds to 20 μm.

The main part of the lobula plate cortex is only weakly stained. Strong T3 expression can be found, however, in large cell bodies close to the medial end of the lobula plate (Figs 5C, 7A), a region in which the cell bodies of the HS and VS cells have been located (Hausen, 1981; Hengstenberg et al., 1982).

The *In(1)omb^{H31}* mutation reduces T3 expression in the embryonic brain, in the optic lobe primordia of the larva and in the adult lobula plate cortex

Defects in the nervous system of the *In(1)omb^{H31}* mutant have so far been only recognized within the adult optic lobes and in the anterior optic tract which is a major connection between the lobula and the central brain. The mutation *In(1)omb^{H31}* removes some 40 kb of optic lobe regulatory region (OLR) from the 3′ end of the *omb* transcription unit, without truncating T3 itself (Pflugfelder et al., 1992; Brunner et al., 1992).

Up to stage 13 (completion of germ band shortening), in situ hybridization did not reveal differences in the distribution of T3 between the two genotypes. With the onset of head involution (stage 14), T3 expression in the brain hemispheres begins to be reduced in *In(1)omb^{H31}* mutant embryos, compared to both younger animals of the same genotype and wild-type embryos of the same age. T3 expression in the ventral nerve chord and in the segmented part of the peripheral nervous system vanishes at the same rate as in the wild type. During the condensation of the ventral nerve chord, T3 expression in wild-type embryos disappears from all but two regions. Just prior to hatching, T3 can only be found at the anterior tip of the embryo in the antennomaxillary complex and in a region of the brain hemispheres (Figs 1J,K). In *In(1)omb^{H31}* embryos, expression in the hemispheres almost completely vanishes toward the end of embryogenesis. However, expression in the anterior, paired clusters of the antennomaxillary complex remains strong as in the wild type and thus can serve as an internal hybridization control (Fig. 6). The differences observed between the two genotypes, therefore, evidently cannot be due to differences in probe accessibility in the whole-mount preparations.

This difference in T3 expression in the brain hemispheres is also observed in third instar larvae and white prepupae (1st and 2nd instar larvae, which express T3 at a much lower level, were not inspected). As at the end of embryogenesis, the *In(1)omb^{H31}* mutant shows less T3 expression than the wild type (Fig. 2C,D). There remains, however, a low but significant level of T3 expression. This signal appears in about the same spatial domains as in the wild type. In the adult brain, there is little overall difference in T3 expression between wild type and *In(1)omb^{H31}*. A difference can, however, be observed at the medial end of the lobula plate cortex where the cell bodies of the HS and VS cells are presumed to be localized. In this area, a cluster of

