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Direct control of neurogenesis by selector factors in the fly eye: regulation of *atonal* by Ey and So

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During eye development, the selector factors of the Eyeless/Pax6 or Retinal Determination (RD) network control specification of organ-type whereas the bHLH-type proneural factor Atonal drives neurogenesis. Although significant progress has been made in dissecting the acquisition of 'eye identity' at the transcriptional level, the molecular mechanisms underlying the progression from neuronal progenitor to differentiating neuron remain unclear. A recently proposed model for the integration of organ specification and neurogenesis hypothesizes that *atonal* expression in the eye is RD-network-independent and that Eyeless works in parallel or downstream of *atonal* to modify the neurogenetic program. We show here that distinct cis-regulatory elements control *atonal* expression specifically in the eye and that the RD factors Eyeless and Sine oculis function as direct regulators. We find that these transcription factors interact in vitro and provide indirect evidence that this interaction may be required in vivo. The subordination of neurogenesis to the RD pathway in the eye provides a direct mechanism for the coordination of neurogenesis and tissue specification during sensory organ formation.

KEY WORDS: Sensory organ, Specification, Neurogenesis, Photoreceptor Development, Eye morphogenesis, *Drosophila*, Atonal, Eyeless, Pax6

INTRODUCTION

Transcription factors of the basic helix-loop-helix (bHLH) type play crucial roles during both peripheral and central nervous system development in organisms as diverse as fly, worm and mouse (Lee, 1997; Brunet and Ghysen, 1999; Vetter and Brown, 2001; Ross et al., 2003; Portman and Emmons, 2000). One of the best-studied models for bHLH neurogenic function is the formation of the sensory organs of the *Drosophila* peripheral nervous system. Here, bHLH proteins function first as 'proneural' factors by selecting proneural precursors (PNPs; cells competent, but not committed, to differentiate as neurons) and then as selector factors by establishing sensory organ precursors (SOPs; cells committed to give rise to the sensory neurons and their accessory cells) (Giangrande and Palka, 1990; Ghysen and Dambly-Chaudierre, 1993).

In the fly auditory organ (Johnston's organ, JO), the stretch receptors (chordotonals, CH) and the eye, this neurogenic role is fulfilled by the bHLH protein Atonal (Ato) (Jarman et al., 1993; Jarman et al., 1994; Jarman et al., 1995). However, because *ato* is crucially required in all three organ types, additional factors must contribute to the specification of sensory organ type. The selector factors controlling regional specificity and the establishment of organ primordia are ideal candidates to play this role. Hence, a co-ordination or integration of the genetic networks controlling neuronal specification and organ identity would be necessary to ensure the correct spatial and temporal deployment of the two pathways and the formation of appropriate sensory structures.

In the case of the fly eye, co-expression of several selectors, including the conserved transcription factors Eyeless/Pax6 (Ey), Sine oculis (So), and Eyes absent (Eya), specifies the pool of cells competent to give rise to photoreceptor neurons and other eye cell types (the eye primordium). These and other factors are linked in a complex genetic cascade called the Ey/Pax6 pathway or Retinal Determination (RD) network. Although the onset of gene expression is initially sequential, their transcription soon becomes subject to extensive cross- and feedback regulation (Pappu and Mardon, 2004; Silver and Rebay, 2005). It is these factors that are thought to determine formation of *ato*-dependent photoreceptor neurons in the eye, rather than mechanoreceptors as in the JO or CH. However, at what level the input from RD factors is integrated with the genetic control of neuronal development is poorly understood and current views vary greatly.

Compelling evidence, collected over the past 12 years, documents the crucial role of the Pax6 pathway in driving eye formation. Among the most dramatic is the observation that misexpression of Ey, or several other RD factors, results in the formation of ectopic eyes (Halder et al., 1995; Bonini et al., 1997; Pignoni et al., 1997; Chen et al., 1997). These and other studies have led to a model of eye specification that places Ey and the RD network at the top, in the role of master controller of eye development (Gehring, 1996; Gehring and Ikeo, 1999). In this model, the RD network would induce expression of *ato* and directly control neurogenesis in the eye, as well as induce additional factors that confer the appropriate neuronal-type specificity.

More recently, an intriguing alternative, that emphasizes the use of *ato* in the formation of diverse sensory organs, has been proposed (Niwa et al., 2004). In this model, *ato* transcription depends on a 'Pax6-independent' enhancer that is utilized in all *ato*-related sensory organs, reflecting the regulation of *ato* in an ancestral protosensory organ. The modification of this *ato*-driven neurogenic program by a Pax6-based head-specification pathway would then result in the formation of the eye and its photoreceptor neurons. Thus, Ey/Pax6 would function parallel to or downstream of Ato expression and sensory organ formation, rather than being an upstream master control gene (Niwa et al., 2004).

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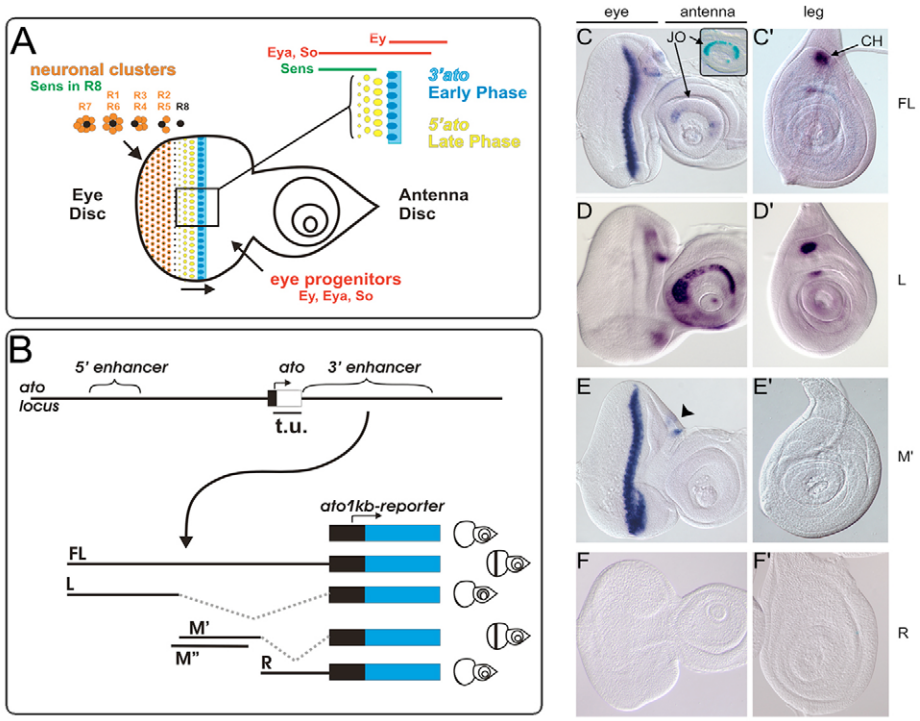

