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Transcriptional regulation of *atonal* during development of the *Drosophila* peripheral nervous system

Yan Sun, Lily Yeh Jan and Yuh Nung Jan*

Departments of Physiology and Biochemistry, Howard Hughes Medical Institute, University of California, San Francisco, San Francisco, CA 94143-0725, USA
*Author for correspondence (e-mail: ynjan@itsa.ucsf.edu)

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SUMMARY

***atonal* is a proneural gene for the development of *Drosophila* chordotonal organs and photoreceptor cells. We show here that *atonal* expression is controlled by modular enhancer elements located 5′ or 3′ to the *atonal*-coding sequences. During chordotonal organ development, the 3′ enhancer directs expression in proneural clusters; whereas successive modular enhancers located in the 5′ region drive tissue-specific expression in chordotonal organ precursors in the embryo and larval leg, wing and antennal imaginal discs. Similarly, in the eye disc, the 3′ enhancer directs initial expression in a stripe anterior to the morphogenetic furrow. These *atonal*-expressing cells are then patterned through a *Notch*-dependent process into initial clusters, representing the earliest patterning event yet identified during eye morphogenesis. A distinct 5′ enhancer drives expression in intermediate groups and R8 cells within and posterior to the morphogenetic furrow. Both enhancers are required for normal *atonal* function in the eye. The 5′ enhancer, but not the 3′ enhancer, depends on endogenous *atonal* function, suggesting a switch from regulation directed by other upstream genes to *atonal* autoregulation during the process of lateral inhibition. The regulatory regions identified in this study can thus account for *atonal* expression in every tissue and essentially in every stage of its expression during chordotonal organ and photoreceptor development.**

Key words: *atonal*, Transcriptional regulation, Photoreceptor, Chordotonal organ, *Notch*, *Drosophila*

INTRODUCTION

All multicellular animals generate their nervous systems in a stereotyped pattern during development. How is this highly reproducible developmental process controlled? Studies of development of the *Drosophila* peripheral nervous system (PNS) have provided useful insights into this question.

Sensory organs that constitute the embryonic or adult PNS of *Drosophila* are derived from the sensory organ precursor (SOP) cells at specific positions in embryos or in imaginal discs. The location of the SOPs is largely determined by the expression pattern of a group of proneural genes. Specification of the SOP cells for different types of sensory organs involves two successive steps (Ghysen et al., 1993). First, cells in proneural clusters start expressing proneural genes and become competent to assume a neuronal cell fate. Shortly afterwards, lateral inhibition mediated by the *Notch* signal transduction pathway leads to repression of proneural gene expression in most cells of the proneural clusters, causing these cells to adopt the epidermal fate, whereas one or a few cells in each proneural cluster strongly upregulate proneural gene expression, thereby becoming committed to a neuronal fate. Since there is little cell migration during PNS development, the final positions of the sensory organs are determined primarily by the initial expression pattern of the proneural genes (Ghysen and Dambly-Chaudière, 1989; Romani et al., 1989; Cubas et al., 1991; Skeath and Carroll, 1991; Campuzano and Modolell, 1992). Hence, regulation of proneural gene expression plays a key role in setting up the stereotyped pattern of the *Drosophila* PNS.

The concept of proneural genes was established from genetic and molecular studies of the role of the *achaete-scute* complex (AS-C) in the development of both the central and peripheral nervous systems (Ghysen and Dambly-Chaudière, 1989; Romani et al., 1989). All four members of the AS-C, i.e. *achaete* (*ac*), *scute* (*sc*), *lethal of scute* (*l'sc*) and *asense* (*ase*), encode the basic helix-loop-helix (bHLH) class of transcription factors (Villares and Cabrera, 1987; Alonso and Cabrera, 1988; Romani et al., 1989). The *ac* and *sc* genes carry the proneural function for development of the external sensory (es) organs in the embryonic and adult PNS. Loss-of-function mutations of *ac* and *sc* eliminate es organs. Conversely, ectopic expression of *ac* or *sc* promotes formation of ectopic es organs. Moreover, the spatiotemporal expression patterns of the *ac* and *sc* genes correlate with the formation of proneural clusters and the subsequent emergence of the SOPs from these clusters.

Transcriptional regulation of *ac* and *sc* has been extensively studied and considerable progress has been made. For instance, enhancer elements for *ac* and *sc* expression in specific subsets of proneural clusters and SOPs in embryos and imaginal discs have been mapped to certain genomic regions of the AS-C (Martínez and Modolell, 1991; Ruiz-Gómez and Ghysen, 1993; Gómez-Skarmeta et al., 1995). Some patterning genes have been found to directly or indirectly regulate *ac* and *sc* expression in regions where specific es organs will form (Skeath et al., 1992; Ramain et al., 1993; Gómez-Skarmeta et al., 1996; Cubadda et al., 1997). The Achaete and Scute proteins can dimerize with the bHLH protein Daughterless (Da) and the resulting heterodimer can activate *ac* and *sc* expression. Inhibitory factors such as *extramacrochaetae* (*emc*) and *hairy* (*h*) have been shown to refine *ac* and *sc* expression precisely to proneural clusters by either sequestering functional Achaete or Scute protein (in the case of *emc*) (Van Doren et al., 1992; Martínez et al., 1993) or repressing their expression in cells outside proneural clusters (in the case of *h*) (Skeath and Carroll, 1991; Ohsako et al., 1994; Van Doren et al., 1994). Within a proneural cluster, lateral inhibition mediated by *Notch* (*N*) signaling pathway silences proneural genes in cells surrounding the SOP via the inhibitory action of the *Enhancer of split* [*E*(*spl*)] complex on *ac* and *sc* expression (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995; Heitzler et al., 1996). Our understanding of transcriptional regulation of the AS-C, however, is still far from complete owing to the complexity of the AS-C genomic region of over 90 kb, the overlapping expression patterns and the functional redundancy of the four AS-C genes.