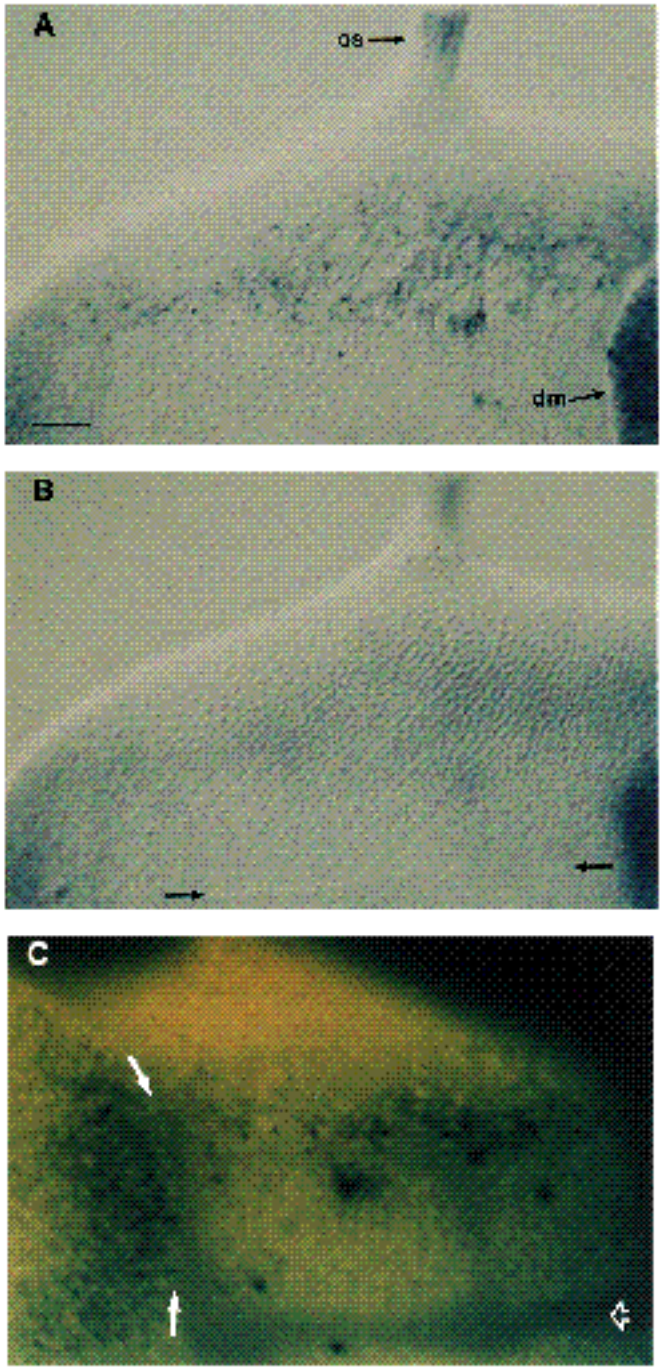

Fig. 4. T3 expression in the eye imaginal disc. In situ hybridization of whole-mount discs (A, B), counterstained with DAPI (C) to visualize nuclei. (A,B) The same disc at a deep (A) and superficial (B) focus. *omb*-transcribing cells mainly reside underneath the disc posterior to the morphogenetic furrow and in the optic stalk (os). There is also expression in the dorsal and ventral disc margin (dm). The morphogenetic furrow is indicated by two arrows. (C) DAPI counterstaining shows the signal to be associated with nuclei. The open arrow points to the morphogenetic furrow. The filled arrows indicate the rim of the disc margin. All preparations were photographed at the same magnification. The bar in A corresponds to 20 μm.

strongly T3-expressing cells can be found in the wild type, but not in *In(1)omb*^{H31} (Fig. 7).

DISCUSSION

T3 expression in the developing optic lobes

In *Drosophila*, the central nervous system (CNS) develops from neuroblast precursors, which delaminate or invaginate after gastrulation. Fate mapping studies (Hartenstein et al., 1985; Technau and Campos-Ortega, 1986) and histological analyses (Hartenstein and Campos-Ortega, 1984; Campos-Ortega and Hartenstein, 1985) led to the recognition of three coherent neurogenic regions: the large ventrolateral neurogenic region from which the neuroblasts of the ventral nerve chord delaminate, and the procephalic neurogenic region and optic lobe anlage in the head region of the embryo. Sensory mother cells of the peripheral nervous system (PNS) develop also in other regions of the embryo.

omb transcription is first observed in stage 8 embryos. The strip of early T3-expressing cells appears to lie within mitotic domain B of Foe (1989), which is characterized by a long interphase after the first 13 rapid synchronous divisions of the developing embryo. As will be discussed below, the length of the *omb* primary transcript precludes T3 expression before the 14th cell cycle and probably also in mitotic domains with short 14th interphases.

The optic lobe anlage invaginates during stage 11 (Campos-Ortega and Hartenstein, 1985). Dorsal and lateral views of stage 12 embryos show that the domain of T3 expression now resides inside the embryo. At blastoderm, the anlage contains about 35 cells and later invaginates apparently without any prior or subsequent cell division, as in first instar larvae the optic anlage is again judged to contain 30 to 40 precursor cells in each hemisphere (Hofbauer and Campos-Ortega, 1990). We estimate the number of T3-transcribing cells in the dorsolateral part of the stage 12 embryo procephalon to be about 100. Thus, T3 expression is not confined to the optic lobe anlage proper, but, on the other hand, does not encompass the entire embryonic brain, as is evident especially in later stages of embryonic development (Fig. 1K).

