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Regulation of atonal expression and the role of proneural genes in neuronal type specification in Drosophila

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REGULATION OF ATONAL EXPRESSION AND THE ROLE OF PRONEURAL GENES IN NEURONAL TYPE SPECIFICATION IN DROSOPHILA

by

YAN SUN

DISSERTATION

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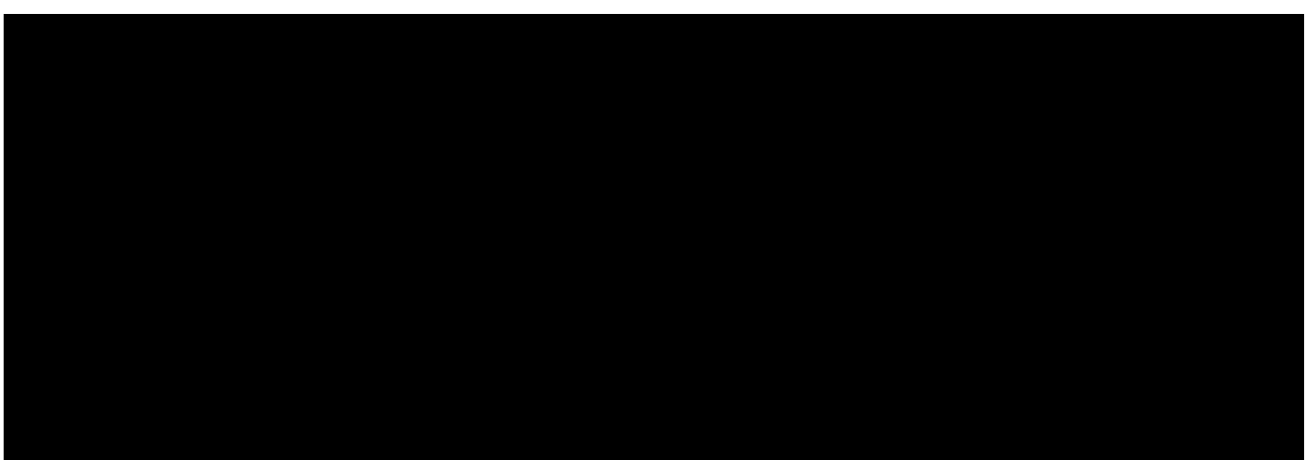

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This dissertation is dedicated to my parents, whose unconditional love and support make my today possible.

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Regulation of *atonal* expression and the role of proneural genes

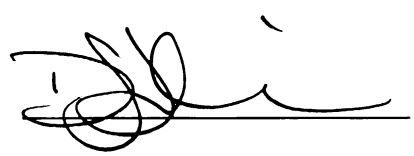
in neuronal type specification in *Drosophila*

Yan Sun

During development of the *Drosophila* peripheral nervous system, expression of proneural genes initiates the formation of sensory organs. The *achaete-scute* complex encodes proneural genes for external sensory organs, whereas *atonal* (*ato*) is a proneural gene for both chordotonal organs and photoreceptors.

The stereotyped expression patterns of proneural genes prefigure the positions of proneural clusters and subsequently the sensory organ precursors (SOPs), thereby determining the locations of future sensory organs. By analyzing various *ato-lacZ* reporter lines, I demonstrated that *ato* expression is controlled by multiple tissue-specific enhancers located 5' and 3' to the *ato* coding sequences. An Ato-independent regulatory region within 5.8 kb 3' to the *ato* coding sequence directs expression in the proneural clusters of chordotonal organs and in the initial stripe anterior to the morphogenetic furrow in the eye disc. In contrast, Ato-dependent modular enhancers located within 9.3 kb 5' to the *ato* coding region drive expression in the chordotonal organ precursors and in intermediate groups and R8 cells in eye discs. Further analysis revealed that the initial stripe of *ato* expression in the eye disc occurs in a prepattern whose formation requires the activity of *Notch*. The regulatory regions identified in this study can account for *ato* expression in every tissue and in essentially every stage of its expression during chordotonal organ and photoreceptor development.