Fig. 1. Independent regulation of *ato* in the eye and other sensory organs. (**A**) Schematic summarizing neuronal morphogenesis in the eye primordium, which begins early in the third larval stage (L3), when eye progenitor cells initiate transcription of *ato*. Development of the ~800 single eyes or ommatidia occurs progressively, as consecutive rows of eye progenitors from posterior to anterior (horizontal arrow) begin to differentiate. This transition is marked by a visible indentation in the epithelium called the morphogenetic furrow (MF). Thus, in the L3 eye disc, one can visualize cells at various stages of development. Anterior to the Ato-positive cells lie retinal progenitors expressing the eye-specification or RD factors Ey, So and Eya. Posterior to the *ato* domain, one finds differentiating neuronal clusters and accessory cells in progressively more advanced stages of development (Wolff and Ready, 1993). The enlarged diagram (upper right) shows the early (blue and dark blue) and late (yellow) phases of *ato* transcription. The position of *ato* expression relative to the Senseless and Eya domains is indicated by the green (Senseless) and red (Eya) bars. (**B**) Construct maps and relationship to the genomic *ato* region. The *3′ato$^{M'}$-βgal* (2.1 kb) and *3′ato$^{M''}$-βgal* (2 kb) constructs, which overlap by 1.5 kb but differ at both ends, drive expression in essentially the same pattern. (**C-F′**) In situ hybridization to *LacZ* mRNA (except for inset in C). In all images, discs are oriented posterior to the left and dorsal up. (C,C′) *3′ato^{FL}-βgal* mRNA reflects *ato* expression in the eye (C), JO (C and C inset) and CH (C′). The inset in C shows overnight staining for β-gal activity. Arrows point to JO in the antenna (C) and femoral CH in the leg (C′). Expression in the antenna occurs at a much lower level than in the eye. (D,D′) *3′ato^{L}-βgal* is expressed in JO (D) and femoral CH (D′). Diffuse low level expression can also be detected upon longer staining anterior to the MF and throughout the antennal disc, but not at the sites of endogenous *ato* expression by the MF or in the ocellar regions. (E,E′) *3′ato$^{M'}$-βgal* drives expression in the eye (E), but not in antenna (E) or leg (E′). Expression is also observed in the ocellar region (arrowhead). (F,F′) *3′ato^{R}-βgal* does not drive expression in eye, antennal or leg discs.

These two models propose different views of how organ specification and neuronal development are integrated during eye formation. However, our current understanding of *ato* regulation is still very rudimentary and a detailed dissection of the *ato* control region is needed to better address this issue.

Expression of the *atonal* gene during eye morphogenesis is very dynamic (Fig. 1A). Gene expression is controlled at the transcriptional level and begins in rows of eye progenitor cells that form a dorsoventral stripe across the eye epithelium (or eye disc) (blue stripe in Fig. 1A). Soon after, the *ato*-positive progenitors begin to regulate *ato* transcription differentially and groups of high-*ato*-expressing cells (called initial clusters) become visible within the stripe. Next, cells located in between the clusters lose *ato* expression completely leading to the appearance of separate groups of *ato*-expressing cells called intermediate clusters. Lastly, the intermediate clusters resolve into single *ato*-positive cells which become R8 neurons, the founder photoreceptor neuron of each single eye or ommatidium (Jarman et al., 1994; Jarman et al., 1995).

This dynamic pattern of *ato* expression is achieved through two separate control regions. Genomic DNA flanking the gene on the 3′ side (*3′ato*) controls the early phase of *ato* expression, i.e. the activation of gene expression in eye progenitor cells as well as formation of the initial clusters (Fig. 1A,B) (Sun et al., 1998). Continued expression, first in intermediate clusters and then in the R8 SOPs, depends instead on regulatory elements that lie 5′ of the *ato* transcription unit and require Ato function (Fig. 1A,B) (Sun et al., 1998). Interestingly, the *3′ato* regulatory region also promotes expression in the JO and the femoral CH (Fig. 1C,C′). It has been proposed that expression in all three sensory organs is driven by a common enhancer (Niwa et al., 2004). However, this hypothesis has not been directly tested.

To better understand the relationship between eye specification and *ato*-driven neuronal development, we chose to investigate the transcriptional activation of *ato* in eye progenitor cells. Through a detailed analysis of the *3′ato* control region, we find that the regulatory elements driving expression in the eye are distinct from those driving expression in JO and CH. Moreover, the RD factors Ey and So directly bind adjacent cis-regulatory sites present within a minimal *3′ato* enhancer region. Ey and So bind in vitro and the insertion of a few base pairs between binding sites disrupts reporter gene expression in vivo. Thus, Ey-So protein-protein interactions may play a significant role in the regulation of *ato* transcription. This study provides insights into the molecular mechanisms that mediate the integration of eye specification and neurogenic pathways, and the developmental transition from organ specification to differentiation during eye morphogenesis.

MATERIALS AND METHODS

Genetics

Fragments of the *3′ato* region were generated by PCR. Mutations, deletions and insertions were introduced using the PCR-Based Site-Directed Mutagenesis Kit (Stratagene). Sequences of all primers and details of cloning are available upon request. All constructs were confirmed by sequencing. DNA sequences were aligned using ClustalW. *P*-element

transformation was performed using vectors pCasper-β-gal and pStinger (Rubin and Spradling, 1982; Barolo et al., 2000). Other fly lines used: *so*^{1}*; eya*^{2}*; UAS-ey; UAS-so; UAS-eya* (Cheyette et al., 1994; Pignoni et al., 1997); and *P{GAL4-dpp.blk1}40C.6* (FBti0002123).

Histology

Standard antibody (Ab), β-gal and in situ protocols were used (Sullivan et al., 2000). Ab used: mAb anti-Elav (1:1000) (Robinow and White, 1991), mAb anti-Eya (1:100) (Bonini et al., 1993), rabbit anti-β-gal (1:1000) (Cappell), guinea pig anti-Sens (1:1000) (Nolo et al., 2000), anti-GFP (1:1000; Upstate Biotech). Anti-mouse, anti-rabbit and anti-guinea pig Cy2-, Cy3- or Cy5-conjugated (Jackson Immuno Research Laboratories) or hrp-conjugated (Bio-Rad) secondary Ab were used at 1:200 dilution. Confocal microscopy was performed using a Leica TCS microscope. DIG-labeled (Roche) in situ probes were generated from pCRII-TOPO-*lacZ*.

