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Role of the proneural gene, *atonal*, in formation of *Drosophila* chordotonal organs and photoreceptors

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SUMMARY

The *Drosophila* gene *atonal* encodes a basic helix-loop-helix protein similar to those encoded by the proneural genes of the *achaete–scute* complex (AS-C). The AS-C are required in the *Drosophila* PNS for the selection of neural precursors of external sense organs. We have isolated mutants of *atonal*, which reveal that this gene encodes the proneural gene for chordotonal organs and photoreceptors. In *atonal* mutants, all observable adult chordotonal organs, and almost all embryonic chordotonal organs fail to form; all adult photoreceptors are missing. For both types of sense organ, this defect is already apparent at the level of precursor formation. Therefore it is a failure in the epidermal-neural decision process i.e. a proneural defect. The failure to form photoreceptors results in atrophy of the *atonal* mutant imaginal disc, due to apoptosis and lack of stimulation of division. Lack of photoreceptors should also eliminate signalling that arises from differentiating photoreceptors and is required for morphogenetic furrow movement in the wild-type eye disc. Nevertheless, a remnant morphogenetic furrow is still observed in the *atonal* mutant disc. This presumably reflects the process of furrow initiation, which would not depend on signals from developing photoreceptors.

Key words: proneural gene, *atonal*, chordotonal organs, photoreceptor, imaginal disc, PNS, *Drosophila*

INTRODUCTION

The *Drosophila* peripheral nervous system (PNS) is composed of four major classes of sensory element: external sense organs (such as bristles), chordotonal organs (stretch receptors), multiple dendritic neurons, and photoreceptors. The stereotyped pattern of the PNS results from the pattern in which neural precursors arise from the ectoderm during development (Ghysen and Dambly-Chaudière, 1989). Neural precursor formation is an important model for the processes of cell specification, neural pattern formation, and assignment of neural subtype identity.

Precursor formation is best understood for external sense organs. In this case, the selection of neural precursors (or sense organ precursors, SOPs) from unpatterned ectoderm is a two-step process (reviewed by Ghysen and Dambly-Chaudière, 1989; Ghysen et al., 1993). First, proneural genes of the *achaete-scute* complex (AS-C) are expressed in patches of ectodermal cells (proneural clusters), and endow these cells with competence to become SOPs (Romani et al., 1989; Cubas et al., 1991; Skeath and Carroll, 1991; reviewed by Campuzano and Modolell, 1992). Lateral inhibition then ensures that only one or a few cells realise this potential, while the remainder adopt the default epidermal fate. This process involves interplay of the proneural genes with the neurogenic genes (such as *Notch* and *Delta*), which encode an inhibitory cell communication pathway (reviewed by Campos-Ortega, 1988; Artavanis-Tsakonas and Simpson, 1991; Ghysen et al., 1993). Ultimately, the chosen SOP suppresses AS-C expression and neural potential in the surrounding cells. The SOP then divides twice to give the neuron and three support cells of the external sense organ.

Two major classes of sensory neuron do not require the genes of the AS-C – chordotonal organs and photoreceptors (Dambly-Chaudière and Ghysen, 1987; Jimenez and Campos-Ortega, 1987). Several lines of evidence suggest that precursors of chordotonal organs arise in a similar way to those of external sense organs (Jarman et al., 1993). Indeed, we previously isolated a candidate chordotonal proneural gene (Jarman et al., 1993). This gene, *atonal* (*ato*), encodes a basic-helix-loop-helix (bHLH) protein that is similar to, but distinct from, the AS-C proteins. *ato* is expressed in the patches of ectodermal cells from which chordotonal precursors arise and ectopic expression results in adventitious chordotonal organs. However, the mutant phenotype of *ato* could only be inferred from the embryonic phenotype of large deficiencies. Such embryos lacked chordotonal organs, but we were unable to show conclusively whether chordotonal organ development required *ato* alone or a complex of related proneural genes. Nor could we judge the requirement for *ato* in the adult PNS, although it was noted that the gene was expressed during formation of the adult chordotonal precursors, as well as the photoreceptors (Jarman et al., 1993).

Recently, we isolated specific mutations of *ato* (Jarman et al., 1994). The elimination of *ato* function is semi-lethal. We

show here that it results in the absence of both larval and adult chordotonal organs. Surviving *ato* mutant adults also lack ommatidia and ocelli, as we have previously described (Jarman et al., 1994). Thus, *ato* is the proneural gene for both chordotonal organs and photoreceptors, the two major AS-C-independent classes of sense organ.

No photoreceptors form in the mutant eye disc, specifically the result of failure to select photoreceptor R8, which is normally the first to be formed (Jarman et al., 1994). Here we describe the detailed consequences of *ato* mutation on eye disc development. In particular, we find that the morphogenetic furrow (MF; Ready, 1989) still forms and apparently even moves. We discuss this in the light of current models of MF movement. We also examine the position of *ato* with respect to other genes involved in early patterning events in the eye disc.

MATERIALS AND METHODS

Fly stocks

ato^{1} is described by Jarman et al. (1994). *ato*^{3} was isolated in an EMS screen for new *ato* alleles. *dpp-lacZ* is described by Blackman et al. (1991) and was obtained from U. Heberlein. *Df(3R)p*^{13} *eya*^{1}, *so*^{1}, *gl*^{1} are from the Bloomington stock center. *Elp* was obtained from E. Grell.

Immunohistochemistry

Antibody staining and in situ hybridization are described by Jarman et al. (1993). For the double labelling, a rabbit anti-ato serum and mouse anti-β-galactosidase (Promega) were used. For the anti-ato serum, ato protein was prepared as follows. The ato reading frame was fused to the His_6 tag of the pRSET bacterial expression vector (InVitrogen). Using this construct, protein was expressed and isolated from bacteria as described (Jarman et al., 1993). This purified protein was used to immunize two rabbits. Serum was used preabsorbed against embryos at a final dilution of 1:5000. Anti-h antibody was a mouse monoclonal, provided by N. Brown. For DAB stainings, avidin-biotin amplification was used (Vector Labs). For immunofluorescence, DTAF- and rhodamine-conjugated secondary antibodies were used (Jackson Immunoresearch).

Acridine orange staining is described by Bonini et al. (1993). BrdU incorporation was essentially as described by Baker et al. (1992), except that incubations for incorporation were generally for 2 hours. For staining the MF, phalloidin-rhodamine was added to the penultimate PBS/Triton X-100 wash after antibody staining (1:50), and a DTAF-conjugated secondary antibody was used to detect the anti-ato antibody.

Microscopic analysis of chordotonal organs

For the antennal sections, fly heads were fixed in 2% glutaraldehyde and 4% formaldehyde in 0.1 M sodium phosphate (pH 7.2) overnight. The tissue was then dehydrated in ethanol, infiltrated with propylene oxide, embedded in epoxy resin (Polysciences), and polymerized in flat embedding molds. Sections were cut in the desired plane at a thickness of 2-3 μm and stained with toluidine blue. For other adult chordotonal organs and for larval chordotonal organs, specimens were dissected and fixed in 4% formaldehyde in sodium phosphate, then mounted in glycerol, and viewed with Nomarski optics without sectioning or staining. To detect the embryonic PNS, the antibody mAb22C10 was used (Zipursky et al., 1984).

RESULTS

ato mutations

ato^{1} was isolated from an EMS screen as described by Jarman et al. (1994). Previously we have shown that deficiencies that uncover *ato* result in embryos that lack almost all chordotonal neurons (Jarman et al., 1993). When stained with mAb22C10 to detect all sensory neurons, both homozygous *ato*^{1} embryos and hemizygous *ato*^{1}*/Df(3R)p*^{13} embryos also show this phenotype (see below). Moreover, we could detect no difference in the extent of chordotonal neuron loss from that of the deficiencies; we deduce that *ato*^{1} is likely to be a genetic null. *ato*^{1} contains missense mutations (Jarman et al., 1994), particularly one in a highly conserved region of the basic domain. Therefore, the protein that is expressed from this allele (see below) is likely to be nonfunctional. Similar mutant phenotypes have been observed for a second allele, *ato*^{3}, which also appears to be a genetic null since no protein is detectable by anti-ato antibodies.