From different proneural clusters, SOPs differentiate into distinct types of sensory organs. To address whether proneural genes affect neuronal subtype specification, I compared the ability of *ato* and *scute* in directing photoreceptor formation when ectopically expressed. I showed that *scute* is capable of inducing ommatidial formation in an *ato* mutant background that otherwise lacks ommatidia, although the photoreceptor composition of the *scute*-induced ommatidia appears to be different from that of *ato*-induced ommatidia. Occasionally, well developed external sensory organs are found in various locations in the *scute*-rescued eyes but not in the *ato*-rescued eyes. These experiments suggest that in addition to their roles in determining the neuronal fate, proneural genes also influence neuronal subtype identity.



Chair, dissertation committee

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CHAPTER 1

INTRODUCTION

Every multicellular organism relies on its cells to acquire appropriate fates during development for its proper performance. How a cell with a distinct fate emerges at an invariant position from a previously equivalent population of cells is a fundamental issue in biology. Studies of neuronal fate determination in the *Drosophila* peripheral nervous system (PNS) have provided valuable insights into understanding cell fate specification, owing to the highly precise and reproducible pattern of the PNS, relative simplicity of the organism, and powerful approaches of molecular and genetic manipulations.

Neuronal fate determination in the *Drosophila* nervous system (NS) is best understood in the case of the sensory nervous system. It is a progressive process involving a series of steps each controlled by distinct groups of genes (Ghysen and Dambly-Chaudiere, 1989), often interacting with each other. This process begins with prepattern genes activating expression of the so-called proneural genes in particular groups of ectodermal cells, the proneural clusters, thereby endowing these cells with neuronal competence. Shortly thereafter, cell-cell interaction results in elevated expression of proneural genes in only one or a few cells, the sensory organ precursor (SOP) cells, within each proneural cluster. As a consequence, these SOP cells fully commit to the neuronal fate. The decision of where and when to make a sensory neuron is controlled primarily by the spatiotemporal expression of proneural genes. In this thesis, I describe an analysis of the transcriptional regulation of one such proneural gene, the *atonal* (*ato*) gene, during the development of the *Drosophila* PNS (Chapter 2). The goal of this work is to understand how prepattern genes and genes involved in cell-cell communication regulate *ato* expression, which leads to neuronal fate specification in a precise fashion.

Once adopting a neuronal fate, the SOPs differentiate into distinct types of sensory neurons, a process previously thought to be controlled mainly by neuronal type selector genes. In Chapter 3, I present evidence that, in addition to neuronal fate, proneural genes

also influence the specification of neuronal subtype identity. I further explore the possibility that the ability of proneural genes to specify distinct types of sensory organs may result from their interactions with different co-factors (Chapter 4).

In this Chapter, I will provide information about the *Drosophila* PNS and its development, with emphasis on the sensory nervous system which is the main experimental system for this work. I will then review our knowledge of the *ato* gene and other proneural genes, on which this project is based.

Development of sensory organs in the *Drosophila* PNS

The Drosophila PNS

Drosophila has both a larval PNS and an adult PNS. The Larval PNS is generated during embryogenesis. Most of its sensory neurons degenerate around pupation. The adult PNS develops during late larval and early pupal stages. Despite the spatiotemporal difference, the two systems bear considerable similarities. Predominantly, many genes involved in development of the larval PNS also play similar roles in development of the adult PNS.

The PNS of *Drosophila* consists of sensory and motor components: the former relays stimuli from the environment or the periphery of the animal to the central nervous system (CNS); the latter innervates the muscles from the CNS. This thesis is focused on the sensory nervous system.

Sensory organs in the Drosophila PNS

There are 4 types of sensory neurons in the *Drosophila* PNS (For review, see Jan and Jan, 1993): external sensory (es) neurons which associate with external mechano- and chemo-sensory structures; chordotonal (ch) neurons, an element in the chordotonal organs which

are the proprioceptor that detect internal stretch and vibration; multiple dendritic (md) neurons which may function as stretch or touch receptors; and photoreceptor cells in the eye which sense and transduce light signals to the CNS. My work mainly deals with the ch neurons and photoreceptor cells, the two types of sensory neurons that are determined by the *ato* gene.

Each unit ch organ is composed of one neuron (ch neuron) and 3 support cells (a ligament cell, a cap cell and a scolopale cell), all of which are derived from a single precursor cell through 3 successive asymmetric cell divisions (Bodmer et al., 1989). Precursors of ch organs arise in stereotyped positions presumably as a result of positional information encoded by prepattern genes. These precursors differ from their neighboring cells as they enlarge and delaminate from the ectodermal layer. They then undergo three rounds of cell division, generating all 4 cells that form each ch organ. Multiple ch organs tend to bundle together. They reside subepidermally in the body wall or at the joint where they sense stretch or vibration.