Another proneural gene, *atonal* (*ato*), was identified based on its sequence similarity to the AS-C genes (Jarman et al., 1993). It acts as the proneural gene for two types of sensory structures, chordotonal (ch) organs and photoreceptors (Jarman et al., 1994, 1995). Flies carrying loss-of-function mutations of *ato* are deprived of almost all ch organs and have highly reduced eyes lacking all photoreceptors. Similar to *ac* and *sc* for es organ development, *ato* is initially expressed in the proneural clusters and subsequently in the SOPs (founder SOPs) for ch organs. Unlike Ac and Sc, Ato protein in the founder SOPs in turn activates the EGF signaling pathway in the surrounding ectodermal cells, resulting in specification and recruitment of additional SOP cells for ch organs (Lage et al., 1997; Okabe and Okano, 1997). Although endowed by the same proneural gene for their developmental potential, photoreceptors in the eye are specified differently from the SOPs for ch organs. During third-instar larval development, the morphogenetic furrow (MF) sweeps across the eye imaginal disc from the posterior to the anterior. While cells anterior to this furrow are undifferentiated and appear unpatterned, cells within and posterior to the furrow begin to assemble into regularly spaced clusters of differentiating photoreceptor cells and later into mature ommatidia (Heberlein and Moses, 1995). Expression of *ato* in the eye disc starts in a dorsoventral stripe immediately anterior to the MF. Within and posterior to the MF, *ato* expression becomes progressively restricted first to a row of evenly spaced intermediate groups of prospective neuronal cells and later to one single cell within each group which differentiates into the R8 photoreceptor cell. Genes such as *hedgehog* (*hh*) (Heberlein et al., 1995), *Notch* (*N*) (Baker et al., 1996; Baker and Yu, 1997) and *rough* (*ro*) (Dokucu et al., 1996) have been shown to affect *ato* expression in the eye. As a prerequisite to mechanistic studies of the dynamic regulation of *ato* expression necessary for embryonic and adult sensory neuron formation, it is important to first identify regulatory regions of the *ato* gene.

In this study, we have located regulatory regions responsible for *ato* expression in every tissue where it is normally expressed. By analyzing the expression patterns directed by various *ato* regulatory regions, we have uncovered a distinct mechanism for transcriptional regulation of *ato*. Moreover, we demonstrate that patterning in the eye takes place before the formation of the intermediate groups of *ato*-expressing cells, previously considered to be the earliest patterning event. We further provide evidence that the neurogenic gene *Notch* is involved in setting up this initial pattern.

MATERIALS AND METHODS

DNA constructs

5′ *ato-lacZ* reporter constructs

A 4.2 kb *Bam*HI-*Bam*HI genomic fragment including 672 bp of the *ato*-coding sequence and 3.5 kb upstream sequence from phage clone λgt 6 was subcloned into the *Bam*HI site of pBluescript to generate pBS.Bm4.2 (a kind gift from Andy Jarman). For 5′F:2.6 reporter construct, pBS.Bm4.2 was digested with *Bgl*II, end blunted with Klenow enzyme and then redigested with *Sac*I to give the 2.6 kb *Sac*I-*Bgl*II fragment. This fragment was ligated to a *Xba*I-*Sac*I adapter (CTAGACGCTGACTACGAGCT / TGCGACTGATGC) first and the resulting insert was cloned into the *Xba*I site and the blunted *Bam*HI site of the pCaSpeR-β-gal transformation vector to generate 5′F:2.6. For constructs with larger fragments of the 5′ regulatory region, a 7.2 kb *Xba*I-*Bgl*II fragment from phage λgt 6 was transferred into *Bgl*II and *Spe*I double-digested pBS.Bm4.2 to generate pBS.XbBm in which the 5′ *Bam*HI-*Bgl*II fragment in pBS.Bm4.2 was replaced by the *Xba*I-*Bgl*II fragment. pBS.XbBm was digested with *Sma*I and *Bgl*II followed by filling in to give a 5.1 kb *Sma*I-*Bgl*II fragment. This fragment was inserted into filled *Bam*HI site of pCaSpeR-β-gal to generate 5′F:5.1. For 5′:F7.2, pBS.XbBm was cut with *Bgl*II, filled in and digested with *Xba*I. The resulting 7.2 kb insert was transferred into *Xba*I and filled *Bam*HI site of pCaSpeR-β-gal. Additional 5′ sequence included in 5′F:9.3 was excised from λgt 6 as a 5.0 kb *Sal*I-*Xho*I fragment (*Sa*lI site was filled in), subcloned into *Sma*I and *Xho*I sites of pBluescript, recovered as a *Xba*I-*Xho*I fragment and inserted into *Xba*I and *Xho*I double-digested 5′F:7.2 to give 5′F:9.3.

3′*ato-lacZ* reporter construct

A 7.2 kb *Sal*I-*Hin*dIII fragment including *ato* open reading frame and 5.8 kb downstream sequence was excised from λgt 6 and subcloned into *Sal*I and *Hin*dIII sites of pBluescript. The insert containing 3′ enhancer region for 3′F:5.8 was recovered as a *Sca*I-*Sac*II fragment and religated to the vector derived from 5′F:2.6, which was digested with *Xba*I, end-filled followed by *Sac*II digestion. The resulting 3′F:5.8 retained a 1.1 kb 5′ sequence immediately upstream of *ato*-coding sequence.

Rescue constructs

The 1.1 kb *ato* promoter region and most of the *lacZ*-coding sequence in the reporter construct 3′F:5.8 was removed by *Sac*II and *Bsi*WI double digestion. After filling in the *Bsi*WI end, a 2.1 kb *Sac*II-*Sca*I genomic fragment from λgt 6 containing the entire *ato* open reading frame and the 1.1 kb 5′ promoter region was ligated to the resulting vector to give pCaSpeR.3′ enhancer-*ato* (3′F:5.8-*ato*). 5′ eye enhancer region was isolated as a 2.6 kb *Bgl*II-*Xba*I fragment from a phage clone λgt 7 overlapping λgt 6. It covers the entire 5′ eye enhancer

region included in the reporter construct 5′F:9.3. This fragment was subcloned into *Bam*HI and *Xba*I sites of pBluescript, reexcised as a *Bgl*II-*Sac*II fragment with *Bgl*II end filled and then ligated with both the 2.1 kb *Sac*II-*Sca*I genomic fragment and *Xba*I, *Bsi*WI digested pCaSpeR-β-gal in which *Xba*I end was filled, resulting in pCaSpeR.5′eye enhancer-*ato* (5′eye-*ato*).

P-element transformation

P-element-mediated transformation of flies was performed as described (Spradling and Rubin, 1982). DNA concentration of the P-element plasmids was 150 ng/μl. pUCπΔ2-3 was used as the helper P-element plasmid at the concentration of 50 ng/μl. At least three independent lines were tested for each construct.

Immunohistochemistry

Generation of a rabbit anti-Ato serum was described by Jarman et al. (1995). For protein double labeling, a mouse anti-β-galactosidase monoclonal antibody (Promega) was used together with the rabbit anti-Ato serum. DTAF- and Rhodamine-conjugated secondary antibodies (Jackson Immunoresearch) were used for immunofluorescent staining.

Whole-mount in situ hybridization

A 3.4 kb *Eco*RI-*Kpn*I fragment from the *lacZ*-coding region excised from plasmid pBS.*khc*:*lacZ* (Giniger et al., 1993) was used as a template for digoxigenin (Boehringer Mannheim) labeling. The labeling was performed according to the manufacturer. Whole-mount in situ hybridization in embryos and in imaginal discs using digoxigenin-labeled probes was described by Tautz and Pfeifle (1989).