Chordotonal organ phenotype of *ato* mutants

All adult and almost all larval chordotonal organs are absent

In homozygous *ato*^{1} embryos and *ato*^{1}*/Df(3R)p*^{13} embryos, all chordotonal organs of the thorax and abdomen are absent except for one or occasionally two neurons that sometimes remain of the abdominal lateral pentascolopidial organ (lch5) (about 25% of segments) (Fig. 1A,B). There is, however, none of the disorganization of the remaining PNS that was seen in embryos containing the synthetic deficiencies (Jarman et al., 1993). This allows us to confirm that the neurons of external sense organs are unaffected, and that a few specific multiple dendritic neurons are also reproducibly absent (vpda and v′td2).

Despite the absence of embryonic sense organs, *ato* mutant larvae can hatch and survive to adulthood. We have dissected *ato*^{1}*/Df(3R)p*^{13} larvae to examine the morphology of the remaining chordotonal neuron of the lateral abdominal organ. In those larvae that survive to third instar, we found that the one or two scolopidia are visible in many abdominal segments (Fig. 1C,D), but their morphology often appears abnormal.

We have analysed the PNS phenotype of *ato*^{1}*/Df(3R)p*^{13} adults. On examination of dissected adult flies, we failed to find any of the chordotonal organs normally associated with the femur, wing base, or ventral abdomen. We also scored a large array of chordotonal scolopidia, Johnston's Organ, in stained sections of the second antennal segment (McIver, 1985). In sections from wild-type flies, some of the many individual scolopidia of this extensive array are apparent (Fig. 1E). In the mutant, sections of this segment are completely devoid of these structures (Fig. 1F). External sense organs are not affected by the mutation. *ato*^{1} homozygous adults have an identical phenotype (not shown).

ato mutant flies are very clumsy on their feet, and they attempt to fly only with extreme reluctance. Other eyeless mutants have few such problems, suggesting these difficulties are a consequence of chordotonal organ loss. Indeed, most chordotonal organs are thought to be proprioceptors of body position (McIver, 1985). Nevertheless, *ato* mutant flies survive. This is consistent with the experimental finding that removal of individual proprioceptors, such as the stick insect femoral chordotonal organ, has very little effect on walking (Bässler, 1973, 1977) [but a strong effect on muscle resistance reflex while stationary (Usherwood et al., 1968)]. This points

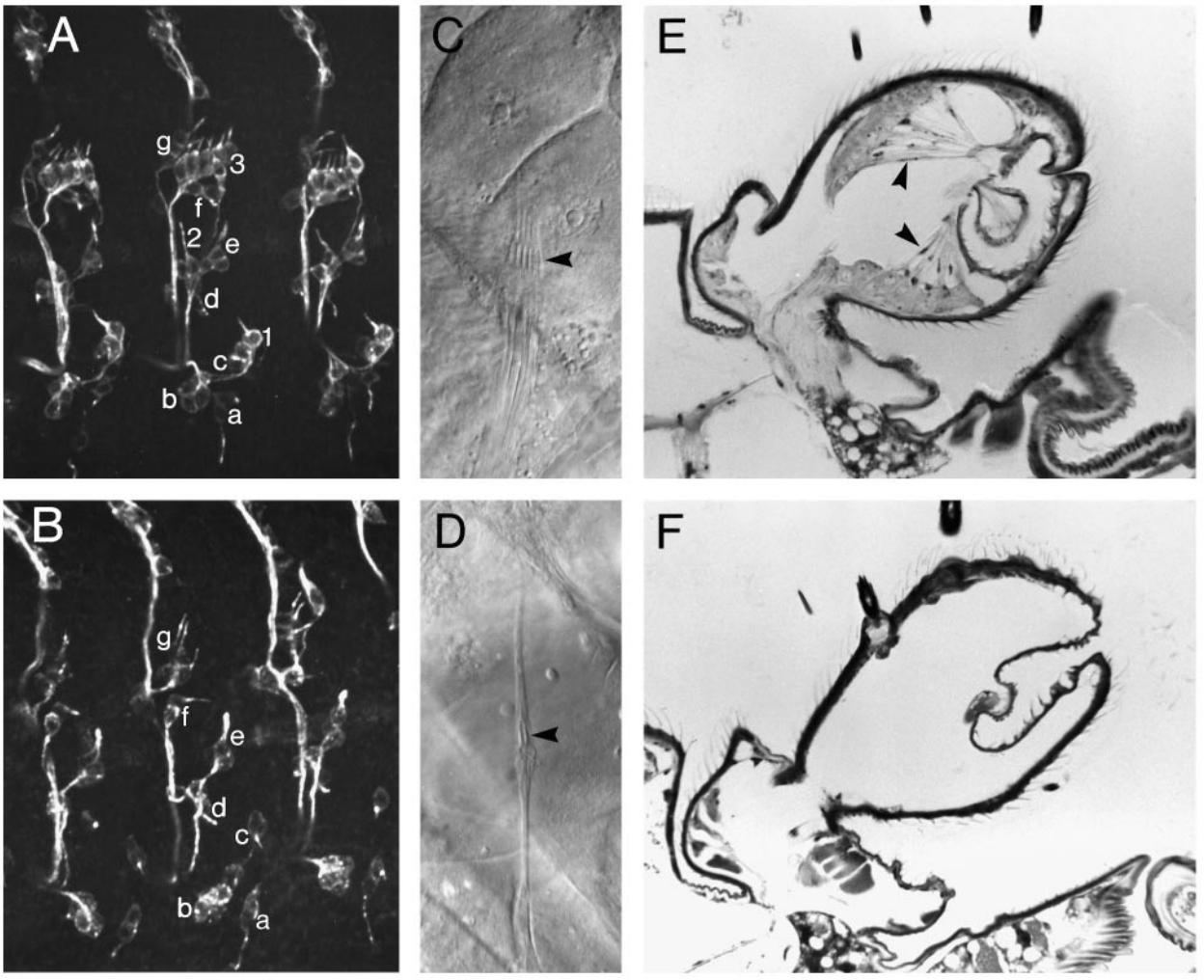

Fig. 1. Chordotonal organ phenotype in *ato*^{1}. (A,B) Portions of late stage embryos stained with mAb22C10 to detect sensory neurons. The ventral and lateral portions of three abdominal segments are shown. (A) Wildtype. (B) *ato*^{1}*/Df(3R)p*^{13}. Letters indicate groups of neurons that are unaffected in the mutant (external sense organ neurons [es] and most multiple dendritic neurons [md]); numbers indicate groups of neurons absent in the mutant (chordotonal organ neurons [ch] and some multiple dendritic neurons). a = vesA,B; b = vmd5; c = vesC; d = v′esA,B, v′ada; e = v′es2, v′pda; f = lesA, ldaA; g = lesB,C, ldaB. 1 = vchA,B, vpda; 2 = v′td2; **3** = v′ch1, lch5 (Ghysen et al., 1986). (C,D) Lateral chordotonal organ (lch5) from third instar larvae. Unstained portions of an abdominal segment viewed with Nomarski optics. (C) Wild type, with arrowhead indicating the five aligned scolopale structures associated with the five neurons. (D) *ato*^{1}*/Df(3R)p*^{13}. The single remaining scolopale is indicated. (E,F) Sections of the adult second antennal segment stained with toluidine blue. (E) Wild-type, with arrowheads indicating some of the scolopales of Johnston's Organ. The neurons of these are seen to the left of and below the scolopales. (F) *ato*^{1}*/Df(3R)p*^{13}, Johnston's Organ is absent.

to redundancy in chordotonal proprioceptive functions with certain external sense organs that are also proprioceptors of limb position (hair plates) and of cuticular stress/muscle loading (sensilla campaniformia).

Failure in chordotonal precursor selection

If *ato* is a proneural gene, we should expect that the loss of chordotonal organs results from a defect of precursor formation. To test this, we stained mutant imaginal discs with antibodies against asense (ase), which detect all SOPs (Brand et al., 1993). All previously located chordotonal organ precursors (Jarman et al., 1993) were clearly and specifically missing in the leg, wing, and antennal discs (Fig. 2A,B and data not shown).