Electrophorectic mobility shift assay (EMSA)

Ey and Toy were produced using a reticulocyte lysate in vitro transcription-translation system (Promega). Either the full-length So protein produced in S2 cells, or a GSTSo^{SixHD}-fusion protein purified from *Escherichia coli* (Six and Homeobox domains) (Kenyon et al., 2005) were used. DNA probes were generated by PCR or by annealing of synthesized oligonucleotides, and were labeled using polynucleotide kinase (NEB) and [γ-^{32}P]ATP (Amersham). The binding reaction was carried out in 15 μl 10 mM HEPES pH 7.9, 35 mM KCl, 10 mM NaCl, 4 mM $MgCl_2$, 20% glycerol, 1 mM DTT, 1 mM EDTA, 0.06 mM PMSF, 0.1% BSA, 10,000 cpm probe, 1 μg poly(dI-dC) (Pharmacia) and 5 out of 50 μl of the in vitro transcription-translation reaction. After incubating 20 minutes at room temperature, samples were analyzed in a 5% non-denaturing polyacrylamide gel followed by autoradiography. For competition experiments, a 40 molar (Ey, Toy) or 100 molar (GSTSo^{SixHD}) excess of unlabeled DNA was added and incubated for 5 minutes at room temperature before adding the radioactive probe.

GST-pull-down

GST and GST-So^{SIXHD} proteins were induced in BL21 cells, and purified with glutathione-agarose beads (Pierce). Full-length [^{35}S]Ey, [^{35}S]luciferase (negative control) or [^{35}S]LexA-Eya^{-} domain-fusion (positive control) proteins were produced by in vitro transcription-translation in a total volume of 50 μl (Promega). For each pull-down experiment, 15 μl of in vitro translated [^{35}S]protein was diluted in 150 μl PBT (PBS, 1% Triton X-100), mixed with either GST or GST-So^{SIXHD} immobilized on glutathione-agarose beads, and incubated at 4°C for 2 hours. Beads were washed four times with PBT and boiled in 20 μl 2×SDS loading buffer before electrophoresis (10% SDS-PAGE) and autoradiography.

RESULTS

The 3′ control region contains multiple cis-regulatory modules

To begin with, we sought to establish whether the activation of *3′ato*-dependent transcription in eye, JO (antenna disc) and femoral CH (leg disc) falls under a common cis-regulatory module (CRM), or whether the 3′ region contains separate, disc-specific enhancers. As a first step, we reproduced the findings of Sun and colleagues (Sun et al., 1998) by generating a *3′ato*^{FL}*-βgal* construct that contains the entire *3′ato* fragment (Fig. 1B) as well as the endogenous *ato* promoter region (a 1039 bp fragment that includes 334 bp of the 5′-UTR). As previously described (Sun et al., 1998; Niwa et al., 2004), expression was observed in the eye disc (Fig. 1C), the antenna (Fig. 1C inset) and the leg discs (Fig. 1C′).

Next, we generated three different reporters containing fragments of *3′ato*^{FL}: a 2.9 kb left fragment (*3′ato*^{L}*-βgal*), a 2.1 kb middle fragment (*3′ato*$^{M'}$*-βgal*) and a 1.8 kb right fragment (*3′ato*^{R}*-βgal*) (Fig. 1B). Multiple independent insertions were recovered and analyzed for each construct (see Fig. S1 in the supplementary material). We found that the *3′ato*$^{M'}$*-βgal* could reproduce all aspects of early *ato* expression within the eye disc (Fig. 1E) and a closely related *3′ato*$^{M''}$ fragment was able to drive transcription in essentially the same pattern (*3′ato*$^{M''}$*-βgal* and *3′ato*$^{M''}$*-GFP* constructs) (Fig. 1B, Fig. 2B and see Fig. S1 in the supplementary material). The M’ and M’’ reporters also showed expression in the ocelli (photoreceptor-containing sensory structures derived from the dorsal region of the eye disc) (Fig. 1E and see Fig. S1 in the supplementary material). No expression was detected in the leg or antennal discs (Fig. 1E,E′).

The expression associated with the JO and CH in antenna and leg discs was observed, instead, in the *3′ato*^{L}*-βgal* lines (Fig. 1D,D′). The level of expression in the antenna was consistently higher in these lines when compared with *3′ato*^{FL}*-βgal.* The retina-related stripe and the ocellar expression domain were absent (Fig. 1D). Lastly, lines carrying the *3′ato*^{R}*-βgal* did not express the reporter in eye, antenna or leg discs (Fig. 2F,F′).

We conclude, therefore, that the 3′ flanking genomic region, similarly to the 5′ regulatory region (Sun et al., 1998), contains multiple CRMs that control *ato* expression in different sensory organs.

A 348 bp fragment contains a ‘core’ eye enhancer

To more precisely identify the region responsible for expression in the eye disc, we further dissected the *3′ato*$^{M''}$ DNA. This analysis resulted in the identification of a 348 bp fragment that is necessary and sufficient to promote reporter gene expression in eye progenitor cells. As summarized in Fig. 2A (and see Fig. S1 in the supplementary material), all reporter constructs that contained this 348 bp region showed expression in a stripe. These include the constructs *3′ato*$^{M''}$*-βgal*, *3′ato*$^{1.2}$*-βgal* and *3′ato*^{348}*-βgal* (Fig. 2B,D,E), *3′ato*^{FL}*-βgal* and *3′ato*$^{M'}$*-βgal (*Fig. 1C,E), *3′ato*$^{M'R}$*-βgal* and *3′ato*^{488}*-βgal* (see Fig. S1 in the supplementary material). On the other hand, all constructs that lacked this region did not activate the reporter in eye progenitors, including *3′ato*$^{M''\text{-}\Delta 348}$*-βgal* (Fig. 2C), *3′ato*^{L}*-βgal*, *3′ato*^{R}*-βgal* (Fig. 1D,F), and *3′ato*$^{M''\text{-}\Delta 488}$*-βgal* (see Fig. S1 in the supplementary material).

Interestingly, the 348 bp region contains two relatively large (A1=99 bp and A2=140 bp) DNA sequences that are highly conserved from *D.* melanogaster to *D. virilis* (Fig. 2A, and see Fig. S2 in the supplementary material). Based on this observation, we also generated constructs containing only A1 or A2 (*3′ato*^{A1}*-βgal* and *3′ato*^{A2}*-βgal)* (Fig. 2A). However, we found that neither region alone was sufficient to drive the stripe of reporter gene expression in the eye disc (see Fig. S3 in the supplementary material).

Based on these results, we conclude that the 348 bp region constitutes a ‘core’ or ‘minimal’ enhancer region for the transcriptional activation of *ato* in eye progenitor cells.