Scanning electron micrography

Fly heads were fixed with 2% glutaraldehyde and 4% formaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) overnight, dehydrated with a graded ethanol series and critical-point dried in CO_2. The samples were sputter coated with 30 nm of gold-palladium and examined with a scanning electron microscope at an accelerating voltage of 5 kV.

Fly stocks

All *Drosophila* stocks were raised on standard cornmeal-yeast-agar medium at 25°C unless otherwise mentioned. ato^1 is described by Jarman et al. (1994, 1995). N^{ts1} is described in Lindsley and Zimm (1992).

RESULTS

Modular arrangement of enhancer elements for embryonic ch organs and adult leg, wing and antennal ch organs

During *Drosophila* embryogenesis, the *ato* gene is transiently expressed at a low level in cells of the proneural clusters for the embryonic ch organs. The expression is quickly refined to a single SOP cell within each cluster. In each hemisegment of a stage 11 embryo, a characteristic row of four to five SOPs (the posterior group) express *ato* following a general dorsal-to-ventral order, i.e., compared to the ventrally located SOPs, *ato* expression in the more dorsal SOP starts earlier and ceases earlier as well (Jarman et al., 1993; Lage et al., 1997). These selected SOPs strongly upregulate *ato* expression and then each divides to produce all cells of a unit ch organ. During larval development, *ato* is expressed in leg, wing, antennal and eye imaginal discs. In leg discs, *ato* is initially expressed in two patches of epidermal cells and subsequently restricted to two small groups of subepidermal cells from which the ch organs in adult legs are generated. In the wing discs, *ato* expression is seen transiently in two small patches corresponding to ch organs in the ventral radius region and the tegula region of the adult wing. In antennal discs, a large ring of *ato*-expressing cells gives rise to Johnston's organ (Jarman et al., 1993).

To identify the enhancer elements that direct these different patterns of *ato* expression, we fused genomic fragments upstream of the *ato*-coding region to a *lacZ* reporter gene. All fusion constructs contained *ato* basic promoter elements provided by a 1.1 kb genomic sequence immediately preceding the coding region, the initiation codon and 15 additional nucleotides of the coding region (Fig. 1). The constructs were introduced into flies via germline transformation and the expression pattern of *lacZ* was studied by double immunofluorescence using anti-β-galactosidase and anti-Ato antibodies (Fig. 2).

Fusion of 2.6 kb of *ato* 5′ sequences to *lacZ* (5′F:2.6) resulted in exclusively embryonic expression in SOP cells of ch organs (Fig. 2B). The expression pattern is very similar but not identical to the expression of endogenous *ato* in these cells (Fig. 2A,C). The differences can be accounted for mostly by a later onset of *lacZ* expression and longer perdurance of β-galactosidase compared to that of Ato (Y. S., unpublished observations). When 5.1 kb of *ato* upstream sequence were fused to *lacZ* (5′F:5.1), the embryonic *lacZ* expression pattern

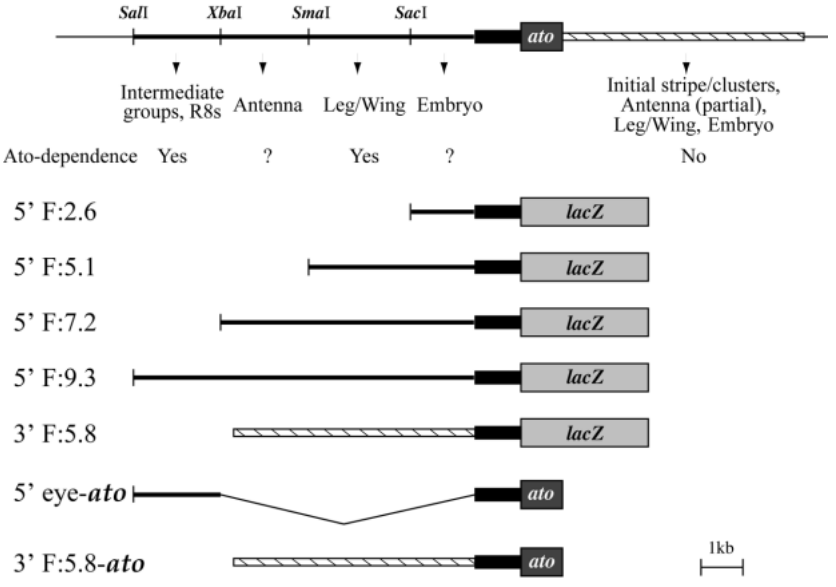

Fig. 1. *ato-lacZ* reporter and *ato* rescue constructs. The genomic region of *ato* is shown above with the 5′ regulatory regions depicted with a thick line and the 3′ region with a hatched bar. The tissue specificity and Ato-dependence of each regulatory region are indicated below. A *lacZ*-coding sequence (missing the first seven codons) (light gray box) used in all reporter constructs was fused in frame to a 1.1 kb genomic fragment (black bar) of *ato* that includes the first 15 nucleotides of the *ato* open reading frame, the start codon and the preceding sequences containing the basic promoter elements. The same genomic fragment was also used in the rescue constructs. The *ato* open reading frame is shown with a dark gray box.