We asked how the expression of *ato* was altered in the mutant. In wild-type imaginal discs, *ato* mRNA expression correlates well with regions from which precursors of chordotonal organs and photoreceptors are chosen. For chordotonal organs, *ato* mRNA is expressed in proneural clusters and later more strongly in the chordotonal organ precursors that arise from these clusters (Fig. 2C,D) (see Jarman et al., 1993). Mutant mRNA and protein are still produced in *ato*^{1} imaginal discs. In *ato*^{1}*/Df(3R)p*^{13} imaginal discs, *ato*^{1} mRNA accumulates in chordotonal proneural clusters as in wild-type discs (Fig. 2F), but there is no subsequent restriction to the individual cells that should become precursors (Fig. 2E). This is also true for ato^{1} protein expression (not shown). This suggests that the refinement of *ato* expression requires ato protein itself (autoregulation) and that the *ato*^{1} mutant does not synthesize functional protein.

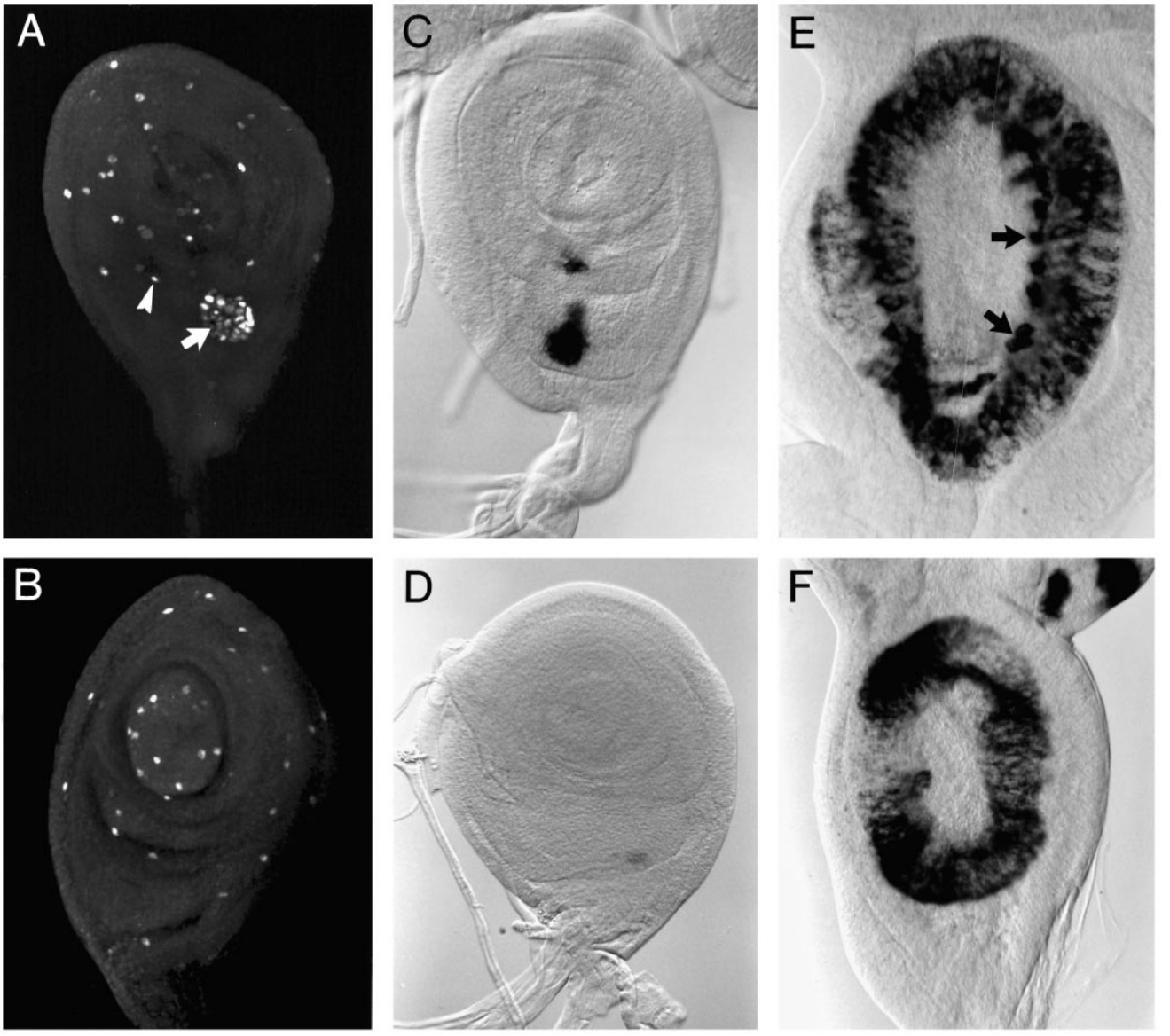

Fig. 2. (A,B) Mesothoracic leg discs stained with antibodies to asense to detect SOPs. (A) Wild type. The arrowhead indicates one of the many solitary external sense organ precursors; the arrow indicates the cluster of precursors that will form the femoral chordotonal organ. (B) *ato*^{1}*/Df(3R)p*^{13}. The chordotonal organ precursors are missing, while external sense organ precursors are unaffected. (C-F) *ato* mRNA expression in wild-type and mutant discs detected by in situ hybridization. (C) Wild-type mesothoracic leg disc, showing two regions of expression. The large area corresponds to the group of precursors of the femoral chordotonal organ (see Fig. 2A); proneural cluster expression has almost ended in this disc at this stage of development. (D) *ato*^{1}*/Df(3R)p*^{13} mesothoracic leg disc. Very faint staining remains in the ectodermal proneural cluster; no staining in potential SOPs is observed. (E) Antennal portion of a wild-type eye-antennal disc. The ectodermal expression corresponds to part of the proneural cluster for the chordotonal organ array of the second antennal segment (Johnston's Organ, Fig. 1E). Arrows point to stronger expression in SOPs that have delaminated from the proneural cluster. (F) *ato*^{1}*/Df(3R)p*^{13} antennal disc. Strong expression is still seen in the proneural cluster, but there is no SOP expression.

ato expression in the eye disc relative to other patterning events

In the eye disc, ommatidial clusters of photoreceptors appear in the wake of the morphogenetic furrow (MF) as it traverses the disc from posterior to anterior (Tomlinson, 1985; Tomlinson and Ready, 1987; Ready, 1989). Within each cluster, the eight photoreceptors appear in a well-defined sequence, starting with R8 and ending with R7 (Tomlinson and Ready, 1987); *ato* is required principly for the selection of photoreceptor R8 (Jarman et al., 1994). But the process of R8 selection, and its link to ommatidial spacing, is complex and incompletely known. To understand *ato*'s role better, we examined its expression relative to other patterning events at the MF.

In the wild-type eye disc, expression of *ato* mRNA and protein begin in a stripe spanning the disc on the anterior edge of the MF (Jarman et al., 1994; Fig. 3A). The RNA expression appears to extend a few cells more anteriorly than the protein