Additional regulatory elements lie outside the 348 bp core region

Subtle but significant differences in expression were apparent between reporter lines. Constructs containing the 1.2 kb DNA region closely mimicked early *ato* expression, including low level expression in a stripe and stronger expression in the initial clusters (*3′ato*^{FL}*-βgal*, *3′ato*$^{M'R}$*-βgal*, *3′ato*$^{M'}$*-βgal*, *3′ato*$^{M''}$*-βgal*, *3′ato*$^{1.2}$*-βgal*) (Fig. 1C,E, Fig. 2B,D,D′, and see Fig. S1 in the supplementary material). On the other hand, reporters containing the core enhancer region but within fragments smaller than 1.2 kb (*3′ato*^{488}*-βgal* and *3′ato*^{348}*-βgal*) reproduced some, but not all, aspects of this pattern. First, and perhaps not surprisingly, the latter constructs consistently displayed a lower level of expression than the larger ones (see Fig. S1 in the supplementary material). Second,

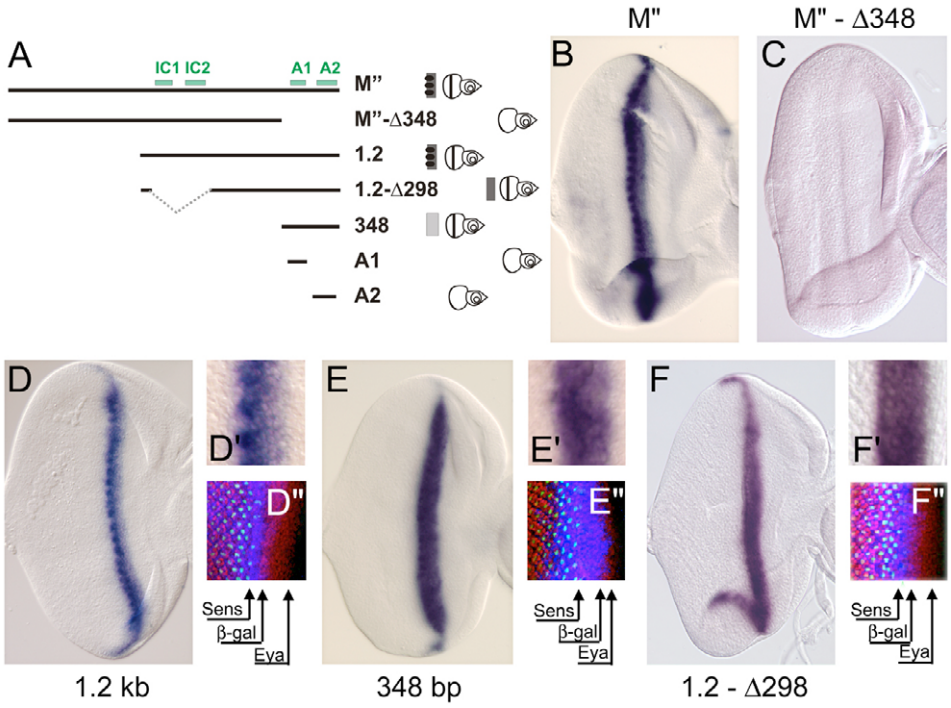

Fig. 2. A 348 bp fragment contains a core module for *ato* activation in eye progenitors. (**A**) Schematic showing the DNA fragments driving reporter gene expression in the different constructs. The green bars IC1, IC2, A1 and A2 mark the relative positions of the evolutionarily conserved DNA sequences investigated in this work. (**B-F″**) All panels (except D″,E″,F″) show in situ hybridization to *LacZ* mRNA. Staining times differ and were optimized for visualization of either the entire stripe (large panels) or the initial clusters (small panels). For a summary of relative levels of expression for all constructs see Fig. S1 in the supplementary material. Panels D″,E″,F″ show confocal images of triple stainings for the Sens (green), β-gal (blue), and Eya (red) proteins. (B) Early phase of *ato* expression as reported by *3′ato$^{M″}$-βgal*. (C) Loss of eye disc expression in *3′ato$^{M″-Δ\ 348}$-βgal*. A faint signal could be detected along the margins of the eye disc after overnight β-gal staining (not shown). (D-D″) *3′ato$^{1.2}$-βgal* is sufficient to drive high level reporter gene expression in the early *ato* pattern including the initial clusters (D,D′). The anterior margin of β-gal protein expression (blue) lies anterior to Sens (green) but posterior to the anterior margin of the Eya domain (red) (D″). (E-E″) *3′ato^{348}-βgal* drives reporter gene expression in a stripe (E). However, the initial clusters do not form (E′). Activation of the reporter gene (blue) occurs in more anterior progenitors as shown by the shift of the anterior margin of β-gal protein expression (blue) farther away from Sens (green) and closer to the anterior border of the Eya domain (red) (E″). (F-F″) *3′ato$^{1.2-Δ298}$-βgal* drives reporter gene expression in a stripe (F). However, the initial clusters do not form (F′). The anterior margin of β-gal protein expression (blue) appears to be minimally affected (F″).

the differential regulation of *ato* expression that leads to formation of the initial clusters was not observed in *3′ato^{488}-βgal* or *3′ato^{348}-βgal*. In these lines, mRNA levels remained relatively uniform across the stripe (Fig. 2E,E′, and data not shown). Third, extra rows of anterior progenitor cells expressed *ato*, thereby generating a broader stripe. This can be clearly seen when comparing the position of the anterior border of the reporter expression domains relative to the domains of two molecular markers: Eya, a protein expressed in all progenitors anterior to the MF, and Senseless (Sens), a protein first expressed just posterior to the early *ato* domain (i.e. at the level of intermediate *ato* clusters) (Fig. 1A, Fig. 2D″). In *3′ato^{348}-βgal* and *3′ato^{488}-βgal,* the anterior margin of the β-gal domain was shifted forward, closer to the anterior border of the Eya domain and farther away from Sens-expressing cells (Fig. 2D″,E″, and data not shown).

Phylogenetic comparison of the 1.2 kb DNA among different *Drosophila* species identified two additional conserved sequences that lie outside the 488 bp region (IC1=88 bp and IC2=133 bp) (Fig. 2A, and see Fig. S2 in the supplementary material). To investigate whether these sequences contained cis-regulatory sites controlling expression level, induction in progenitor cells and/or formation of the initial clusters, we tested the effect of removing a 298 bp region that spans these sequences from the 1.2 kb fragment. Reporter gene expression driven by a *3′ato$^{1.2-Δ298}$-βgal* construct was similar to expression of the *3′ato^{348}-βgal* transgenes in that the level of expression was lower and the initial clusters did not form (Fig. 2, compare E′,F′,D′). However, the reporter did not appear to be as precociously activated as *3′ato^{348}-βgal* (Fig. 2, compare E″,F″,D″).