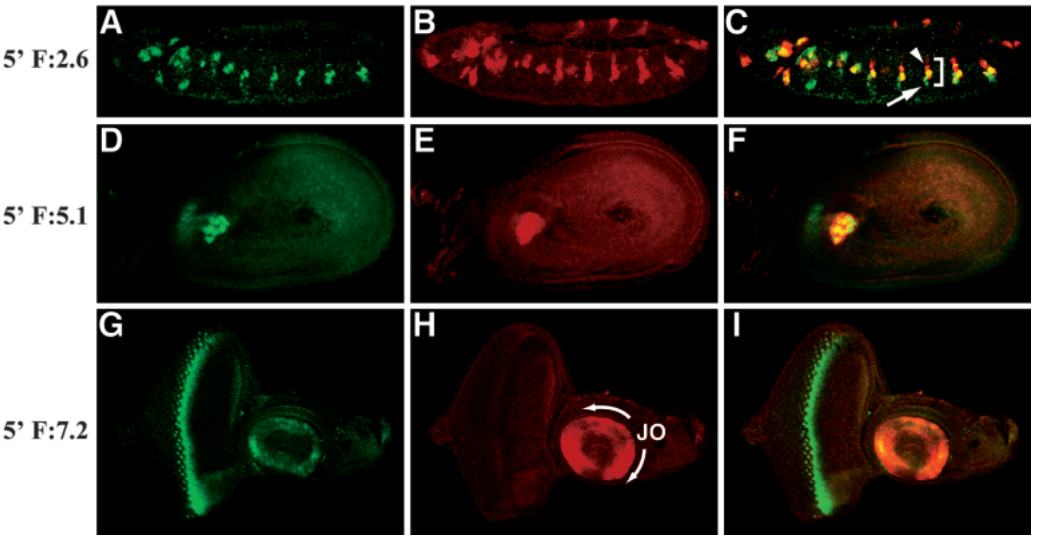

Fig. 2. Expression of the 5′ *ato-lacZ* fusion genes in embryos and imaginal discs. Embryos and imaginal discs from third instar larvae carrying various 5′ *ato-lacZ* fusion genes were double stained with antibodies against Ato (green) and β-galactosidase (red). (A-C) Lateral view of a stage 11 embryo containing the 2.6 kb *ato-lacZ* fusion gene (5′F:2.6). *ato* expression is seen in a row of 4 to 5 SOPs (A; bracket in C) selected from proneural clusters in each hemisegment (Jarman et al., 1993; Lage et al., 1997). β-galactosidase appears later and persists longer compared to endogenous Ato. Therefore, in this case, β-galactosidase is not yet in the ventral-most SOPs, which have just emerged from the proneural clusters (arrow in C), but is still present in the dorsal-most SOPs in which endogenous Ato has already disappeared (B; arrowhead in C). (D-F) A leg disc of a third instar larva containing the 5.1 kb *ato-lacZ* fusion gene (5′F:5.1). A large Ato-expressing cluster (D) gives rise to precursors of the femoral ch organs. β-galactosidase expression (E) coincides with endogenous *ato* expression (F). A small double-stained patch was also observed in a different focal plane. (G-I) An eye-antennal disc from a third instar larva carrying the 7.2 kb *ato-lacZ* transgene (5′:F7.2). A large ring of *ato* expression in the second antennal segment (G) consists of precursors for Johnston's organ (JO), which also express *lacZ* (H,I). No *lacZ* expression is observed in the eye disc (H).

was identical to that observed with 5′F:2.6 (data not shown), but additional expression was detected in leg (Fig. 2E) and wing imaginal discs (data not shown). Here, *lacZ* expression closely mirrored the endogenous *ato* expression (Fig. 2D,F). Strong *lacZ* expression was also seen in a large crescent and a few small groups of cells in each brain lobe (data not shown), a pattern that is reminiscent of *ato* expression (Jarman et al., 1993). Weak expression in antennal discs was also detected occasionally in some fly lines (data not shown). Finally, when 7.2 kb of *ato* upstream region were fused to *lacZ* (5′F:7.2), in addition to expression in embryos, leg discs and wing discs, the fusion gene was also strongly expressed in antennal discs in a pattern identical or very similar to that of *ato* (Fig. 2G-I). We conclude that successive modular enhancers located within a 7.2 kb interval 5′ to the *ato*-coding region direct its expression in embryos, leg discs, wing discs and antennal discs.

Distinct enhancer elements drive *ato* expression anterior or posterior to the morphogenetic furrow (MF) in eye discs

In eye imaginal discs, *ato* is first expressed in a stripe of cells located just ahead of the MF. As the MF progresses anteriorly sweeping past these *ato*-expressing cells, *ato* expression is progressively restricted first to regularly spaced intermediate groups within the MF and later to single R8 cells within and behind the MF (Jarman et al., 1994, 1995) (Fig. 3A). While the 5′F:7.2 fusion gene is not expressed in eye discs (Fig. 2H), eye expression is detected when 9.3 kb of upstream region are fused to *lacZ* (Fig. 3B). However, this 5′F:9.3 fusion gene is only expressed in intermediate groups and R8 cells within and posterior to the MF. In fact, a 2.6 kb fragment from –7.1 kb to –9.7 kb is sufficient to drive expression in intermediate groups and R8 cells (data not shown). No expression anterior to the furrow was observed using the 9.3 kb or a larger 15.8 kb upstream fragment. In contrast, when we fused a 5.8 kb genomic fragment downstream of the *ato* open reading frame to *lacZ* (3′F:5.8), we detected *lacZ* expression in a stripe immediately anterior to the MF (Fig. 3C), similar to that of endogenous *ato*. Both 5′ and 3′ enhancers also direct expression in ocelli precursors (Fig. 3B,C). We conclude that *ato* expression in intermediate groups and R8 cells is directed by a 5′ enhancer located between 7.2 and 9.3 kb upstream of the coding region whereas expression in the initial stripe is directed by a 3′ enhancer located in a 5.8 kb fragment downstream of the coding region.

Both eye enhancer elements are required for rescue of the *ato* mutant eye phenotype

To test which of the two enhancer elements is necessary for *ato* function in the eye, we fused the 2.6 kb 5′ eye enhancer region or the 5.8 kb 3′ enhancer region to the *ato* open reading frame and tested the transgenes for their ability to rescue the *ato* mutant phenotype as shown in Fig. 4B. 1.1 kb of genomic sequence immediately preceding the coding region were included in both transgenes to provide basic promoter elements (Fig. 1). This 1.1 kb fragment alone, when fused to the *lacZ* reporter gene, drives no significant expression except for two clusters in the embryonic head region (data not shown). When introduced into an ato^1 mutant background (Jarman et al., 1994), two copies of either the 5′ eye-*ato* or the 3′F:5.8-*ato* were unable to rescue the mutant eye phenotype. In some rare cases, 9 or fewer ommatidia (as compared to about 750 ommatidia in a wild-type eye as shown in Fig. 4A) were observed (data not shown). This limited rescue may result from some residual activity of the Ato^1 mutant protein, which can function partially only when wild-type Ato is provided either anterior to or within the MF. In contrast, flies carrying one copy of each construct typically had eyes containing 40% of the normal number of ommatidia (data not shown) and flies carrying two copies of each transgene often had nearly normal

eyes, albeit slightly rough (Fig. 4C). Thus, transgenes carrying the 5′ enhancer or the 3′ enhancer alone fused to the *ato*-coding region are not able to substantially rescue the *ato* phenotype, but both transgenes together can cause dramatic rescue of the *ato* mutant eye phenotype. Furthermore, they were able to restore *ato* expression to an almost wild-type pattern in an *ato*^{1} mutant eye disc (Fig. 4D-F).