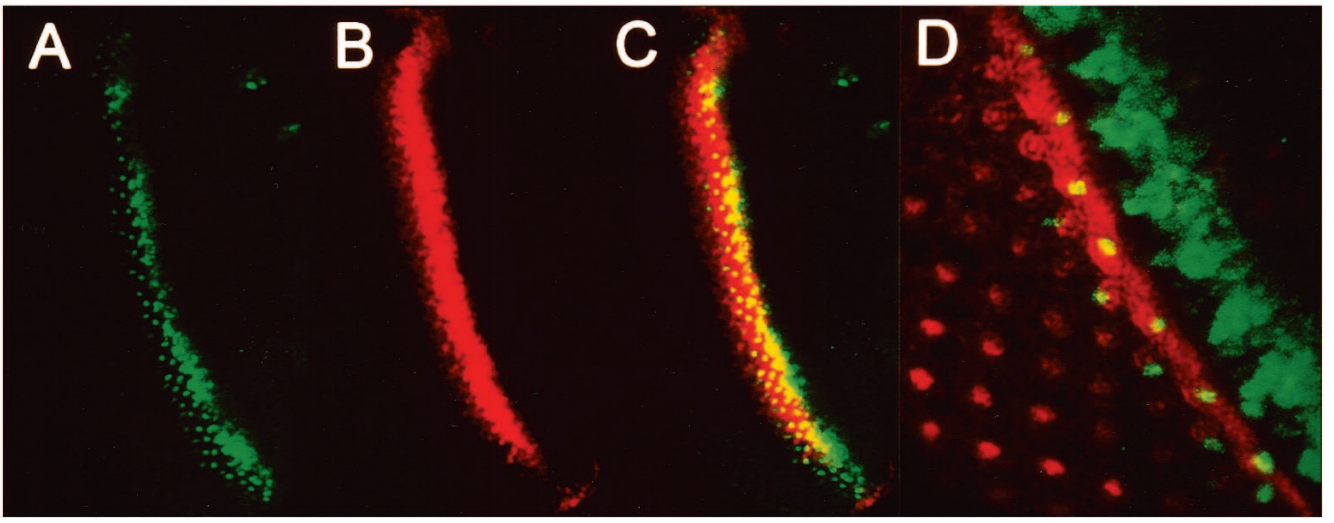

Fig. 3. *ato* expression in wild-type eye disc. (A-C) Eye portion of eye-antennal disc from third instar larva that contains a *dpp-lacZ* insert, stained with antibodies to ato (green) and β-galactosidase (red). (A) ato expression begins in a stripe. Posterior to (left of) this, expression is confined firstly to regularly spaced groups of cells (intermediate groups), and then to rows of isolated cells, the precursors of the R8 photoreceptor. Expression is also seen in two other sites (top right) that correspond to areas in which ocelli precursors form. (B) In this *dpp-lacZ* line, β-galactosidase is expressed in a stripe marking the MF. (C) ato expression begins about 2 cell diameters before the MF, and refinement to R8 precursors occurs in the middle of the MF. (D) Eye disc stained with antibodies to ato (green) and with phalloidin (red) to detect cell apical shape changes. ato becomes refined to R8 at about the stage that the rosettes become detectable by phalloidin.

and is stronger in this stripe. Slightly later, stronger protein expression is seen in small groups of cells on the posterior edge of this band (referred to here as the intermediate groups). This expression then becomes abruptly confined to isolated, regularly spaced columns of cells, the precursors of R8, where it persists for about three columns. We have positioned this process relative to the expression of *decapentaplegic* (*dpp*) as a marker of the MF. *dpp* is expressed in the deepest part of the MF (Heberlein et al., 1993; Ma et al., 1993), a pattern that is faithfully replicated by a *dpp-lacZ* fusion gene (Blackman et al., 1991) (Fig. 3B). Double labelling with anti-ato and anti-β-galactosidase antibodies in a line containing the *dpp-lacZ* fusion gene shows that ato protein expression begins just anterior (about 2-3 cells diameters) of β-galactosidase. Refinement to the intermediate clusters takes place at the anterior edge of the β-galactosidase expression, and confinement to future R8 cells occurs in the deepest part of the furrow (Fig. 3B,C).

This time course suggests that patterning of *ato* expression precedes the patterning events revealed by histological techniques such as cobalt sulphide, lead sulphide, or phalloidin staining (Tomlinson and Ready, 1987; Baker et al., 1990; Wolff and Ready, 1991b). In the wild-type eye disc, the cells in the MF show strong apical phalloidin staining, coinciding with the apical constriction and shortening of the cells (Fig. 3D). As the MF traverses the undifferentiated ectoderm of the eye disc, 'rosettes' of about 10-20 cells organise on its posterior edge, forming a precise lattice pattern that prefigures the final spacing of ommatidia (Wolff and Ready, 1991b) (Fig. 3D). Two columns later, the rosettes become refined to preclusters of five cells, containing the precursors of photoreceptors R8 and R2-5. Neural differentiation of R8 begins shortly after this. Upon double staining with phalloidin and anti-ato antibodies, we find that the ato intermediate groups are seen within the phalloidin furrow stripe, apparently preceding the emergence of phalloidin-detectable organization on the posterior edge of the MF (Fig. 3D). Shortly after, ato becomes restricted to R8 precursors just as the rosettes bud off from the MF (column 0-1, Tomlinson and Ready, 1987). This time course of patterned ato expression is very similar to that described for *scabrous* (*sca*), since sca expression in groups of cells and its restriction to R8 precursors also precedes histological patterning (Baker and Zitron, 1995). Comparison of the data strongly suggests that sca expression coincides with the later patterned component of ato expression (i.e. all but the initial stripe). Therefore, the restriction of ato to the intermediate groups is, with *sca*, the earliest patterning event yet identified, preceding that revealed by histological techniques. Similarly, ato and sca expression identify the R8 cell before it is recognisable by other markers or histological staining.

Expression of ato in eye mutants

We have examined the changes in ato expression in some mutants of eye development. Flies mutant for *eyes absent* (*eya*) or *sine oculis* (*so*) are completely eyeless. Both genes are required in the eye disc prior to the patterning events associated with the MF (Bonini et al., 1993; Cheyette et al., 1994). The eye discs from these mutants show strong atrophy, and there is neither furrow nor photoreceptor formation. Consistent with this, *ato* is not expressed in eye discs from *so* or *eya* larvae, except in the region of the ocelli precursors (Fig. 4A-C).

The *Ellipse* (*Elp*) mutant is characterized by a large reduction in the number of ommatidia formed, although those formed are mostly of normal construction (Baker and Rubin, 1992). Expression of *ato* is altered in *Elp* eye discs in two ways (Fig. 5A,C). Firstly, very few ato-expressing R8 cells appear, as expected from the reduced number of ommatidia. Secondly,

although ato is initially expressed in a stripe anterior to the MF, there is no apparent refinement to the intermediate groups; instead, expression terminates abruptly in the furrow in all but the R8 precursors. This is reminiscent of the effect of *Elp* on sca expression – the R8 cells express sca, but there is no prior expression in the cell groups that are seen in the wild type (Baker and Rubin, 1992).

In *glass* (*gl*) mutants, patterning and neural differentiation begins as normal in the eye disc, but the cells never express photoreceptor-specific markers and eventually die (Moses et al., 1989). In these eye discs, we find that *ato* expression is normal up to and including restriction to R8 (Fig. 5B). But subsequently, the R8-specific expression remains on longer (for at least 4-6 columns instead of the normal 3). This may indicate that negative feedback from *gl* normally helps to shut down ato expression.

Photoreceptors are absent, but a partial morphogenetic furrow remains in mutant eye discs

We previously showed that no photoreceptors are formed in the *ato* mutant eye disc (Jarman et al., 1994). Now we describe the consequences of this for eye disc development. While photoreceptor formation depends on the furrow, the impetus for furrow progression across the undifferentiated disc comes from feedback from differentiating photoreceptors behind the furrow (Ma et al., 1993; Heberlein et al., 1993). Thus, *hedgehog* (*hh*) provides a signal from newly formed photoreceptors that stimulates *dpp* as the primary determinant of MF movement. In 'furrow stop' mutants, these signals are absent, and the furrow arrests.

In *ato* mutants, no photoreceptors are formed. Nevertheless, we find that the MF is not completely absent from the mutant imaginal disc. A shallow crease that appears to correspond to a remnant MF persists across the posterior of the disc. Phalloidin (Fig. 6A-C) or cobalt sulphide (not shown) staining consistently show this cellular apical shortening, but the heavily stained apical constrictions characteristic of the wild-type MF are not seen.

The conclusion that this crease is the MF comes from observation of certain furrow-associated markers. Firstly, *dpp*, the primary agent for furrow movement, is expressed in a weak, broadly interrupted stripe extending down the crease (mRNA in Fig. 7A,B), a pattern that strongly resembles its expression in furrow-stop mutants (Heberlein et al., 1993). Secondly, mutant *ato* RNA and protein are still expressed in a stripe on the anterior edge