At the time of pupariation, the T3 expression pattern, as reconstructed from whole mounts and sectioned material, is complex. Derivatives of both the outer and inner optic anlage express T3. In addition, cells on both sides of the boundary between the primordia of the optic lobes and the central brain contain the *omb* transcript. During pupal development, the location and arrangement of cells at this boundary changes drastically. In spite of their different provenance, the T3-expressing cells from this region are probably all involved in visual pathway formation. It is known that many of the cells projecting medially from the lateral protocerebrum are associated with visual centers of the central brain (Strausfeld, 1976). Some of the T3-expressing cells in the developing lamina of the white prepupa appear to be glia.

T3 expression in the embryonic germ band

T3 expression in the germ band begins later than in the procephalic region and, unlike the latter, is transient. The lat-

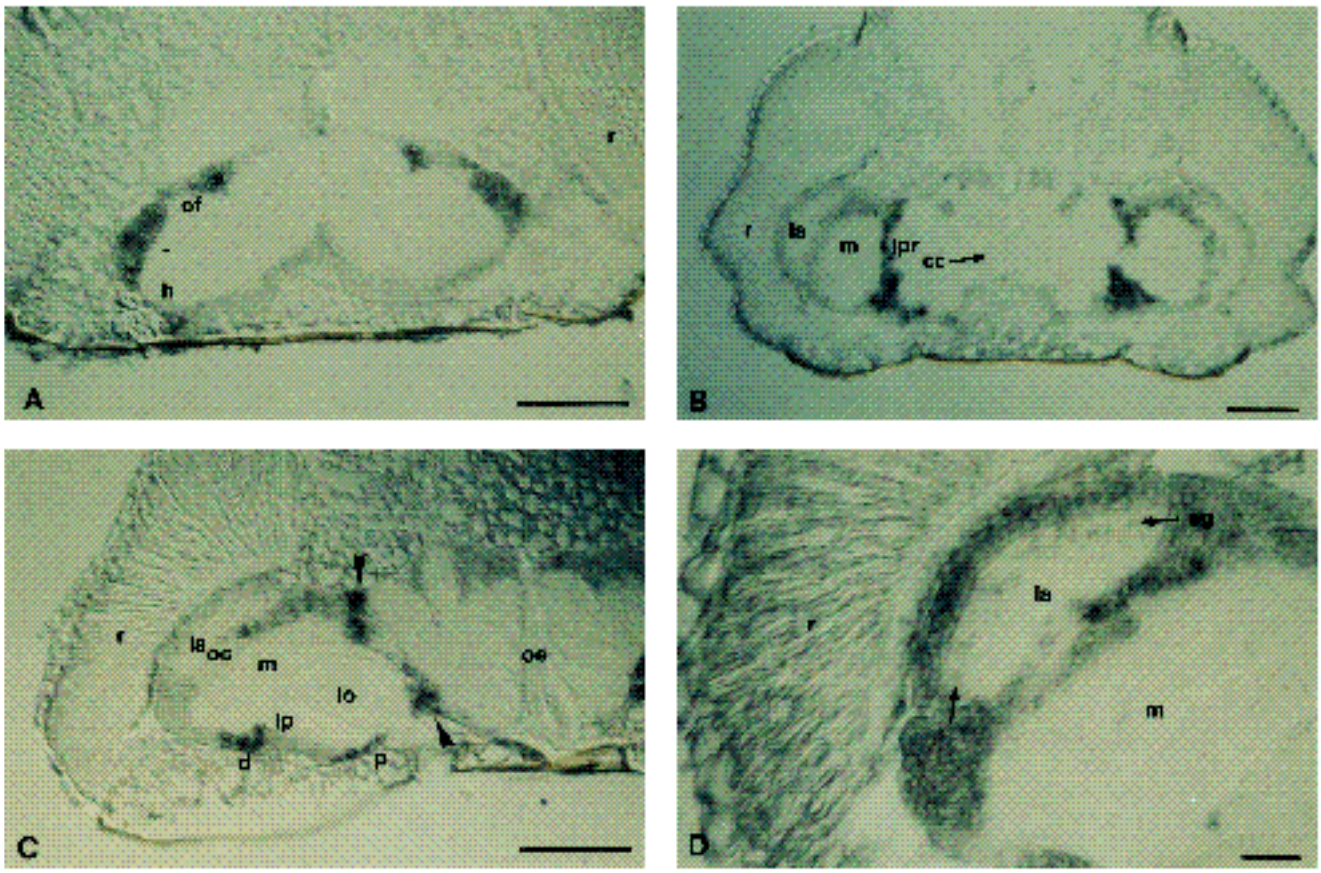

Fig. 5. T3 expression in the adult brain. In situ hybridization to 10 μm horizontal cryostat sections. The sections are oblique horizontal with the plane more ventral anteriorly. Anterior is up. (A,B) Section through the most dorsal protocerebrum (A) and at the level of the central complex (cc; B). Labeled cells are concentrated in the cell body rind around the dorsal lateral horn of the protocerebrum (h), anterior to the anterior optic foci (of), and in the area between the dorsal medulla (m) and the lateral protocerebrum (lpr). Additionally, labeled cells are scattered in the medulla cortex surrounding the medulla neuropil and between the medulla (m) and the lamina (la). r, retina. (C) Horizontal section through the visual system at the level of the oesophagus (oe). There is strong labeling at the boundary between optic lobes and the central brain (arrowheads), and proximal (p) and distal (d) to the lobula plate (lp). There are labeled cells scattered in the anterior medulla cortex between medulla and lamina neuropil, and anterior to the center of the outer chiasm (oc). lo, lobula. (D) Horizontal section through the lamina at the level of the oesophagus. The position of the stained elements (between arrows) in the distal lamina neuropil indicates staining of epithelial glia cells (eg). The bars in A to C correspond to 100 μm, in D to 20 μm.