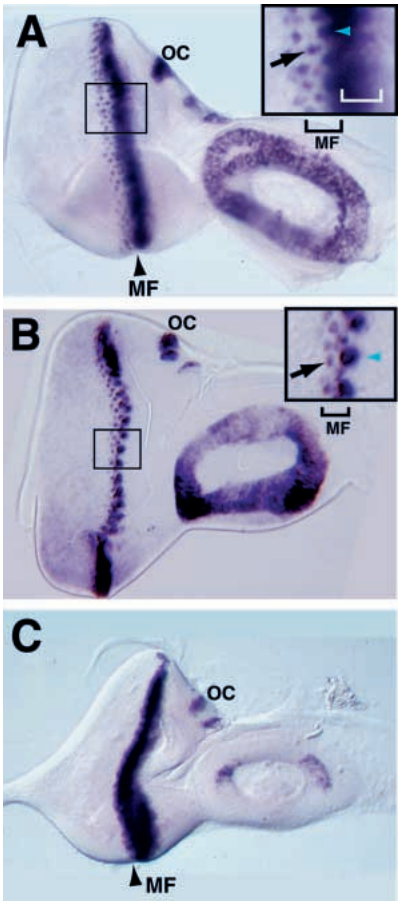

Fig. 3. Two distinct enhancer elements direct *ato* expression in eye discs. Expression of *ato* (A) or *ato-lacZ* fusion genes (B,C) was detected by whole-mount in situ hybridization with digoxigenin-labeled probes in eye-antennal discs from third instar larvae. Eye discs in all figures are shown with posterior to the left. The insets show the boxed regions at a higher magnification. (A) A wild-type disc hybridized to an *ato* probe. Within and posterior to the MF, expression is first seen in regularly spaced intermediate groups (arrowhead in inset), and then refined to rows of isolated R8 photoreceptor cells (arrow). Anterior to the MF, *ato* is expressed in a stripe (initial stripe, bracket). Two small patches mark the ocellar region (OC). (B) A disc containing a transgene with a 9.3 kb 5′ genomic fragment fused to *lacZ* (5′F:9.3) was hybridized with a *lacZ* probe. *lacZ* expression was observed only in the intermediate groups (arrowhead) and the R8 photoreceptor cells (arrow) within and posterior to the MF. No expression anterior to the MF was detected. Expression is also seen in the ocellar region (OC). (C) *lacZ* expression in a disc carrying a transgene with a 5.8 kb 3′ genomic fragment fused to *lacZ* (3′F:5.8). Expression in the eye disc occurs exclusively in the initial stripe anterior to the MF and in ocellar precursors. Partial expression in the antennal region is often observed.

Based on the reporter gene expression pattern and the phenotype rescue, we conclude that *ato* expression in eye discs is directed by two distinct regulatory regions, which are located 5′ and 3′ to the *ato*-coding sequence. Both regions are required for normal *ato* function. This arrangement suggests that the initiation of *ato* expression in a stripe anterior to the MF and its restriction into intermediate groups and R8 photoreceptor cells within and posterior to the MF rely on separate transcriptional events.

Expression of the *ato* 5′ eye enhancer but not the 3′ enhancer requires *ato* function

Transcriptional autoregulation has been observed for several bHLH transcription factors (Skeath and Carroll, 1992; Van Doren et al., 1992). To examine the function of the Ato protein in the regulation of *ato* expression, we analyzed the *lacZ* expression patterns of the 3′ and 5′*ato-lacZ* reporter genes in an *ato*^{1} mutant background. The *ato*^{1} mutation causes several amino acid substitutions in the Ato DNA-binding domain and has been characterized as a strong hypomorphic or null allele (Jarman et al., 1994, 1995). While the 3′ enhancer directed *lacZ* gene expression anterior to the MF in the *ato*^{1} mutant (Fig. 5A), the 5′ enhancer failed to drive *lacZ* expression posterior to the MF in this mutant (Fig. 5B). These experiments suggest that *ato* expression is initiated anterior to the MF via the 3′ enhancer element by an Ato-independent mechanism; autoregulation of the *ato* gene via the 5′ eye enhancer element is then responsible for maintaining and enhancing its expression in the intermediate groups and R8 photoreceptor cells within and posterior to the MF.

In addition to expression in the eye, the 3′ enhancer region directs *ato* expression in embryos, in leg and wing discs, and partially in antennal discs (data not shown). Unlike in the eye disc, however, the expression patterns in leg and wing discs directed by the 3′ enhancer region closely resemble those controlled by the 5′ leg and wing enhancer regions. In embryos, *lacZ* expression driven by the 3′ enhancer was often seen in clusters of cells surrounding the *ato*-expressing SOP cells, corresponding presumably to the proneural clusters for the ch organs (data not shown). By contrast, the 5′ enhancer-driven expression of *lacZ* is mainly observed in the embryonic SOP cells, indicating that the 3′ enhancer acts earlier than the 5′ enhancer. We also analyzed the Ato-dependence of the 5′ and 3′ enhancers in embryos, leg and wing discs, and antennal discs. In leg discs, *lacZ* expression driven by the 3′ enhancer was unchanged in the *ato*^{1} mutant background (Fig. 5C), while expression from the 5′ enhancer was completely eliminated (Fig. 5D). Our results suggest initiation of transcription directed by the 3′ enhancer followed by Ato-dependent refinement via the 5′ region as a common scheme for regulating *ato* expression that is shared by photoreceptor and leg ch organ development. However, we observed *lacZ* expression in embryos and antennal discs driven by the 5′ enhancers in slightly altered patterns in the *ato*^{1} mutant background (Fig. 5B and data not shown), suggesting that either distinct Ato-independent mechanisms may operate in the refinement of *ato* expression in embryos and antennal discs, or residual activity of the Ato^{1} protein is sufficient to modulate reporter gene expression while failing to direct complete development of ch organs.

Clusters prefiguring ommatidia are formed anterior to the MF

Pattern formation in *Drosophila* eye discs is thought to occur within and posterior to the MF. Morphological pattern formation in these regions can be revealed by histological stainings as 'rosettes' forming along the posterior edge of the MF (Tomlinson and Ready, 1987; Wolff and Ready, 1991). This is preceded by the formation of evenly spaced *ato* (and *sca*)-expressing intermediate groups covering roughly the anterior half of the MF, the earliest known patterning event in eye morphogenesis (Jarman et al., 1995; Baker et al., 1996; Baker and Yu, 1997). The initial expression of *ato* anterior to the MF was thought to occur in a homogeneous dorsoventral stripe (Jarman et al., 1994, 1995). However, upon closer examination of *lacZ* expression in 3′ enhancer-*lacZ* reporter lines, we discovered that this initial stripe is in fact composed of two elements (Fig. 6): in addition to a weak band of homogeneous *ato* expression, we found one row of regularly spaced clusters (referred to as initial clusters thereafter) located on the posterior edge of the homogenous band immediately anterior to the MF at exactly the positions where the next row of intermediate groups will form later (Fig. 6A). These initial clusters can only be observed in lightly stained samples. More intense staining causes these clusters to be concealed by expression in surrounding cells. This may explain why they were not seen before. The existence of the initial clusters located anterior to the intermediate groups in wild-type eye discs was confirmed by both in situ hybridization using an *ato* probe (Fig. 6B) and immunocytochemical staining with an anti-Ato antibody (Fig. 6C). This prepattern of initial clusters represents the earliest patterning event yet described during *Drosophila* eye development and we conclude that pattern formation during eye development takes place before cells enter the morphogenetic furrow.