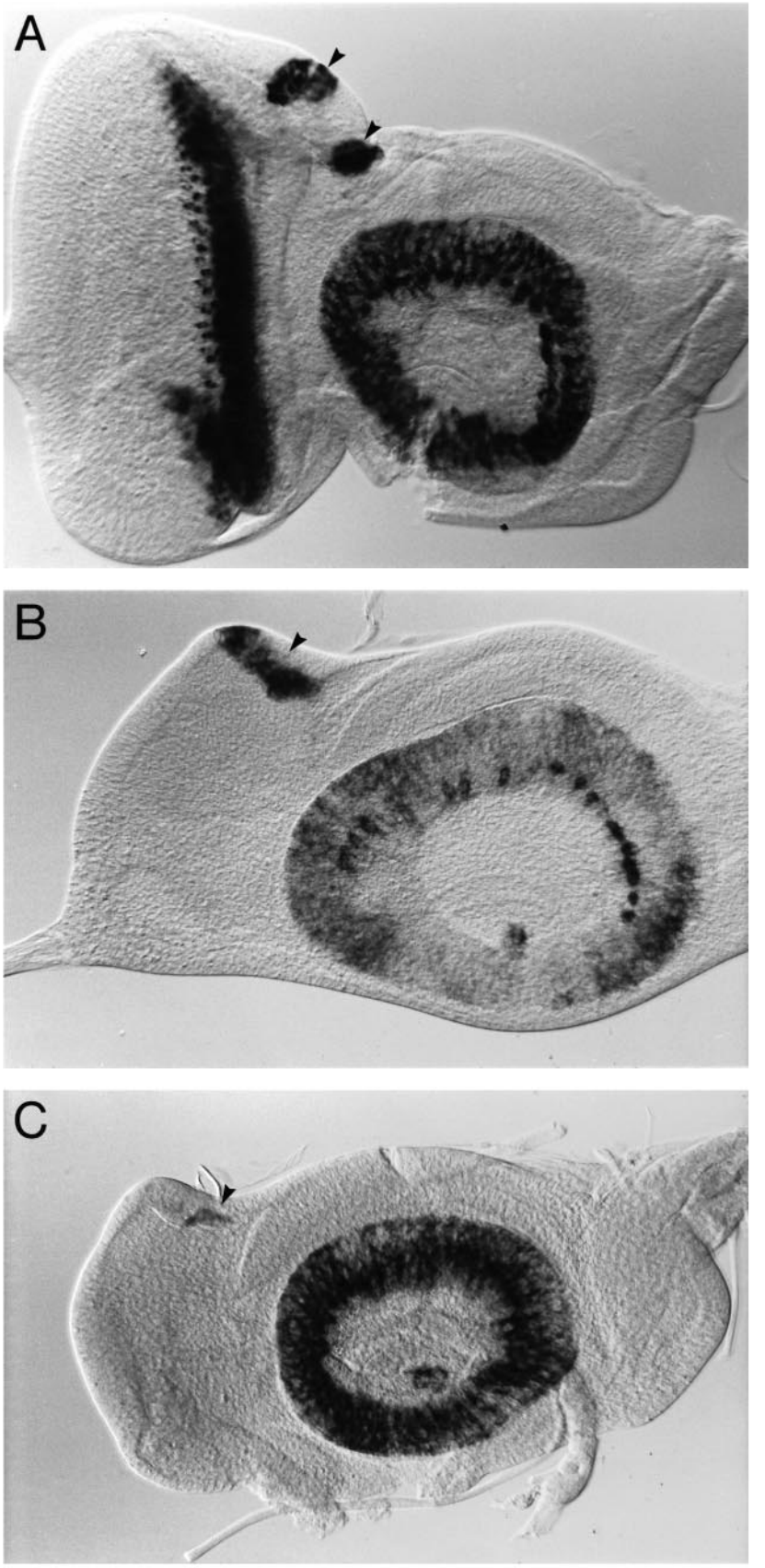

Fig. 4. Effect of eye mutations on *ato* expression. Eye-antennal discs from third instar larvae hybridized to detect *ato* mRNA. (A) Wildtype. (B) *eya* mutant. No *ato* expression is seen in the eye disc, although expression remains in the ocelli and antennal regions (arrowheads). (C) *so* mutant. Similar effect to *eya*.

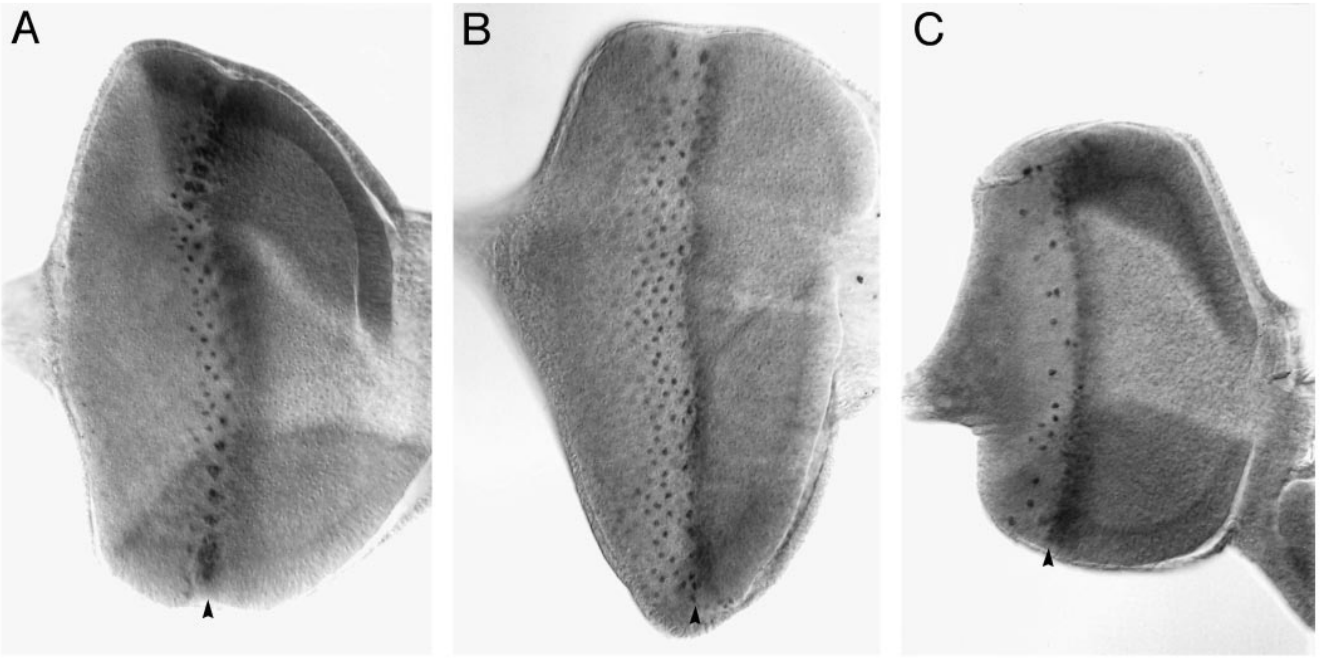

Fig. 5. Effect of eye mutations on ato expression. Eye-antennal discs from third instar larvae stained with anti-ato antibodies. (A) Wild type. (B) *gl* mutant. The dynamics of ato expression proceed as normal, except that expression in R8 precursors perdures for 4-5 columns instead of the usual 3 columns. (C) *Elp* mutant. The stripe of expression is still seen, but refinement to R8 precursors is sporadic, and no intermediate groups are seen. Arrowhead marks the posterior edge of the MF.