Although endowed by the *ato* gene as well for their developmental potential, photoreceptors in the eye are specified in a manner different from that of ch organs and es organs (Tomlinson and Ready, 1987; Wolff and Ready, 1993). Some eight hundred unit eyes, the ommatidia, arranged in a crystalline array, constitute the compound eye. Each ommatidium is a hexagonal structure composed of 8 light-sensing photoreceptor cells (R1-R8) and 12 accessory cells (pigment cells and lens-secreting cone cells). The compound eye develops during the late larval and early pupal stages from the eye imaginal disc, a monolayer epithelium formed by invagination of the ectoderm during embryogenesis. Ommatidial development begins at the posterior tip of the eye disc and propagates anteriorly, the leading edge is marked by the morphogenetic furrow (MF), a dorso-ventral indentation which sweeps from posterior to anterior as eye morphogenesis

proceeds. While cells anterior to the MF 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 following a precise order starting with R8, then R2/5, R3/4, R1/6, R7 and finally the accessory cells (Tomlinson and Ready, 1987; Wolff and Ready, 1993)

proneural genes initiate neuronal development

Development of es organs is initiated with the expression of proneural genes in the *achaete-scute* complex (AS-C) (Campuzano and Modolell, 1992; Ghysen and Dambly-Chaudiere, 1988). The AS-C comprises four structurally similar genes [*achaete* (*ac*), *scute* (*sc*), *lethal of scute* (*l'sc*) and *asense* (*ase*)] most of which are necessary for the formation of es organs and some md neurons (Alonso and Cabrera, 1988; Cabrera, 1990; Romani et al., 1989). The stereotyped distribution of the transcripts prefigures the invariant positions of future es organs. The AS-C genes are initially expressed in clusters of ectodermal cells (the proneural clusters) from which future SOP cells emerge. Moreover, loss-of-function mutations in the AS-C genes result in the elimination of es organs. Conversely, ectopic expression of AS-C genes leads to the formation of ectopic es organs (for review, see Campuzano and Modolell, 1992; Ghysen and Dambly-Chaudiere, 1989; Jan and Jan, 1990). The genetic and phenotypic studies indicate that the AS-C genes confer neuronal potential to clusters of cells that express them. This event precedes the final commitment to a neuronal fate of the SOPs singled out from proneural clusters. Hence the AS-C genes were entitled "proneural" genes (Ghysen and Dambly-Chaudiere, 1989).

The stereotyped expression patterns of the AS-C genes are achieved through the actions of "prepattern" genes which link the gross body plan, set up by early patterning genes, and neuronal development, determined by expression of proneural genes. The existence

of prepattern genes was first postulated by Stern (1954). It has been substantiated by the finding that, in the embryo, the positions of proneural clusters are initially specified by combinatorial actions of pair-rule genes along the anterior-posterior (A/P) axis and dorsoventral (D/V) patterning genes. The positions of the proneural clusters are later maintained by segment polarity genes (Skeath, 1992). In addition, a group of genes including *araucan* (*ara*) and *caupolican* (*caup*) of the *iroquois* (*iro*) complex (Gomez-Skarmeta et al., 1996; Gomez-Skarmeta and Modolell, 1996), *pannier* (*pnr*) (Ramain et al., 1993) and *u-shaped* (*ush*) (Haenlin et al., 1997; Cubadda et al., 1997) have recently been found to regulate *ac* and *sc* expression during adult es organ development. Another means by which prepattern genes restrict sensory organ formation to precise locations is to induce the expression of the inhibitory *extramacrochaetae* (*emc*) gene (Garrell and Modolell, 1990) in a pattern complementary to that of proneural genes.

All four members of the AS-C encode proteins with basic helix-loop-helix (bHLH) motifs. Biochemical and x-ray crystallographic studies have shown that the HLH domain is required for dimerization, whereas the basic region mediates sequence-specific DNA binding via consensus sites of E boxes (CANNTG) (Blackwell and Weintraub, 1990; Murre et al., 1989; Murre et al., 1989). The AS-C proteins dimerize with the ubiquitously expressed Daughterless (Da) protein which also belongs to the bHLH family (Cabrera and Alonso, 1991; Caudy et al., 1988). The resulting heterodimers regulate transcription of downstream target genes including those involved in lateral inhibitory signaling (Singson et al., 1994), the AS-C genes themselves (Jarman et al., 1993; Van Doren et al., 1992), and possibly other genes responsible for the formation of neuronal precursors. The Emc protein sequesters functional proneural gene products from binding to DNA through heterodimerization (Martinez et al., 1993; Van Doren et al., 1992).