Initial clusters are formed in a *Notch*-dependent process

Restriction of proneural gene expression from proneural clusters to SOPs is usually *Notch* (*N*) dependent. During eye development, *N* is known to function within and posterior to the MF in restricting *ato* expression to R8 cells within intermediate groups (Baker et al., 1996; Baker and Yu, 1997). Anterior to the morphogenetic furrow, *N* has been shown to promote *ato* expression (Baker and Yu, 1997). To test the function of *N* in the formation of the *ato* prepattern anterior to the MF and in regulating the 3′ enhancer, we examined *lacZ* expression from the 3′ enhancer-*lacZ* reporter gene in a temperature-sensitive *N* mutant background (N^{ts1}) (Lindsley and Zimm, 1992).

When larvae carrying the N^{ts1} allele and the 3′ enhancer-*lacZ* fusion gene were shifted to the restrictive temperature for 2 hours, the 3′ enhancer-directed *lacZ* expression anterior to the MF became continuous and appeared broader and stronger than that in wild type, and the initial clusters normally seen within the initial stripe failed to form (Fig. 7A,B). The endogenous *ato* gene responded to *N* inactivation similarly in the initial stripe (Fig. 7C,D). We conclude from these experiments that *N* is involved in refining *ato* expression anterior to the MF from a continuous band to patterned initial clusters that prefigure the future ommatidia.

DISCUSSION

During *Drosophila* PNS development, transcriptional regulation of proneural genes determines the position of proneural clusters and subsequently the selection of a single SOP cell from a proneural cluster, thereby prefiguring a future sensory organ. To understand the mechanism of this transcriptional regulation, we have carried out a promoter analysis of the proneural gene *ato*. Our results demonstrate that (1) modular enhancer elements located within 9.3 kb upstream of the *ato*-coding region direct *ato* expression in the ch organ precursors in the embryo, the leg and wing discs, the antennal disc and in intermediate groups and R8 cells in the eye disc, respectively. Another regulatory region, located within 5.8 kb downstream of the *ato*-coding sequence, is responsible for the initial phase of *ato* expression in the embryo, in the leg disc, in the wing disc, partially in the antennal disc and in the initial stripe anterior to the MF in the eye disc. (2) The 3′ enhancer functions in an Ato-independent fashion, whereas the 5′ elements employ Ato-dependent regulatory mechanisms in leg

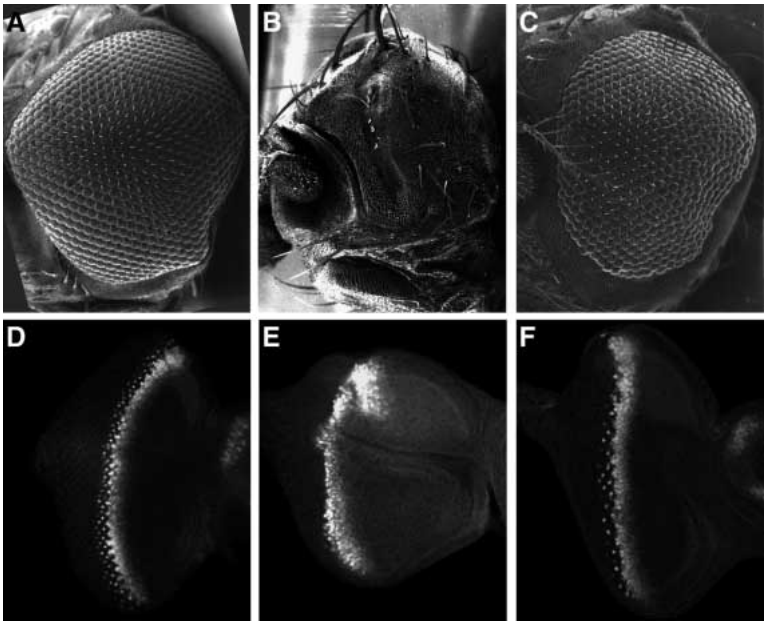

Fig. 4. Rescue of ommatidia in ato^1 mutants by 3′ and 5′ eye enhancer-directed *ato* expression. (A-C) Scanning electron micrographs of compound eyes. (A) Wild-type. (B) ato^1. No ommatidia are formed in the presumptive eye field except for a few pigment cells and bristles (Jarman et al., 1994). Rescues with either the 5′ eye enhancer-*ato* or the 3′ enhancer-*ato* (3′F:5.8-*ato*) alone normally show similar eye phenotypes as ato^1 (data not shown). (C) ato^1 eye rescued with two copies each of the 5′ eye enhancer-*ato* and 3′F:5.8-*ato*. Rescued eyes are of variable sizes. Slight overall roughness was observed, suggesting that additional fine adjustment is required for complete rescue. (D-F) Eye discs in which *ato* expression was detected by immunostaining with an anti-Ato antibody. (D) Wild-type disc. (E) ato^1 disc. The initial stripe of *ato* expression anterior to the MF is present with weaker expression in the central region (also see Jarman et al., 1995). (F) An ato^1 disc, which also contains two copies each of 5′ eye enhancer-*ato* and 3′F:5.8-*ato*. A nearly wild-type expression pattern is restored.

and eye discs and possibly in other tissues where *ato* is expressed. (3) Our experiments also demonstrate that the initial stripe of *ato* expression in the eye is not homogeneous, but occurs in a prepattern whose formation requires the activity of *Notch*.

Modular arrangement of tissue-specific enhancers in the *ato* 5′ regulatory region

By studying mutants bearing genomic alterations (for instance, deletions, insertions) in the AS-C, some enhancer elements for subsets of *ac* and *sc* expression patterns have been mapped to discrete sites extending over a large region (about 90 kb) of the AS-C (Martínez and Modolell, 1991; Ruiz-Gómez and Ghysen, 1993; Gómez-Skarmeta et al., 1995). Among these enhancers, only a few have been further defined by molecular means and no modular organization of tissue-specific enhancers has been established. In contrast, the regulatory region for *ato* expression is much smaller (about 15 kb) and has a much simpler organization. The genomic region located 5′ to the *ato* open reading frame contains modular enhancers each of which determines *ato* expression in sensory organ precursors in a specific tissue type for the formation of ch organs or photoreceptors. We also demonstrate that 3′ regulatory region recapitulates approximately the normal pattern of *ato* expression in embryos, in leg discs, in wing discs, partially in antennal discs, and in the initial stripe in eye discs. It is possible that the 3′ regulatory region is also composed of independent modules. Each module may respond to distinct combination of positional information and initiate *ato* expression in particular tissues. Taken together, the modular organization of the *ato* regulatory region determines spatial control of *ato* expression in different tissues.