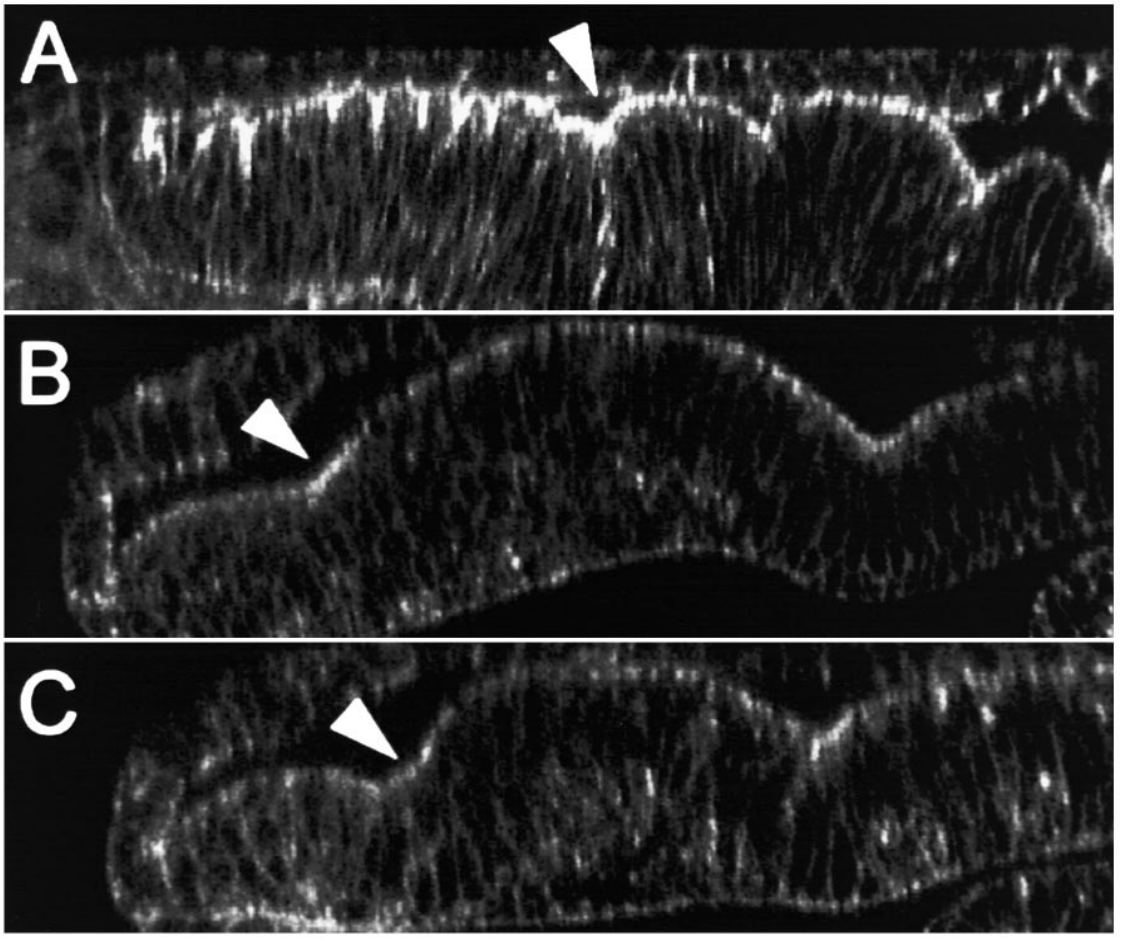

Fig. 6. The MF in *ato* mutant discs. Confocal cross-sections of eye discs stained with phalloidin. (A) Wild type. The MF is revealed in cross-section by cell shortening and strong apical staining (arrowhead). Differentiating photoreceptor clusters are also detected by periodic strong staining posterior (left) of the MF. (B,C) Two examples of $ato^{1}/Df(3R)p^{13}$ mutant disc in cross-section. An indentation is reproducibly seen in the posterior of the disc (arrowhead). We propose that this represents the MF. This is weakly stained, and no photoreceptor clusters are seen. A second indentation in the anterior of the disc (right) marks the border between the eye and antennal portions of the disc.

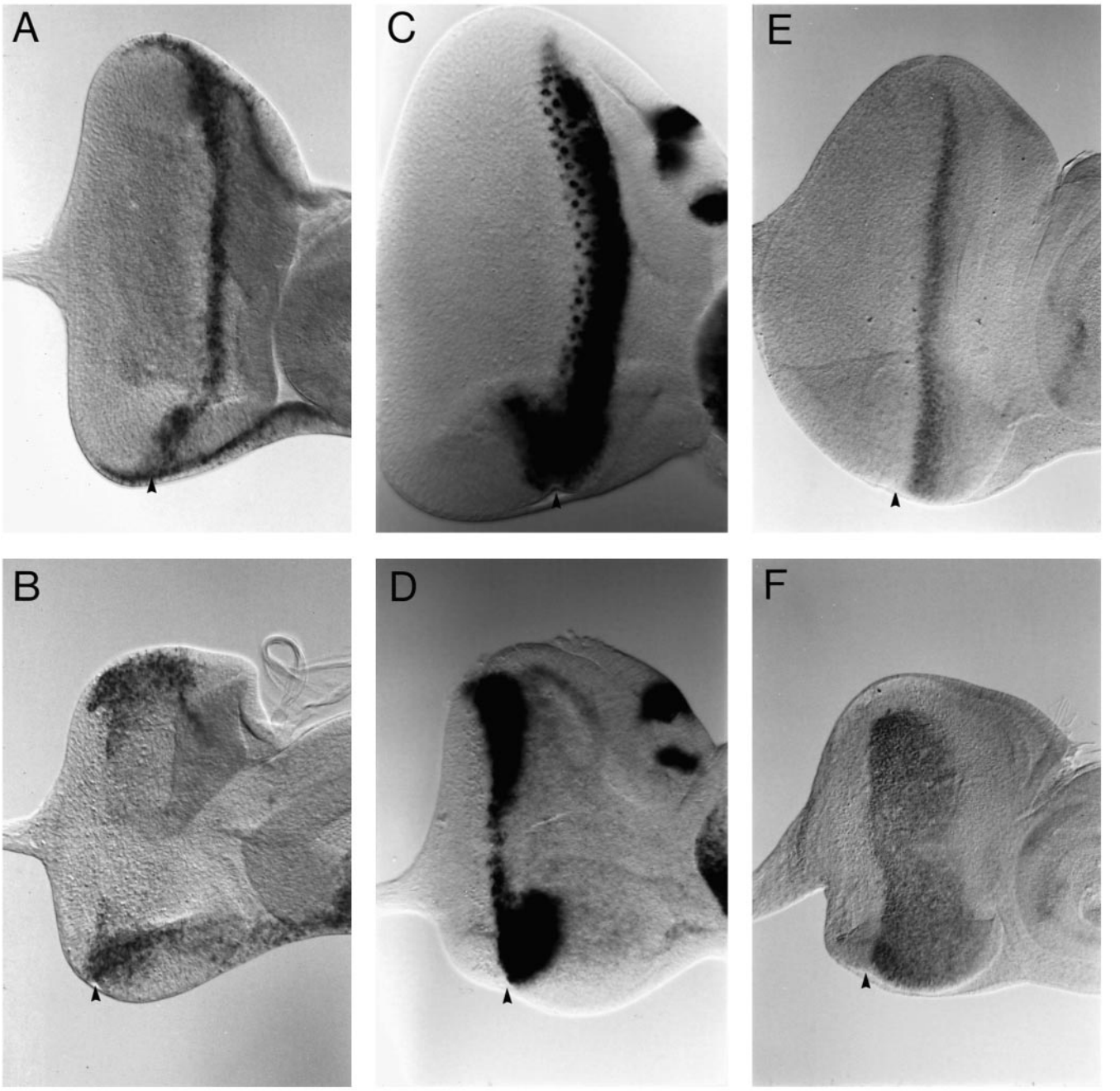

Fig. 7. Molecular attributes of the MF in the mutant. (A,B) Eye discs in which *dpp* mRNA is detected by in situ hybridization. (A) Wild-type disc. The stripe marks the MF. (B) $ato^{1}/Df(3R)p^{13}$. An interrupted stripe of weak *dpp* expression is associated with the indentation. (C,D) Eye discs in which *ato* mRNA is detected by in situ hybridization. (C) Wildtype. (D) $ato^{1}/Df(3R)p^{13}$. The stripe of expression anterior to the MF remains, although it is weaker in the centre. (E,F) Detection of *h* expression using anti-h antibodies. (E) Wild type. h is expressed in a stripe just anterior of the ato stripe. (F) $ato^{1}/Df(3R)p^{13}$. The stripe of h expression is still present, but much broader than normal. Arrowhead marks the posterior edge of the MF.

of the crease, although there is no refinement to intermediate groups or to R8 precursors (Fig. 7C,D). Expression is usually weaker in the middle of the disc (particularly for the protein, not shown). Moreover, expression of mRNA, but not protein, often smears into the anterior portion. This effect is even more pronounced in the expression of *hairy* (*h*). *h* is normally expressed in a stripe just anteriorly to *ato* (Brown et al., 1991; Fig. 7E). In the mutant disc this stripe is still seen anterior to the crease, although it extends much farther anteriorly (Fig. 7F). Therefore, this crease has molecular attributes of the MF, expressed in appropriate positions. We conclude that it is the remains of a MF that forms despite the absence of photoreceptors. Moreover, that there are cells posterior to this remnant MF suggests that it must have moved at least initially. On the other hand, the remnant stripe of *dpp* suggests that the MF has already arrested at the stage of development examined.