Another bHLH gene, *atonal* (*ato*), was identified as a proneural gene for ch organs and photoreceptor cells (Jarman et al., 1993; Jarman et al., 1994; Jarman et al., 1995). I will summarize our knowledge about *ato* in a separate section in this chapter, since most of the work included in this thesis deals with regulation of *ato* expression and its activity during ch organ and photoreceptor development.

Neurogenic genes restrict proneural gene expression to one or a few SOPs within each proneural cluster

A class of genes were named "neurogenic" genes because of their mutant phenotype: inactivation of any of these genes leads to striking hypertrophy in the CNS (Lehmann et al., 1981; Lehmann et al., 1983). Members of this gene family include *Notch* (*N*) (Kidd et al., 1986; Wharton et al., 1985), *Delta* (*Dl*) (Vässin et al., 1987), *Suppress of Hairless* [*Su*(*H*)] (Schweisguth and Posakony, 1992), genes in the *Enhancer of split* complex [*E*(*spl*)-C] (Klambt et al., 1989; Knust et al., 1992), *mastermind* (*mam*) (Smoller et al., 1990), *neuralized* (*neu*) (Boulianne et al., 1991; Price et al., 1993), and *big brain* (*bib*) (Rao et al., 1990). Later, it was found that mutations in these genes also affect PNS development at various steps (Cagan and Ready, 1989; Hartenstein and Campos-Ortega, 1986). Furthermore, many of the neurogenic genes also influence a variety of developmental processes outside the nervous systems such as oogenesis (Ruohola et al., 1991), mesoderm differentiation (Corbin et al., 1991), epithelial development (Hartenstein et al., 1992), axon guidance (Giniger et al., 1993) and cell proliferation (Go et al., 1998; Johnston and Edgar, 1998).

The *N* gene encodes a transmembrane receptor (Kidd et al., 1986; Wharton et al., 1985) with a large extracellular domain at its N-terminus. This extracellular domain contains 36 tandem epidermal growth factor (EGF)-like repeats responsible for ligand binding and three cysteine-rich Notch/Lin-12 (*Notch* homolog in *Caenorhabditis elegans*) repeats

thought to be involved in receptor activation. The intracellular domain of *N* has a RAM domain and 6 tandem CDC10/Ankyrin repeats, both of which have been implicated in interacting with cytoplasmic components of the signaling cascade. In addition, the intracellular domain contains two nuclear localization sequences and a PEST sequence which plays a role in *N* protein turnover. The *Dl* gene encodes a transmembrane protein (Vässin et al., 1987; Kopczynski et al., 1988) which serves as a ligand for the Notch receptor. It also contains multiple EGF repeats and a cysteine-rich DSL (Delta-Serrate-Lag-2) region in its N-terminal extracellular domain, both of which appear to be important for binding to the N receptor. No function has been assigned to the intracellular domain of Dl.

One cytoplasmic component that relays signals from the N receptor on the cell surface to nuclear events is the product of the *Su(H)* gene, another neurogenic locus. The Su(H) protein is highly related to a family of mammalian transcription factors named RBP-J_k (Recombination signal sequence Binding Protein for J_k gene) (Schweisguth and Posakony, 1992) and has been shown to interact with the Ankyrin repeats of N (Fortini and Artavanis-Tsakonas, 1994). Like its mammalian counterparts, Su(H) has been demonstrated to regulate gene expression through direct DNA binding. The downstream target genes include members of the *E(spl)*-C (Lecourtois and Schweisguth, 1995) which also belong to the family of neurogenic genes. The *E(spl)*-C comprises seven closely related bHLH genes (Klambt et al., 1989; Knust et al., 1992). They function in response to *N* signaling to suppress neuronal fate by repressing the transcription or activity of proneural genes (Bray, 1997; Nakao and Campos-Ortega, 1996; Oellers et al., 1994).