These results showed that in addition to the control of gene activation (which resides within the 348 bp region), at least two more enhancers are present within the 1.2 kb fragment, including a clusters formation/expression level regulatory region and an anterior repression module.

The core region is regulated by RD factors

To further explore the relationship between the RD network and *ato*, we focused on the core region responsible for activation. Genetic evidence strongly suggests that the RD factors are required, directly or indirectly, for transcriptional activation of *ato*. Several strong mutant alleles of the RD genes display ‘eyeless’ phenotypes in the adult and lack expression of *ato* in the L3 eye disc (Bonini et al., 1993; Cheyette et al., 1994; Jarman et al., 1995). Hence, we chose to investigate how the *3′ato^{348}-βgal* construct responded to loss or gain of RD network function and assayed reporter gene expression in: (1) discs lacking the activity of the RD factors So or Eya; (2) discs in which Ey, or So and Eya, are ectopically expressed and induce ectopic eyes; and (3) *eya* or *so* mutant discs in which Ey is ectopically expressed but can not induce ectopic eyes.

In order to severely reduce RD network function, we opted to use strong mutant alleles of the *eya* and *so* genes: *eya^{2}*, a 100% penetrant eye-specific null allele, and *so^{1}*, an eye-specific allele that displays the null eyeless phenotype with ~95% penetrance. Expression of the *3′ato^{348}-βgal* reporter was absent in all *eya^{2}/eya^{2}* discs and in nearly all *so^{1}/so^{1}* discs (Fig. 3A-C).

Conversely, in gain of function experiments, we observed ectopic induction of the reporter in the wild-type but not in *eya* or *so* mutant backgrounds. To drive Ey or So+Eya expression in tissue other than

the eye, we employed the Gal4/UAS system (Brand and Perrimon, 1993). *UAS-ey* or *UAS-So+UAS-eya* transgenes were ectopically induced under the control of the *dpp-Gal4^{blk}* driver and reporter gene expression was assayed within the antenna, leg and wing epithelia. When Ey or So+Eya were expressed in a wild-type background, *3′ato^{348}-βgal* expression was detected ectopically in all three discs (Fig. 3D-F, and data not shown). However, *Gal4*-driven expression of Ey in *so^{1}* or *eya^{2}* mutant discs did not result in activation of the reporter (Fig. 3G, and data not shown).

We conclude that the core eye enhancer is regulated as would be expected for the endogenous *ato* gene by the RD network.

Ey and So bind to cis-sites required in vivo for reporter gene expression

As mentioned above, deletion of the 348 bp region (*3′ato$^{M'-\Delta 348}$-βgal*) resulted in loss of reporter gene expression in eye progenitor cells (Fig. 2C). In addition, removal of the A1 conserved region (*3′ato$^{M''-\Delta A1}$-βgal*) was sufficient to prevent reporter gene expression (see Fig. S1 in the supplementary material). Interestingly, within the A1 region, we identified sequences matching the consensus binding sites for the transcription factors Pax6/Ey (paired domain) and So (Fig. 4A and see Fig. S2 in the supplementary material) (Niimi et al., 1999; Ostrin et al., 2006; Pauli et al., 2005). The presence of potential binding sites raised the possibility that Ey and/or So directly regulate *ato* expression.

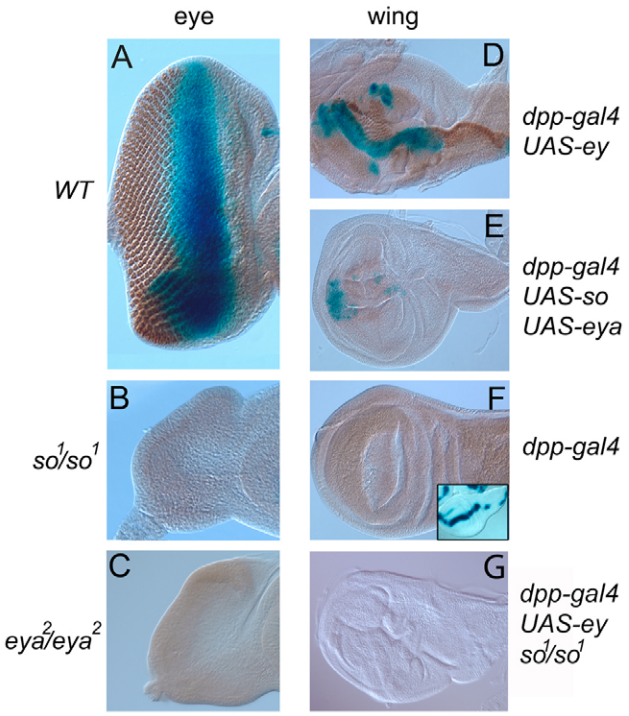

Fig. 3. Genetic control of *3′ato^{348}-βgal* expression by RD factors. (**A-C**) Wild-type L3 eye discs (A) stained for β-gal (blue) to detect expression of the *3′ato^{348}-βgal* reporter, and for the pan-neural marker Elav (brown) to detect developing neuronal clusters. The *3′ato^{348}-βgal* reporter is not expressed and neurons do not form in *so^{1}* (B) or *eya^{2}* (C) mutant discs. (**D-F**) *dpp-Gal4*-driven expression of Ey (D) or So+Eya (E) in the wing disc activates expression of the *3′ato^{348}-βgal* reporter. A negative control (*dpp-gal4* alone) is shown in F; the inset shows the pattern of Gal4 expression in a *dpp-Gal4 UAS-lacZ* wing disc. (**G**) *dpp-Gal4*-driven expression of Ey (D) is not sufficient to induce the *3′ato^{348}-βgal* reporter in the *so^{1}* mutant.

To test this hypothesis, we carried out electrophoretic mobility shift assays on DNA fragments spanning a ~500 bp region around these sites (Fig. 4B). The fragment containing the two sites, probe III, could be shifted by either Ey, So or GST-So^{SixHD} (see Materials and methods) (Fig. 4, lanes 1-7 in C,D, and data not shown). In all