eral pattern in stage 12/13 embryos is similar to the known PNS structure (Campos-Ortega and Hartenstein, 1985; Hartenstein, 1988; Ghysen et al., 1986; Ghysen and O'Cane, 1989). We directly demonstrated T3 expression in the PNS by doublestaining with anti-HRP antiserum which, in the PNS, binds to sensory and most accessory cells (Jan and Jan, 1982; Campos-Ortega and Hartenstein, 1985; data not shown) and by in situ hybridization of *daughterless* (*da*) embryos.

The dorsal-to-ventral expansion of the lateral T3 expression domain corresponds to the formation-pattern of PNS precursors (Bodmer et al., 1989) and to sensory organ development (Ghysen et al., 1986; Hartenstein, 1988). The last divisions of PNS precursors occur during the third mitotic wave after blastoderm formation in defined, segmentally repeated positions underneath the epidermis, coinciding with germ band retraction (Bodmer et al., 1989). At this time, sensory organ position is already specified. Even though *omb* transcript can be detected during the last divisions of PNS precursors, the omb protein is probably formed too late to still affect fate decisions of these cells (see below). Genes that have been shown to govern fate decisions during PNS formation are expressed earlier in development (e.g., *cut*: Blochlinger et al., 1990; *numb*: Uemura et al., 1989).

Shortly after the onset of germ band retraction, an increasing number of cells in the ventral nervous system begins to transcribe T3. The ventral nervous system is formed from neuroblasts that delaminate in three phases from the ventral neurogenic region during the last stages of germ band extension (stages 9 and 10; Campos-Ortega and Hartenstein, 1985). As discussed below, in rapidly dividing neuroblasts (e.g., Truman and Bate 1988; Ito and Hotta 1992), *omb* transcription is unlikely because of the large size of the T7 primary transcript. The expression of T3 in the ventral nervous system during germ band retraction therefore is probably in ganglion mother cells, neurons, or possibly glia cells (Klämbt and Goodman, 1991).