Distinct enhancer elements direct *ato* expression in proneural clusters and SOPs with different Ato-dependence

Two separate regulatory regions located 5′ or 3′ to the *ato*-coding sequence are seemingly redundant. However, further analysis revealed that, in addition to the spatial regulation determined by modularly arranged enhancer elements, these two distinct regulatory regions impose sequential temporal control of *ato* expression in every tissue. This is best represented in the eye due to the asynchrony of ommatidial development. Neurogenesis in the eye involves a stepwise restriction of *ato* expression from a continuous band (initial stripe) to intermediate groups and then to individual R8 cells, which in turn recruit neighboring cells to become photoreceptors. Our results demonstrate that the 3′ enhancer directs *ato* expression in the initial stripe in an Ato-independent manner. A 5′ autoregulatory enhancer element drives expression in intermediate groups and R8 cells. Enhancers for *ato* expression during development of ch organs in leg discs follow the same arrangement and Ato-dependence. Although the very transient nature of proneural clusters for ch organs made it difficult to determine the temporal order of expression from these two sets of enhancers, there appears to be a common theme for temporal regulation of *ato* expression in different tissues. Patterning genes mediating positional information could initiate *ato* expression via the 3′ element at the places where ch organs or photoreceptors are to be formed. During lateral inhibition, this initial expression would be restricted to a single SOP cell, which then switches to autoregulation via the 5′ element. In fact, a similar mechanism also operates at least in one case for *ac* expression in specific domains during neuroblast formation in the embryonic CNS, except that other gene products instead of Ac are responsible for the enhanced expression in the neuroblasts (Skeath et al., 1994). Indeed, autoregulation (or cross-regulation) of *ac* and *sc* functions to specify some other SOPs in the PNS and neuroblasts in the CNS (Van Doren et al., 1992), indicating that the AS-C and *ato* share some basic regulatory mechanisms, such as the use of distinct enhancer elements to direct expression in proneural clusters and SOP cells and the involvement of autoregulation.

The 5′ enhancer elements for *ato* expression in leg discs and eye discs require *ato* to be active since, in the ato^1 mutant background, expression from these enhancers is absent. This effect of *ato* on its own expression could be direct or indirect, but the presence of E boxes, the consensus binding sites for bHLH transcription factors, in the 5′ eye enhancer region (data not shown) is consistent with direct binding of Ato to its own promoter region. However, this autoregulation alone can not account for the tissue specificity of *ato* expression. For example, in leg discs containing the 5′ eye-*lacZ* transgene, the endogenous Ato is not sufficient to drive *lacZ* expression there (data not shown). This tissue specificity could be achieved by three means. First, bHLH proteins are known to heterodimerize and different binding partners could be required in different tissues. However, Ato has been shown to form transcriptionally active complexes with Daughterless (Jarman et al., 1993; Rosay et al., 1995) and the ubiquitous presence of this protein argues against this possibility. Alternatively, the activity of the Ato-Da heterodimer could be regulated by tissue-specific activating or repressing cofactors, which target the complex to a specific enhancer element. Finally, individual enhancer elements could be activated by their interaction with other tissue-specific transcription factors in addition to the binding of Ato-Da heterodimer to E boxes.

The transcriptional regulation of *ato* in embryos and antennal discs appears different from that in leg discs and eye discs. We detected *lacZ* expression driven by the 5′ embryonic and antennal enhancers in the ato^1 mutant. There are two alternative explanations for this observations. First, these 5′ enhancers are Ato-dependent; although insufficient to lead ch organ development, residual activity retained in Ato^1 mutant protein is sufficient to induce reporter gene expression. It has been demonstrated that *ato* expression is initiated in proneural clusters but fails to resolve into SOP cells in ato^1 mutant (Jarman et al., 1995). Consistent with this, β-galactosidase from the 5′ embryonic enhancer-*lacZ* reporter gene is often detected at a lower level in clusters of ato^1-expressing cells in the ato^1 mutant, instead of in isolated SOP cells as in wild-type embryos. Alternatively, an Ato-independent mechanism may operate to restrict *ato* expression from proneural clusters to the SOP cells in the embryo and the antennal disc.

Ommatidial patterning begins ahead of the MF through a *Notch*-dependent process

The stereotyped pattern of the *Drosophila* compound eye is prefigured in the eye disc by regular arrays of developing ommatidia posterior to the MF, which were first revealed as evenly spaced ‘rosettes’ of 10-20 cells (Tomlinson and Ready, 1987; Wolff and Ready, 1991). *ato* expression in the

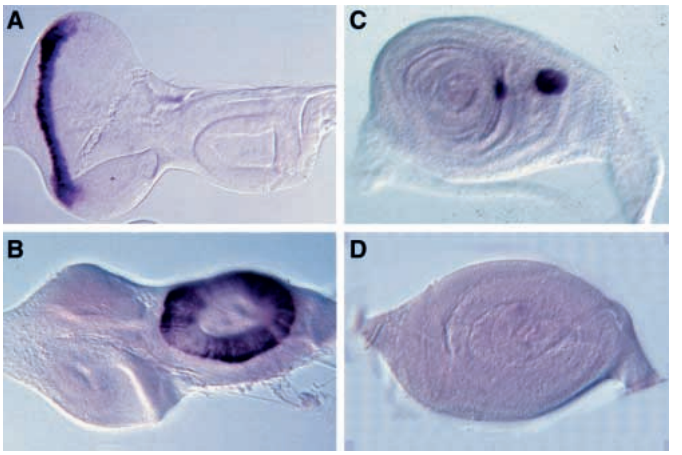

Fig. 5. Expression driven by 5′*ato* enhancers but not 3′ enhancers requires *ato* function. An eye-antennal disc (A) and a leg disc (C) from 3′F:5.8; ato^1 fly. *lacZ* expression directed by the 3′ enhancer is still present in an ato^1 mutant background as revealed by in situ hybridization. An eye-antennal disc (B) and a leg disc (D) from 5′F:9.3; ato^1 fly. *lacZ* expression driven by 5′ enhancers is abolished in the same ato^1 background in leg and eye discs including the ocellar region. Note that expression in the antennal disc remains.