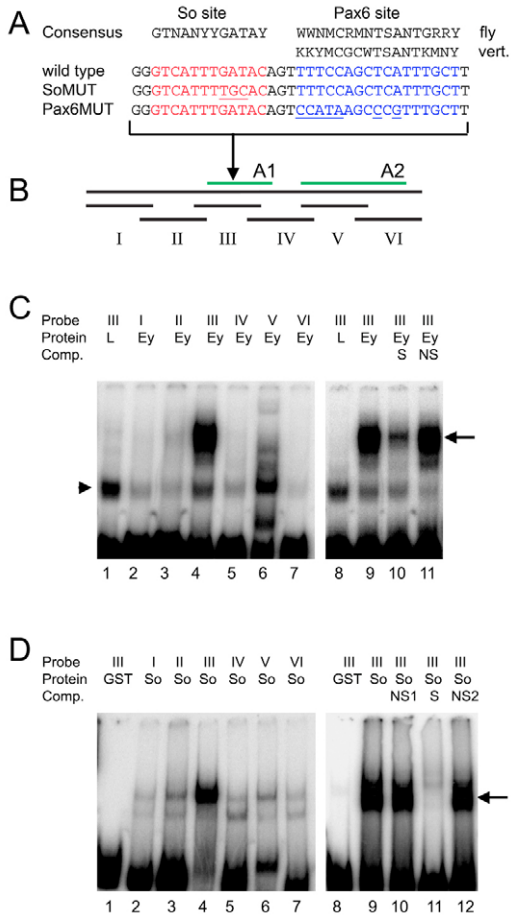

Fig. 4. Ey and So bind specifically to the core eye-enhancer. (**A**) Sequence of two potential binding sites for So (red) and Pax6 (blue) present in probe III. Consensus sequences are shown above. The sequence changes introduced to disrupt protein-DNA interactions are underlined. (**B**) Schematic showing the six overlapping probes (I-VI) used in EMSA. Green bars mark the conserved sequences A1 and A2. The relative position of the So and Pax6 sites within A1 is indicated. (**C**) EMSA with Ey protein. The position of the shifted Ey-III complex is marked by an arrow. Ey shifts probe III (lane 4) but not other probes (lanes 2,3,5,6,7). The binding of Ey to probe III can be competed by unlabeled III DNA (lane 10) but not by unlabeled Pax6^{MUT}-III DNA (lane 11). Probes (I-VI), protein (Ey) and/or competitor DNA (S,NS) added to each reaction are listed above each lane. S, specific: non-labeled probe III DNA. NS, non-specific: non-labeled probe III with the Pax6^{MUT} site. Reticulocyte lysate (L) only was added in the negative controls (lanes 1,8). The lower band (arrowhead) reflects a non-specific shift due to the lysate. (**D**) EMSA with So protein (GSTSo^{SixHD}). The position of the shifted GSTSo^{SixHD}-III complex is marked by an arrow. GSTSo^{SixHD} shifts probe III (lane 4) but not the other probes (lanes 2,3,5,6,7). The binding of So to probe III can be competed by unlabeled DNA with a So-binding site, but not by unlabeled So^{MUT}-III DNA. Probes (I-VI), protein (GSTSo^{SixHD}) and/or competitor DNA (S,NS1,NS2) added to each reaction are listed above each lane. GST was added to the negative controls (lanes 1,8). S, unlabeled so^{AE} oligo [So-binding site from Pauli et al. (Pauli et al., 2005)]; NS1, unlabeled probe II DNA; NS2, unlabeled probe III with the So^{MUT} site.

cases, competitive binding assays showed that protein binding was specific. Binding was competed by unlabelled probe III DNA or by oligomers containing consensus binding sites, but not by probe III DNA in which either binding site had been mutated (Fig. 4, lanes 8-11 in C and lanes 8-12 in D).

As might be expected, Toy, another Pax6-type factor that functions in eye development, also binds the Pax6 consensus site specifically (see Fig. S4 in the supplementary material). However, it is unlikely that Toy plays a crucial role in directly regulating *ato* expression in vivo because the *3′ato*^{348}*-βgal* reporter could be induced in the absence of Toy protein. In fact, misexpression of Ey induces ectopic eyes and the *3′ato*^{348}*-βgal* reporter (Fig. 3D-F), but not expression of *toy* (Czerny et al., 1999). By contrast, ectopic Ey activates transcription of the *so* gene and So+Eya induces *ey* (Pignoni et al., 1997; Halder et al., 1998). Hence, Ey and So would both be present to activate the *3′ato*^{348}*-βgal* reporter.

To test whether the Ey- and So-binding sites were required in vivo, we generated reporter constructs containing either a mutated Pax6 (*3′ato*^{348Pax6MUT}*-βgal*) or a mutated So (*3′ato*^{348SoMUT}*-βgal*) site, as well as a 1.2 kb construct in which both sites were altered (*3′ato*$^{1.2Pax6\text{-}SoMUT}$*-βgal*). The base pair changes introduced had been reported to reduce binding affinity to consensus sites (Ostrin et al., 2006; Pauli et al., 2005; Punzo et al., 2002; Niimi et al., 1999). In all cases, reporter gene expression was severely affected, from strongly reduced to completely absent (Fig. 5 A-D).

In summary, Ey and So can specifically bind sites within the core eye element in vitro and these sites are required for reporter gene expression in vivo.

So and Ey directly bind in vitro and may interact in vivo

Strikingly, the So and Pax6 sites are located adjacent to one another (Fig. 4A), being separated by exactly 3 bp in all sequenced *Drosophila* species (see Fig. S2 in the supplementary material and http://insects.eugenes.org/species/). This arrangement raised the possibility that bound So and Ey may interact with each other and that such interactions are required in vivo. In order to explore these hypotheses, we tested for direct binding in vitro and investigated the significance of the spacing between their cis binding sites in vivo.

For the in vitro assay, Gst-pull-down assays were carried out. In these experiments, a GST-So^{SixHD} fusion protein containing the evolutionarily conserved domains showed a clear interaction with full-length Ey protein (Fig. 5E). As negative controls, GST alone did not bind Ey, and GST-So^{SixHD} did not bind luciferase (Fig. 5E). Thus, Ey and So can directly interact at the protein level.

To explore the in vivo significance of this interaction, we reasoned that the conserved 3 bp spacing between sites is likely to be crucial for such interaction. Due to the helical structure of the DNA, differences in spacing between binding sites affect the spatial arrangement of bound factors not only by altering the distance between the proteins, but, perhaps more importantly, by altering the orientation of the bound factors relative to one another. Since ~11 bp span a complete (360°) rotation of the helix, the insertion of only a few bp in between sites is all that is needed to achieve misorientation. Thus, we generated two constructs: one with a 3 bp insertion between sites (*3′ato*$^{348+3A}$*-βgal*) and one with a 6 bp insertion (*3′ato*$^{348+6A}$*-βgal*), thereby introducing a rotation between sites of ~90° and ~180°, respectively. We predicted that, if interactions between So and Ey were not crucial, both insertions (3 and 6 bp) would be likely to have little or no effect on reporter gene expression. On the other hand, if Ey-So interactions were essential, the 6 bp insertion, and possibly the 3 bp insertion as well, would disrupt transcriptional regulation. We found that the latter was indeed the case. We detected a modest but consistent drop in expression levels in the *3′ato*$^{348+3A}$*-βgal* transgenic lines (Fig. 5F). Moreover, a severe reduction in reporter gene expression was observed with the *3′ato*$^{348+6A}$*-βgal* construct (Fig. 5G).