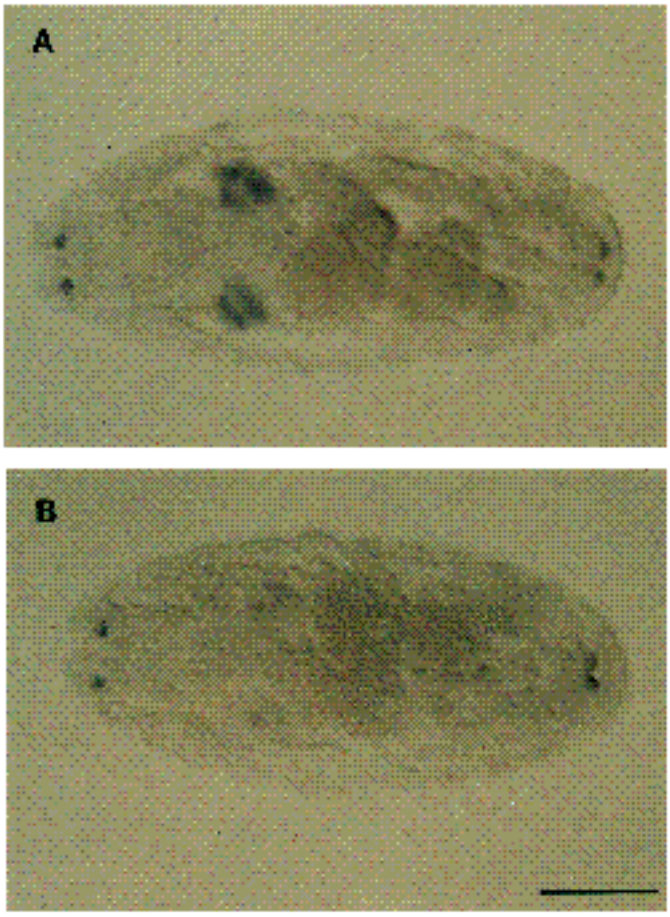

Fig. 6. Difference in T3 expression between wild type and *In(1)omb*^{H31} at the end of embryonic development. (A,B) Dorsal view of stage 17 embryo whole mount; (A) wild type, (B) *In(1)omb*^{H31}. Anterior is left. In wild-type embryos, T3 persists only in the anterior antennomaxillary complex and within the brain (cf. Fig. 1J and K). In *In(1)omb*^{H31} mutant embryos, only expression in the antennomaxillary complex remains. The posterior signal is artefactual staining of the posterior spiracles. The bar in B corresponds to 100 μm.