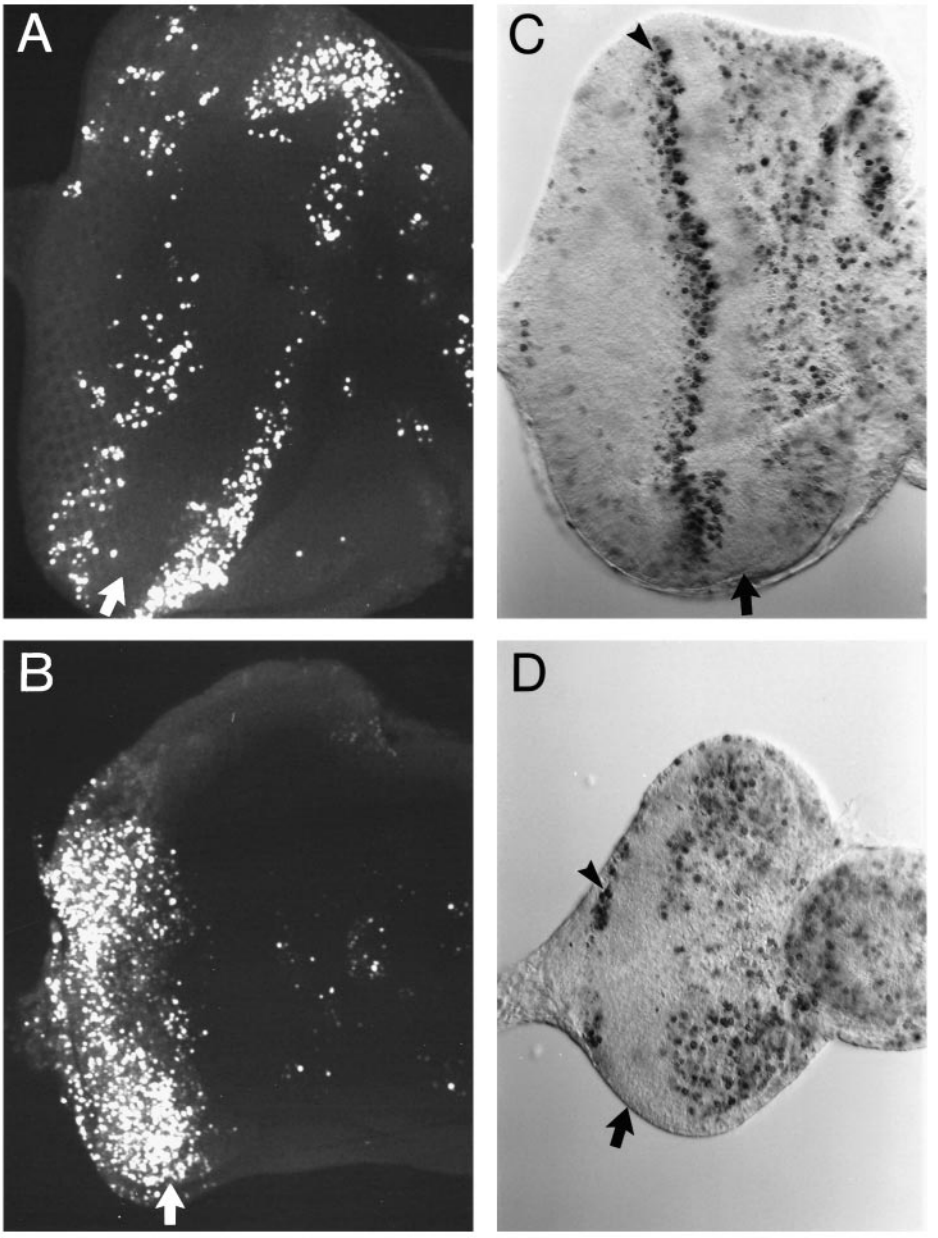

Fig. 8. Cell death and DNA synthesis in the mutant eye disc. (A,B) Acridine orange staining to detect apoptosis. (A) Wild type. Acridine orange detects cell death mostly in two phases on either side of the MF (as reported by Wolff and Ready, 1991a). (B) *ato*^{1}/*Df(3R)p*^{13}. Extensive cell death is seen in and posterior to the MF. (C,D) DNA synthesis detected by BrdU incorporation followed by staining with antibodies to BrdU. (C) Wild type. Asynchronous replication in the anterior of the disc becomes synchronized before the MF, is arrested in G1 within the MF (arrow), and then there is a second synchronized wave of synthesis in all cells outside the photoreceptor preclusters after passage of the MF (arrowhead). (D) *ato*^{1}/*Df(3R)p*^{13}. DNA synthesis occurs in the anterior of the disc, and there is apparent arrest within the remnant MF (arrow). Posterior to this, the second wave of weak synthesis is observed in this example (arrowhead), but not in all cases.

The eye discs of *ato*^{1}/*Df(3R)p*^{13} third instar larvae are reduced in size. Atrophy is pronounced in the posterior. This could be due to cell death and/or lack of cell division. Extensive refractile blebbing posterior to the MF indeed suggests apoptosis (Bonini et al., 1993). Staining with acridine orange, which specifically labels cells undergoing programmed cell death, confirms that a massive amount of death is occurring (Fig. 8A,B). Unlike the cell death reported in *eya* mutants; (Bonini et al., 1993), but similar to that of *Elp* (Baker and Rubin, 1992), the death in *ato* seems to be concentrated within and posteriorly to the MF. Thus, the three cell fates we observe in the *ato* eye are those thought (in the wild-type disc) to be the default or ground state fates for cells not chosen to be photoreceptors: pigment cell, interommatidial bristle, and cell death (Cagan and Ready, 1989; Wolff and Ready, 1991a; Baker and Rubin, 1992). The difference is that we see extensive cell death in the larval eye disc posterior to the morphogenetic furrow rather than in the late pupa.

We have also examined DNA replication by BrdU incorporation (Baker and Rubin, 1992). In the wild-type eye disc (Fig. 8C), there is unsynchronized BrdU incorporation ahead of the MF, but cells within the furrow are in G1 arrest and do not incorporate BrdU; posteriorly, there is a second round of DNA replication and mitosis of all cells outside of the photoreceptor preclusters (Woff and Ready, 1991b; Thomas et al., 1994). In the mutant eye disc, the cell cycle arrest ahead of the MF is still seen, but events behind the furrow are more variable (Fig. 8D). Often, no reinitiation of BrdU incorporation is seen, but occasionally there is a broadly interrupted stripe of replicating cells (as in the figure). We propose that this reflects the advanced stage of furrow arrest in the third instar eye disc, such that the second round of replication indeed occurs in the mutant but only as long as the furrow is moving. It has been postu-

lated that the progression into S phase is stimulated by photoreceptor preclusters (Wolff and Ready, 1991b), but studies on *Elp* suggest that only mitosis, and not DNA replication, require the presence of photoreceptors (Baker and Rubin, 1992). Our results appear to confirm this.

DISCUSSION

Our results demonstrate that a proneural stage is a general feature of early neurogenesis in the *Drosophila* PNS. For external sense organs, the expression of proneural genes of the AS-C create neurally competent groups of ectodermal cells from which SOPs are selected. We have shown that chordotonal organs (this paper) and photoreceptors (Jarman et al., 1994) also require a proneural gene (*ato*). The AS-C and *ato* can account for the origin of almost the entire PNS. There are, however, certain exceptions. In the embryo, two multiple dendritic neurons are unaccounted for, despite their dependence on *daughterless* (*da*), the presumed dimerization partner of the proneural genes (Jarman et al., 1993). With a viable *ato* mutant, we now know that certain adult external sense organs are also largely unaffected in mutants of AS-C or *ato*, particularly the stout row of bristles on the wing margin and the chemosensory organs of the second antennal segment. Other proneural gene(s), presumably of the bHLH family, therefore remain to be detected.

A single proneural gene for chordotonal organs

We previously presented strong evidence for *ato* being the counterpart of the AS-C in the formation of chordotonal organs (Jarman et al., 1993). This was based on sequence analysis, expression pattern, DNA-binding properties, and the effect of misexpression. But without specific mutations of *ato*, we could only gauge the requirement for *ato* by analysing the phenotype of embryos that contained synthetic deficiencies of the locus. This analysis was consistent with *ato*'s proposed proneural role (chordotonal neurons were missing from such embryos). However, we were unable to prove decisively that a single gene, rather than a complex of redundant genes, was responsible for the phenotype. Our isolation of a specific EMS-induced lesion of *ato* with a phenotype comparable to that of the deficiencies strongly supports our original conclusions, and suggests that a single proneural gene exists in the region of the *ato* locus. In contrast, of the many AS-C lesions known (Lindsley and Zimm, 1992), none are point mutations of individual genes. This is presumably due to extensive redundancy within this gene complex (Jiménez and Campos-Ortega, 1990). Despite their very different functions, one observation suggests there may even be some redundancy between *ato* and AS-C. A few chordotonal neurons remain in the *ato* mutant embryo. The AS-C are not normally required for their formation (Dambly-Chaudière and Ghysen, 1987), but these neurons are absent in an *ato* and AS-C double mutant (Jarman et al., 1993). We note that *scute* is indeed expressed during the formation of the first precursor of the lateral chordotonal organ (Vaessin et al., 1994).