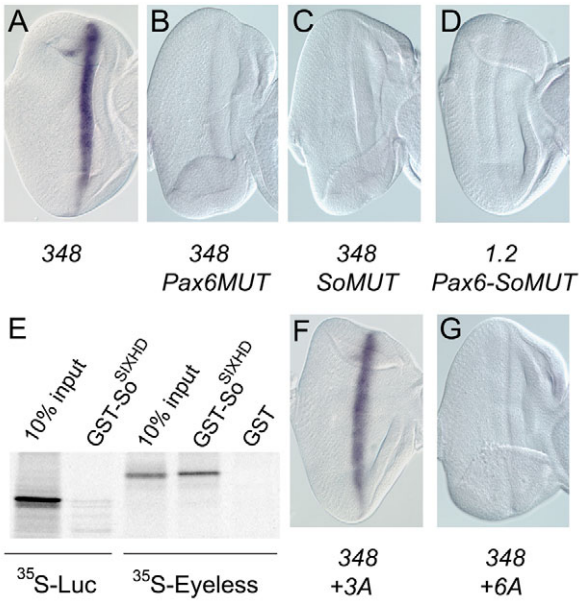

Fig. 5. In vivo requirement for cis sites and direct interaction between Ey and So. (**A-D**,**F**,**G**) Detection of *LacZ* mRNA by in situ hybridization; in all cases, the staining reaction was carried out for 3 hours. (A) *3′ato*^{348}*-βgal* reporter gene expression. (B) The introduction of several base-pair changes in the Ey-binding site results in loss of reporter gene expression in *3′ato*^{348Pax6MUT}*-βgal*. (C) The introduction of several base-pair changes in the So-binding site results in loss of reporter gene expression in *3′ato*^{348SoMUT}*-βgal*. (D) The introduction of multiple base-pair changes in both So- and Ey-binding sites results in loss of reporter gene expression in *3′ato*$^{1.2Pax6\text{-}SoMUT}$*-βgal*. A faint signal as a stripe and/or along the margins of the eye disc was detected in some of the transgenic lines after overnight β-gal staining (not shown). (**E**) Ey and So can directly interact at the protein level. A GST-So^{SixHD} fusion protein (containing the Six and homeobox domains) binds full-length Ey protein. GST alone does not bind Ey, and GST-So^{SixHD} does not bind luciferase. 10% of the ^{35}S-labelled proteins and 100% of the pulled-down yields are shown. (F) Insertion of three As between the So- and Ey/Pax6-binding sites in *3′ato*$^{348+3A}$*-βgal* results in a reduction in reporter gene expression. (G) Insertion of six As between the So- and Ey/Pax6-binding sites in *3′ato*$^{348+6A}$*-βgal* severely affects reporter gene expression. A weak signal was detected in some of the transgenic lines after overnight β-gal staining (not shown).

These results show that Ey and So interact directly in vitro. The observation that small insertions between their binding sites affect reporter gene expression is consistent with transcriptional regulation by an Ey-So complex in vivo.

DISCUSSION

Modular control of early *ato* expression in the eye

The regulatory elements controlling the early phase of *ato* expression in the eye lie within a 1.2 kb region located 3.1 kb downstream of the *ato* transcription unit. The early phase of *ato* transcription results from the integration of multiple regulatory inputs through separate cis-regulatory modules present within the 1.2 kb region (Fig. 6A).

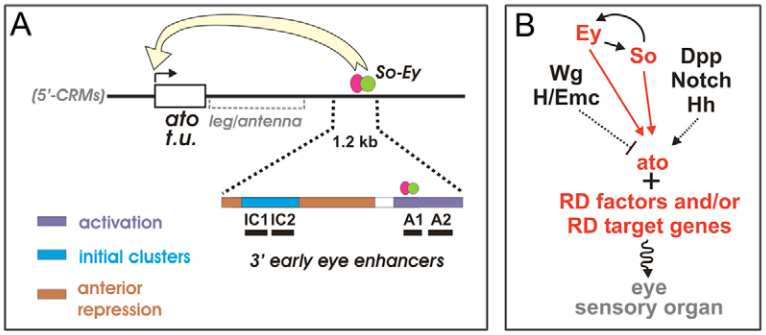

Fig. 6. Regulation of *ato* and integration of the *ey/Pax6* and *ato* pathways. (**A**) The regulatory elements controlling the early phase of *ato* expression in the fly eye lie within a 1.2 kb region located 3.1 kb downstream of the *ato* transcription unit (t.u.). The early-*ato* stripe and its intermediate clusters result from the integration of multiple regulatory inputs through separate enhancers. A likely model for the regulation of *ato* by the RD network involves the formation of an Ey-So (or Ey-So-Eya) complex onto adjacent Pax6 and So cis-regulatory sites present within the evolutionarily conserved A1 box. (**B**) Proposed integration of eye specification and neurogenic inputs during eye development. The RD network directly controls *ato* transcription in eye progenitor cells and, most likely indirectly, modifies the neurogenetic program downstream of *ato*. Black arrows indicate regulatory interactions based on genetic and molecular evidence (solid) or genetic evidence only (dashed).

Cis-regulatory elements essential for gene activation map to the last 348 bp of the 1.2 kb region and include the So- and Ey-binding sites. Other factors undoubtedly bind to sequences within A1-A2 and regulate gene expression as neither A1 nor A2 alone are sufficient to drive expression in the eye disc. Genetic evidence suggests that signaling by the Bmp4-type factor decapentaplegic (Dpp) also contributes to *ato* activation and two putative binding sites for Mad (a transcription factor shown to activate Dpp pathway targets) appear to be required for *ato* expression in all discs (Niwa et al., 2004). However, a Mad consensus site present in the A2 box does not correspond to either one of the two elements identified by Niwa and colleagues. Moreover, both the previously identified sites lie within the L fragment well upstream of the M'-M" interval containing the eye-disc enhancers. A potential source for this discrepancy lies in the use of different promoters. Whereas Niwa and colleagues used the heterologous *hsp43* promoter, we relied on the endogenous *ato* promoter region (~700 bp of 5′ genomic DNA). Future analyses of 3′ enhancer-promoter interactions may resolve this issue.