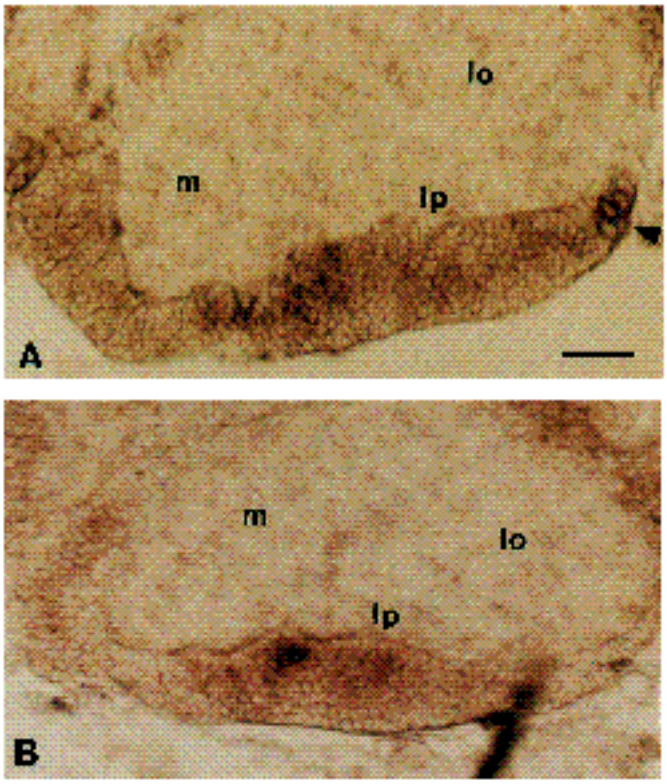

Fig. 7. Difference in T3 expression between wild type and *In(1)omb*^{H31} in the adult lobula plate cortex; (A) wild type, (B) *In(1)omb*^{H31}. Horizontal section through imaginal brains at the level of the oesophagus. Lateral is left, medial right and anterior at the top. Neuropil regions of medulla (m), lobula (lo) and lobula plate (lp) are indicated. The arrowhead in A points to a cluster of large T3-expressing cell bodies which cannot be found in the mutant (B). The bar in A corresponds to 20 μm.

omb null mutants are pupal lethals in which development to pupariation is lengthened to nearly twice the normal duration. Late mutant embryos, completely deficient in *omb*, have no apparent morphological defects in their peripheral or ventral nervous systems, as visualized by mab22C10 immunocytochemistry (data not shown). As the 6 kb *omb* transcript is not maternally transmitted, we conclude that *omb* is not required for the morphogenesis of the basic features of the peripheral and ventral nervous systems.

omb is transcribed in the adult brain

In the fly central nervous system, the larval and pupal development of entire neuropil areas with their corresponding cell body rinds is relatively well known, especially in the optic lobes (Meinertzhagen, 1973; White and Kankel, 1978; Kankel et al., 1980; Hofbauer und Campos-Ortega, 1990). It is thus possible to recognize in several brain areas a certain continuity in the *omb* transcription pattern between the white prepupa and the imago even though we do not yet know if this continuity also holds at the level of the individual cell. Thus, in the imaginal lamina, cells associated with the distal and proximal boundary of the neuropil appear to be glia cells. In the prepupa, at least part of the lamina staining appears to derive from glial T3 expression. It is thus possible that some cells express T3 throughout their lifetime suggesting a continuous requirement for *omb* function. A correlation between the prepupal and imaginal expression also seems to exist for cells along the boundary between the optic lobes and the central brain. Such a correlation does not hold for all T3-expressing cells. In the adult, a group of cell bodies between the medulla and lobula plate cortex, probably encompassing the cells C_2, C_3, T_2, T_3 and Y (Fischbach and Dittrich, 1989), show strong *omb* transcription. This cell population in the larval brain is part of the central cell plug and located near the lamina. It appears only slightly labeled in stainings of the larval brain.

Surprisingly, T3 is differently expressed in regions of the adult cortex for which there had been no hints as to structural and functional specializations. For example, in the imaginal medulla cell body rind, a group of cells anterior but not posterior to the center of the outer chiasm shows strong T3 expression.

In view of the missing HS and VS cells in *In(1)omb^{H31}* mutant flies, it was of interest to determine whether T3 is expressed in the cell bodies of these cells. This question, unfortunately, is difficult to answer unambiguously without double staining experiments. The cell bodies of the HS and VS cells are connected to their large axonal trunks by thin neurites (Hausen, 1981) and show considerable positional variation between individuals (S. Schneuwly, personal communication). Clearly, there is strong T3 expression in several large cell bodies medial to the lobula plate cortex. The lack of such cells in *In(1)omb^{H31}* suggests that the T3-expressing cells in this area indeed are the HS and VS lobula plate giant neurons.

omb is transcribed in glia cells in the developing retina-lamina complex

Part of the T3 expression pattern in the developing and mature lamina as well as in the eye imaginal disc clearly derives from glia cells. Of the six types of glia cells underneath the retina and in the lamina that have been recognized so far (Boschek, 1971; Trujillo-Cenóz and Melamed, 1973; Shaw 1977; Saint Marie and Carlson, 1983), the developmental roots of only one are known. Cagan and Ready (1989) observed the delamination of 'subretinal cells' behind the morphogenetic furrow of the eye imaginal disc, which they consider to be the precursors of the pigmented glia of the adult retina.