Regulation of the proneural genes of the AS-C is believed to occur in two stages (Van Doren et al., 1992; Ghysen et al., 1993). First, expression is activated in ectodermal proneural clusters by a prepattern of positional information (Ghysen and Dambly-Chaudière, 1989; Skeath et al., 1992). Later, autoregulation augments expression, particularly in the cells destined to become SOPs. Our isolation of an *ato* point mutant gives us an opportunity to observe *ato* expression in the absence of *ato* function, thus uncoupling the role of initial activation from subsequent autoregulation. Consistent with the proneural-neurogenic model, we see that initial activation occurs normally, but there is no increase of expression in future precursors. A later marker, *asense*, also shows that the precursors never form.

Role of *ato* in pattern formation in the eye

In the formation of the ommatidium, it is believed that R8 is the first photoreceptor to be determined, which then recruits other photoreceptors and accessory cells in a series of inductive steps (Tomlinson and Ready, 1987; Banerjee and Zipursky, 1990; Basler and Hafen, 1991; Rubin, 1991). We previously showed that *ato* is the proneural gene for photoreceptors, being specifically required for R8 formation (Jarman et al., 1994). N. Brown and S. B. Carroll (personal communication) have recently shown that other neural HLH genes also function in photoreceptor formation. Mutations in *ato*'s in vitro dimerization partner, *daughterless*, have a similar effect to *ato* on photoreceptor formation, while the negative regulators *extramacrochaetae* and *h* appear to prevent premature photoreceptor formation ahead of the MF. Since these genes are required for correct AS-C function in other parts of the PNS (Botas et al., 1982; Caudy et al., 1988; Skeath and Carroll, 1991; Van Doren et al., 1992; Cubas and Modolell, 1993), it is likely that their role in the eye is also to modulate proneural gene (*ato*) function.

Whilst much interest has focused on the cell-cell interactions involved in progressive recruitment within the ommatidium, less is known of how the initial specification of R8 relates to ommatidial origin and spacing. Two possibilites have been considered: the process of R8 specification may itself also determine ommatidial spacing (Baker et al., 1990; Basler and Hafen, 1991), or else R8 specification may take place only after ommatidial spacing has been laid down by another mechanism (Cagan and Zipursky, 1992; Cagan, 1993). The question therefore arises of whether the genes for R8 specification (including *ato*) are also required for ommatidial spacing. The first possibility was based on the finding that the lateral inhibition gene *sca* is required for correct ommatidial spacing (Baker et al., 1990), and that most cells in the MF become R8-like cells when lateral inhibition is prevented (Cagan and Ready, 1989; Baker et al., 1990; Baker and Zitron, 1995). In this model, the entire stripe of *ato* expression anterior to the MF might represent the zone of neural competence, and the refinement of this expression to the intermediate groups may be intermediate stages of refinement in a continuing process of long range lateral inhibition, ultimately resulting in selection of equally spaced R8 cells. In this case, *ato* refinement would be an early event in ommatidial patterning, and *ato* function would be central to ommatidial origin as well as R8 specification.

More recently, it has been suggested that R8 selection occurs only after ommatidial spacing has been laid down (Cagan and Zipursky, 1992; Cagan, 1993; Thomas et al., 1994). Ultimately, each R8 would be selected from a small, independent 'equivalence group' of cells (perhaps the *ato* intermediate clusters) by local lateral inhibition interactions and *ato*

function. However, the initial spacing of these groups may depend on a separate, unknown long range mechanism. This was based on the original observation that R8 identity was apparent only after the regular spacing of the rosettes and later preclusters is detectable histologically (Tomlinson and Ready, 1987; Wolff and Ready, 1991b; Cagan and Zipursky, 1992). We find, however, that refinement of *ato* expression, both to intermediate groups and then to R8, appears to precede rosette formation; this refinement is in fact the first known patterning event in the MF (along with patterning of *sca* expression). We also find that neither the *ato*-expressing intermediate groups nor the histological rosettes are formed in the *ato* mutant MF, suggesting that they are not the result of some prior *ato*-independent spacing mechanism. Thus, *ato* may play a role both in spacing and then in R8 selection within spaced clusters. Recent work suggests that *sca* may also play roles in both precluster formation and R8 selection (Ellis et al., 1994). Baker and Zitron (1995) propose that the groups of *sca*-expressing cells in column 0 may act to pattern the next row of ommatidia by setting up periodic fields of inhibition, perhaps by imposing pattern within the continuous stripe of *ato* expression.

Effect of lack of photoreceptors on eye disc development

The failure of photoreceptor formation has secondary effects on the developing eye disc. In the wild-type eye, movement of the MF (and photoreceptor formation) is signalled by *dpp* expression, which in turn depends on a signal (*hh*) produced by photoreceptors behind the MF (Heberlein et al., 1993; Ma et al., 1993). Yet, the MF still forms in the *ato* mutant despite the lack of photoreceptors. *ato* essentially resembles a 'furrow stop' mutant, since the MF of the third instar larva has characteristics associated with the arrested MF in such mutants (notably a weak, interrupted stripe of *dpp*; Heberlein et al., 1993). Furrow formation, therefore, apparently occurs in the absence of the mechanism for furrow progression. The most likely explanation is that this reflects the mechanism of furrow initiation, which must precede photoreceptor formation and be independent of it, and therefore is presumably intact in the *ato* mutant. Such a mechanism has been recognized as necessary (Ma et al., 1994), but it was unclear what MF initiation might entail. Based on behaviour of the MF in the *ato* mutant, we suggest that the mechanism entails a photoreceptor-independent pathway of activating *dpp* expression in the posterior extremity of the disc.

It is interesting that this mutant MF is some way anterior from the posterior edge of the eye disc. It is possible that that is the position in which the MF is first formed, and that the cells posterior to it are not destined to be part of the compound eye. That is, not only is the mutant MF arrested in the third instar, but it also never moved before this. However, there are indications that the MF originally forms more posteriorly, and moves anteriorly prior to its arrest. Most critically, *ato* mutants are not completely eyeless: although there are no ommatidia, a stripe of pigment cells and interommatidial bristles remains. Thus, some cells are adopting 'post-furrow' non-ommatidial fates that would only be seen after passage of the MF. Moreover, some stimulation of cell division is seen posterior to the MF; this again is normally only observed in cells after they emerge from the MF. We speculate that photoreceptor-independent *dpp* activation in the posterior may not only promote MF formation but also allow the MF to begin moving anteriorly. The MF may move far enough to allow initial photoreceptor formation via *ato* activation, in turn triggering perpetuation of MF movement (progression) via *hh* expression. It is notable that *dpp* expression is stronger at the edges of the *ato* mutant MF, where photoreceptor-independent reinitiation must occur repeatedly. Moreover, the expression of *ato* itself reflects this pattern, suggesting that *dpp* directly activates the stripe of *ato* expression. Although the furrow halts in the posterior of the disc, we also see some signs that some aspects of the posterior to anterior progression of events is still occurring, notably the extension of *ato* and *h* expression into the anterior of the disc.

Neural subtype determination

We previously showed that the proneural genes influence the neuronal subtype identity of the SOPs that they produce (Jarman et al., 1993). Thus, generalized expression of AS-C genes results only in ectopic external sense organs, whereas *ato* yields predominantly chordotonal organs. Now we find that *ato* is required for two very different classes of sense organ. The formation of photoreceptors in one place and chordotonal organs in another must result from additional factors that modulate any identity function of *ato*. For instance, *glass* (*gl*) may be the regional factor that distinguishes the eye disc from other imaginal discs. This gene encodes a zinc-finger protein that is expressed posteriorly to the morphogenetic furrow (Moses and Rubin, 1991). In *gl* mutants, photoreceptor precursors are formed and begin neural differentiation, but they never express photoreceptor-specific markers (Moses et al., 1989). Another modulating factor may be the eye-specific paired-box/homeobox product of the gene, *eyeless* (Quiring et al., 1994).

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