Separate cis-regulatory elements located within the conserved DNA regions IC1 and IC2 control initial clusters formation. This feature of *ato* expression has been shown to require Notch (N) function. Sequence analysis of the IC1-IC2 region does identify a binding site for the effector of N signaling Suppressor of Hairless [Su(H)]. However, contradictory reports have been published on how Notch controls *ato* expression. Sun and colleagues (Sun et al., 1998) found that transcription of *ato* is uniformly upregulated upon inactivation of Notch in N^{ts1} mutant discs. By contrast, early Ato protein expression is severely reduced in null Notch mutant clones (Baonza and Freeman, 2001; Li and Baker, 2001). Since these experiments made use of different genetic reagents, it is difficult to interpret these results. Notch signaling may independently regulate *ato* expression at the mRNA and protein levels. Alternatively, the source of the discrepancy may lie in the use of different alleles, one hypomorphic (N^{ts1}), and the other null (N^{54l9}).

Lastly, activation of the *3′ato*^{348}*-βgal* reporter (core element) occurs prematurely as compared with endogenous *ato*. The *3′ato*^{348}*-βgal* mRNA is also found in cells lying just anterior to the proneural domain. Eye progenitors from this region are at a developmental stage referred to as pre-proneural and are characterized by the expression of the transcription factor Hairy (H) in addition to RD proteins (Greenwood and Struhl, 1999). In the absence of Hairy and its partner Extra Macrochaetae, neurogenesis begins precociously within the eye disc (Brown et al., 1995). Thus, Hairy contributes to the downregulation of *ato* expression and prevents precocious neurogenesis. Activation of the reporters *3′ato*^{348}*-βgal* and *3′ato*^{488}*-βgal* (but not *3′ato*$^{1.2}$*-βgal* or *3′ato*$^{1.2\text{-}\Delta 298}$*-βgal*) in pre-proneural cells suggests that cis-elements mediating anterior repression lie within the 1.2 kb DNA fragment but outside the IC and A boxes. Although a search for canonical Hairy-binding sites does not identify potential regulatory elements, additional short stretches of evolutionarily conserved DNA are present and may contribute to this and/or other aspects of *ato* regulation.

Direct control of *ato* by a potential Ey-So complex

Over the last few years, Ey and So have been shown to play a crucial role in the deployment and maintenance of the RD network by directly regulating the transcription of several eye-specification genes [*ey*, *so*, *eya*, *dachshund* (*dac*) and *optix*] (Niimi et al., 1999; Punzo et al., 2002; Ostrin et al., 2006; Pappu et al., 2005; Pauli et al., 2005). However, little is known about downstream targets of the RD cascade. Although So also activates the post-MF expression of *hedgehog* and *lozenge* (Yan et al., 2003; Pauli et al., 2005), this gene regulation is likely to reflect the late, differentiation-related functions of So (Pignoni et al., 1997). Thus, it is unclear how the RD factors induce eye formation and what aspects of the morphogenetic program they control directly.

Our results strongly suggest that the transcription factors Ey and So control activation of *ato* expression. This is the first example of a gene required during eye morphogenesis that is directly regulated by the RD network. The direct control of *ato* by Ey and So is a likely reason why ectopic eye induction by Eya+So or Dac depends on the activation of their upstream regulator *ey* (Pignoni et al., 1997; Chen et al., 1997). Other downstream targets may also be similarly controlled by multiple RD factors.

The in vitro and in vivo evidence presented here also suggest that Ey and So may form a complex when bound to the adjacent cis-regulatory sites in the *3′ato* core element (Fig. 6A). Together with the previously reported interactions of Eya-So and Eya-Dac (Pignoni et al., 1997; Chen et al., 1997), this finding raises the possibility that additional multimeric complexes involving several RD factors may also be involved in driving the transcriptional program for eye development. As originally proposed by Curtiss and Mlodzik (Curtiss

and Mlodzik, 2000), the observation that normal eye development is severely disrupted when one or another RD factor is over-expressed suggests that the RD proteins must be present at an appropriate level relative to one another. As all four proteins, Ey, Eya, So and Dac, have now been shown to interact in various combinations, the formation of such complexes and the recruitment of additional shared co-factors are likely to be sensitive to the relative concentration of RD factors present in eye progenitor cells.

The model of gene regulation exemplified by the control of *ato* transcription provides a strong rationale for the feedback regulatory loops that link late and early RD gene expression. This regulation is likely to play a crucial role in ensuring the presence of appropriate levels of all four RD factors to optimize complex formation and co-regulation of downstream targets.

Co-ordination of selector and neurogenic pathways and the evolution of *ato/Ath*-dependent sensory organs

As summarized in the introduction, current models for the co-ordination of organ identity and neurogenesis in the eye place the Pax6 pathway either upstream of, or in parallel to, the control of neurogenesis. The findings presented in this paper favor the former model. We have identified separate regions for the regulation of *ato* transcription in the eye versus other sensory organs (JO and CH). In addition, the presence of Ey- and So-binding sites that are required in vivo for reporter gene activation strongly suggests that endogenous *ato* expression is directly regulated by these factors. Thus, the RD network does not merely modify sensory organ development within the eye disc, but does, in fact, directly control it (Fig. 6B). In doing so, it also contributes to the co-ordination of selector and neurogenic inputs required to generate complex sensory structures such as the eye.

Is this regulatory relationship between Ey-So and *ato* ancestrally derived? That is, was the direct link between ancestral Pax- and Ath-like genes already established in the protosensory organ that gave rise to today's *ato*-dependent sensory structures? The association of Pax-, Six- and Ath-type factors with sensory perception is not restricted to photic sensation but extends to mechanoreception in diverse organisms including mouse, jellyfish and mollusks (Treisman, 2004; Piatigorsky and Kozmik, 2004; Fritzsch and Piatigorsky, 2005; Tessmar-Raible et al., 2005). In the jellyfish *P. carnea,* which lacks eyes but responds to a variety of environmental stimuli including light, expression of a putatively ancestral-like *PaxB* gene, *Six1/2*, *Six3/6* and *atonal-like 1* is associated with neuronal precursors found in the medusa tentacles (Groger et al., 2000; Kozmik et al., 2003; Stierwald et al., 2004; Seipel et al., 2004; Tessmar-Raible et al., 2005). Although the studies carried out in more basal metazoa consist mostly of analyses of gene expression and not function, this evidence does suggest that the association of Pax/Six/Ath-type factors and sensory organ development is ancient and may have been retained over more than 600 million years of evolutionary history.

It is possible that the mechanisms of transcriptional regulation uncovered between *Pax* and *Six* genes (Niimi et al., 1999; Pauli et al., 2005; Ostrin et al., 2006) and between *Pax/Six* and *ato* (this work) may have arisen early during evolution. Such regulatory interactions may have favored the continued association of *Pax/Six/Ath* as various modifications of their genetic cascades led to the development of more complex and diverse sensory organs. The investigation of *ato/Ath* gene regulation in other sensory organs and in basal metazoans is likely to clarify the evolutionary relationship among these pathways and the sensory modalities they control.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/24/4881/DC1

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