At the larval and prepupal stage, T3 expression is observed, among other regions, in the eye imaginal disc, the optic stalk and the developing lamina. The irregular pattern of *omb*-transcribing cells in the posterior part of the imaginal disc in which a regular ommatidial pattern has already formed, as well as the position of the transcribing nuclei on the basal side of the disc preclude an identity of these cells with any of the ommatidial cells. Their appearance among the outgrowing retinula axons makes a glial function likely. Probably, these T3-expressing cells correspond to the subretinal cells of Cagan and Ready (1989). In case the cells in the optic stalk should also derive from the eye anlage, it could be envisioned that also some of the lamina glia cells are of imaginal disc origin, as is suggested by the results of Melamed and Trujillo-Cenóz (1975). In the adult lamina, T3 appears to be expressed in marginal and epithelial glia and in one or more types of distal glia cells.

Studies in vertebrates suggest that glia cells fulfil important functions in the development of the visual system (Johnson and Gooday, 1991; Kljavin and Reh, 1991) and in behaviour (Laming, 1989). *omb* may prove a valuable tool to investigate developmental aspects of glial function also in the insect visual system.

omb is transcribed in both neuronal and glial cells. In insects and vertebrates, it has been shown that both can derive from common precursors (Edwards, 1970; Williams et al., 1991) and there is precedence for the expression of certain transcription factors in both cell types (Monuki et al., 1989; He and Rosenfeld, 1991).

Localization of T7/T3 in *omb*-transcribing cells

As emphasized in the introductory section on *omb* transcription, in situ hybridization signals observed in this study inevitably are the sum of hybridization to T3 and its 75 kb precursor T7. On northern blots, exonic probes always detect T7 in addition to the mature transcript T3 (Pflugfelder et al., 1990, 1992a). T7 processing is apparently either slow or often incomplete. Nonetheless, the staining intensities in in situ hybridizations faithfully render the developmental expression profile of T3 as it is observed by northern blot analysis. In most of the stages and tissues studied, the hybridization signal is predominantly localized in the nucleus, and there it is concentrated in one or two spots. Nuclear localization was verified in the eye imaginal disc by counterstaining with DAPI. In sections of third instar larval and adult brains, T3 appears, however, mainly localized in the cytoplasm. This is not a methodological artefact. Figs 3A and 4 show that, in both sections and whole mounts, T3/T7 is nuclearly localized in optic stalk cells. On the same section (Fig. 3A), T3 in brain cells is found in the cytoplasm. The hybridization analysis therefore suggests a differential regulation of the nucleocytoplasmic transport in different tissues and at different stages of development. The antisera that we have raised so far were not of sufficient quality to investigate the question of differential gene expression at the protein level.

A discrete subnuclear transcript localization has previously been described in both *Drosophila* and vertebrate cells (e.g., Shermoen and O'Farrell, 1991; Huang and Spector, 1991). Shermoen and O'Farrell (1991) argued that the nuclear in situ signal derives from hybridization to template-bound nascent transcripts and that mitosis leads to an abortion of these transcripts. In the *Drosophila* embryo, the RNA polymerase II transcription rate has been estimated to between 1.1 and 1.4 kb/minute (Thummel et al., 1990; Irvine et al., 1991; Shermoen and O'Farrell, 1991). To transcribe the T7 transcript of the *omb* gene completely would thus require approximately one hour. Indeed, no *omb* transcript could be detected during the first 13 short and synchronous cell cycles of the *Drosophila* embryo.

Generally, in the embryo, two dots per transcribing nucleus were observed for both *Ubx* (Shermoen and O'Farrell, 1991) and *omb*. At later stages, we found only one dot per nucleus. This argues for the somatic pairing of homologous chromosomes. Such pairing has been invoked to explain transvection phenomena that occur in the *Ubx* locus (Tartof and Henikof, 1991; Micol et al., 1990). Transvection may also operate within the *omb* locus (B.P. and G.O.P., unpubl. observations).