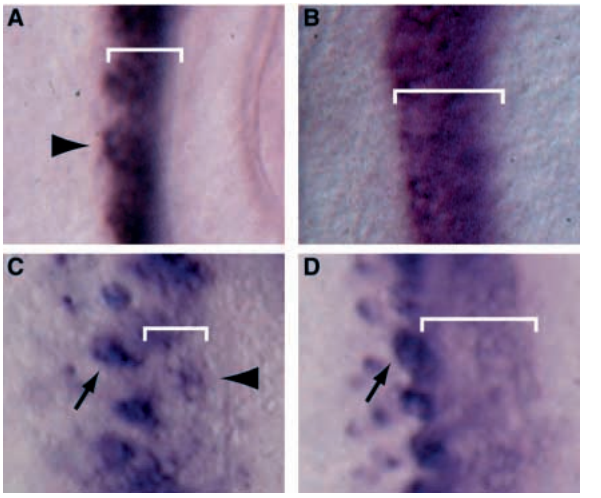

Fig. 7. Initial clusters are formed in a *Notch*-dependent process. Third instar larvae were shifted to the restrictive temperature (31.5˚C) of N^{ts1} for 2 hours followed immediately by in situ hybridization with digoxigenin-labeled probes. (A) 3′F:5.8/+ disc and (B) N^{ts1}/Y; 3′F5.8/+ disc hybridized with a *lacZ* probe. Initial clusters (arrowhead in A) anterior to the MF fail to form when *N* activity is reduced (B). The initial stripe in N^{ts1} mutant (bracket in B) appears broader than that in wild type (bracket in A), and the expression level of *lacZ* in N^{ts1} mutant (B) is comparable to the enhanced expression in the initial clusters in wild type (A). (C) Wild-type disc and (D) N^{ts1} disc hybridized with an *ato* probe. Endogenous initial clusters (arrowhead in C) of *ato* also disappear in N^{ts1} disc (D) after the temperature shift. Expression in the initial stripe (bracket in D) appears broader and at a higher level than that in wild type (bracket in C). The intermediate groups of N^{ts1} mutant (arrow in D) resemble those in wild type (arrow in C).

intermediate groups within the MF precedes these morphological clusters by two rows (Jarman et al., 1995). It was therefore believed that the transition from non-patterned anterior cells to patterned posterior clusters takes place within the MF as represented by the intermediate groups. However, upon close examination, we found that *ato* expression in the initial stripe anterior to the MF occurs originally as a weak homogeneous dorsoventral band, but is then refined into one row of regularly spaced initial clusters located ahead of the intermediate groups. These initial clusters also exhibit a slight upregulation of *ato* expression. This result contrasts a previous model of pattern formation during eye development, which assumes that patterning occurs within the MF, with cells anterior to the furrow being unpatterned and equal in their gene expression pattern. Where does the positional information for patterning these initial clusters come from? Since these clusters always form at positions immediately anterior to intermediate groups and R8 cells, patterning signals may come from those more posterior cells. Indeed, recent experiments have suggested such a signaling mechanism, possibly involving the genes *argos* and *rhomboid* (Roush, 1997). How this mechanism influences *ato* expression, however, remains to be determined.

Besides its function in ensuring that only one cell in each intermediate group takes on the R8 fate posterior to the MF (Cagan and Ready, 1989; Baker et al., 1996), we show that *Notch* is required for the emergence of *ato*-expressing initial clusters from a homogeneous band of *ato*-expressing cells anterior to the MF. When *Notch* is inactivated, initial clusters fail to form and *ato* is transcribed at a uniform and somewhat higher level in all cells within the initial stripe. This *Notch*-dependent transition from an early weaker and wider expression to higher expression in only a subset of cells is reminiscent of lateral inhibition during neurogenesis. Therefore, other molecules in the *Notch* signaling pathway such as *suppresser of Hairless* [*su*(*H*)] and *E*(*spl*) are likely to participate in regulating initial cluster formation. Recently, Baker and Yu (1997) reported that *Notch* inactivation results in decreased level of Ato protein in the initial stripe. It thus appears that *Notch* exerts opposite effects on *ato* RNA and protein. The molecular mechanism for the post-transcriptional regulation of *ato* by *Notch* awaits further investigation.

Transplantation experiment of eye disc fragments revealed

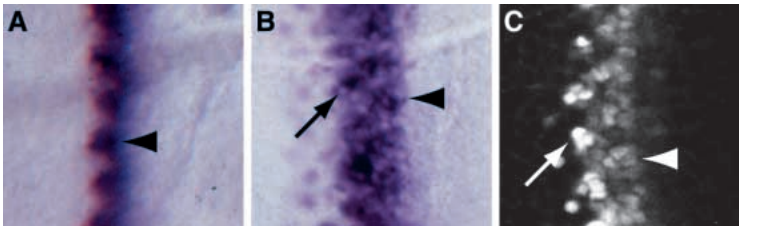

Fig. 6. Pattern formation in the eye disc occurs anterior to the MF. (A) 3′F:5.8 disc. *lacZ* expression was detected by in situ hybridization. Initial clusters (arrowhead) anterior to the MF are formed on the posterior edge of the initial stripe. (B) Wild-type disc hybridized with an *ato* probe. Initial clusters (arrowhead) prefigure the following row of intermediate groups (arrow). (C) Wild-type disc stained with an antibody against Ato. Initial clusters (arrowhead) and intermediate groups (arrow) are both evident.

that cells immediately anterior to the MF have already acquired the potential to differentiate into photoreceptors in the absence of the MF (Lebovitz and Ready, 1986). Besides, precocious differentiation of photoreceptors induced by local activation of the *hh* signaling pathway in the anterior eye disc takes place primarily in a domain adjacent to the MF (Heberlein et al., 1995). Therefore, a 'competence zone' for photoreceptor differentiation has been proposed, although the molecular nature and the boundary of this zone are not clear (Ma et al., 1993; Heberlein and Moses, 1995; Heberlein et al., 1995). The initial stripe of *ato* expression co-localizes with this competence zone. Given that enhanced proneural gene expression in SOP cells that have emerged from proneural clusters is normally associated with the establishment of neuronal fate in these cells, it is likely that *ato* expression in the initial stripe provides cells with the competence retained in the 'competence zone' to differentiate into photoreceptors and the increased expression in initial clusters within the initial stripe represents the commitment to a photoreceptor fate.

Our results show that the *ato* regulatory region contains a relatively simple modular arrangement of several independent enhancer elements that direct expression in different tissues and cell types. This modular architecture provides good resources for studying the molecular events that are associated with selecting a single neural precursor from an initially homogeneous population of cells. Further dissection of the identified promoter elements and identification of factors that bind to these elements will enhance our understanding of neurogenesis.

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