The altered pattern of T3 expression in *In(1)omb^{H31}* and the developmental defects in *omb* mutants

In *In(1)omb^{H31}*, the HS and VS giant fibers of the lobula plate are lacking (Heisenberg et al., 1978). Several enhancer trap lines, which selectively express a β-galactosidase marker in the HS and VS cell bodies, lack staining in these cells when crossed into the genetic background of *In(1)omb^{H31}* (S. Schneuwly and S. Kerscher, personal communication). This strongly argues that, in *In(1)omb^{H31}*, the entire HS and VS cells and not just their fibers are missing, suggesting that the mutation *In(1)omb^{H31}* either prevents the proliferation of the respective precursor cells, changes their fate to a less noticeable cell type or is required in cell maintenance.

Proliferation of optic lobe imaginal neuroblasts begins already in the first larval instar. The differentiation of ganglion cells starts in the third larval and pupal stages (Meinertzhagen, 1973; Gundersen and Larsen, 1978; White and Kankel, 1978; Hofbauer and Campos-Ortega, 1990). Geiger and Nässel (1981, 1982) succeeded in eliminating the HS and VS cells by laser ablating a small target area in 28 hour *Musca* larvae (1st or 2nd instar; West, 1951). In no case was an incomplete ablation of the set of HS and VS cells observed, suggesting that these cells derive from a single precursor cell that has not yet proliferated at the time of ablation.

Null mutations in *omb* affect optic lobe development more drastically. *l(1)omb* mutants are pupal lethals with 40% survival to the end of pupal development. At this stage, in 10% of all cases, virtually no optic lobe neuropil can be found. In the remaining cases, neuropil is discernible but severely reduced in volume and deranged in structure. The two sides of the brain express the defect independently (Pflugfelder et al., 1992a). Of the four optic lobe neuropil regions, only the lamina can generally be clearly discerned in *l(1)omb* mutants but appears fragmented into smaller patches of neuropil whose number approaches the number of lamina cartridges in a given section. This fragmentation appears to derive from a reduced cohesiveness in the lamina; a defect that might be caused by a lack of glia cells or their function. The remaining neuropil structurally has little resemblance to the wild-type optic lobes. This spectrum of phenotypes is already established at the 3rd larval instar (G. O. P., unpublished data).

The available data on the development of the giant neurons of the lobula plate point to an early cause of the $In(1)omb^{H31}$ phenotype. This study shows that T3 expression in the optic lobe anlage of the embryo is conspicuously changed in the mutant. Until mid-embryogenesis, mutant and wild-type *omb* transcription are indistinguishable. At the end of embryogenesis, T3 expression in the mutant larval brain is close to the detection limit, while it is still prominent in the wild type.

The drastic difference in T3 expression between wild type and the regulatory mutant $In(1)omb^{H31}$ with its subtle and specific neuroanatomical defects is surprising in view of the fact that null mutations in *omb* have such severe consequences for optic lobe development. The following hypothesis can reconcile these observations. We assume that *omb* expression during the first half of embryogenesis is an important factor in the commitment for optic lobe formation. In its absence (null alleles of *omb*), no optic lobes are formed in at least 10% of all animals. In $In(1)omb^{H31}$ animals, T3 expression during late embryogenesis and during optic lobe differentiation is severely reduced relative to the wild type. However, the residual level of *omb* expression appears to suffice for nearly normal differentiation of the optic lobes, leading only to the specific neuroanatomical defects described in the introduction. Our experiments cannot, however, exclude a crucial role of *omb* in HS and VS cell ontogeny also later in development.

Adult *omb* expression suggests an involvement of the gene in visual function or in the plasticity of the visual system. Long-term changes have been demonstrated in adult *Drosophila* both at the level of visual behaviour (Hirsch et al., 1990) and brain structure (reviewed in Heisenberg, 1989). The reverse genetic approach made possible by the cloning of the locus will allow to define the roles of *omb* in the various cells and during the stages of its normal expression.

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