As revealed by the above molecular data, extensive genetic studies have placed *Dl*, *N*, *Su(H)* and *E(spl)* sequentially in a signaling cascade responsible for various local intercellular communications (for review, see Artavanis-Tsakonas et al., 1995). During

sensory organ development, this pathway operates to control the cell fate choice between a neuronal fate and an epidermal fate by regulating proneural gene expression within each proneural cluster. It should be noted that the molecular mechanism by which *N* signaling activates the expression of its downstream target genes is still mysterious. One early model states that, in the absence of N-Dl binding, Su(H) is retained in the cytoplasm through its interaction with the intracellular RAM domain (Tamura et al., 1995) and the Ankyrin repeats of N (Fortini and Artavanis-Tsakonas, 1994). Activation of *N* signaling via N-Dl interaction triggers the release and subsequent nuclear translocation of the Su(H) protein. Modification of the Su(H) protein may also be involved. As a transcription factor, Su(H) activates the expression of the *E(spl)*-C genes (Lecourtois and Schweisguth, 1995; Lecourtois and Schweisguth, 1997), whose products in turn inhibit transcription of the proneural genes (Bray, 1997; Nakao and Campos-Ortega, 1996; Oellers et al., 1994), thus suppressing neuronal fate in cells where the above signaling events take place. Recently, several groups (Kopan et al., 1996; Lecourtois and Schweisguth, 1998; Schroeter et al., 1998; Struhl and Adachi, 1998) provided genetic, molecular and biochemical data indicating that upon activation by Dl, N is proteolytically cleaved, resulting in the release of its intracellular domain (NICD). The NICD then enters the nucleus and activates transcription either directly or indirectly in concert with other factors such as Su(H) (for review, see Chan and Jan, 1998).

While preventing the neighboring cells from taking on the same fate through the inhibitory *N* signaling pathway, cells sending the signal through Dl continue to express proneural genes and become SOPs committed to a neuronal fate. The difference between the signaling and receiving cells may originate from stochastic fluctuations in the levels or activity of Dl and N and is later augmented by a regulatory loop involving the *N* signaling cascade and the proneural genes (Heitzler et al., 1996).

The molecular mechanisms by which other neurogenic genes (*mam*, *neu* and *bib*) affect neurogenesis remain elusive. Whether they participate in the *N* signaling pathway is not clear, although some genetic interactions with *N* have been reported (Doherty et al., 1997).

N signaling has been implicated in a wide variety of cell fate decisions which involve all three germ layers (Cagan and Ready, 1989; Corbin et al., 1991; Giniger et al., 1993; Go et al., 1998; Hartenstein et al., 1992; Johnston and Edgar, 1998; Ruohola et al., 1991; Xu et al., 1992). Upon examining *N* function on *ato* expression in the eye (Chapter 2) and comparing the results with observations from other groups (Baker et al., 1996; Baker and Yu, 1997), I discovered a novel function for *N* in post-transcriptional regulation of *ato*, which may add new insight into our understanding of the *N* pathway.

Neuronal type selector genes determine neuronal subtype identity

SOPs are morphologically indistinguishable, but once formed, they undergo stereotyped cell divisions and differentiation processes to generate sensory organs of distinct types, depending on their positions in the body. There are formally two possibilities for how SOPs may acquire different identities. First, SOPs might be born with multiple developmental potentials. In this case, proneural genes would only assign a generic neuronal identity to SOPs and would not provide direct information about the type of sensory organs the SOPs will generate. The subtype identity would later determined by the actions of other genes such as neuronal type selector genes. Second, proneural genes would not only specify SOP formation, but also impose subtype information on SOPs. The reality, however, appears to lie in between these two possibilities.

As the name suggests, neuronal type selector genes specify the subtype identity of the sensory organ to which a SOP will give rise. Two such genes have been identified: *cut* and *pox-neuro*.

The *cut* gene encodes a large nuclear homeodomain-containing protein (Blochlinger et al., 1988). Several lines of experiments indicate that *cut* specifies the es organ identity as opposed to the ch organ identity during PNS development. First, loss-of-function mutations of *cut* result in transformation of es organs into ch organs (Bodmer et al., 1987). Conversely, ubiquitous expression of *cut* transforms ch organs into es organs (Blochlinger et al., 1991). Moreover, *cut* is expressed in the SOPs and the differentiated cells of es organs but not those of ch organs (Blochlinger et al., 1990). Thus, *cut* functions as a binary switch to control an es/ch bipotential SOP to development into either an es organ or a ch organ. It is believed that *cut* expression is activated by proneural AS-C genes and *da* and later maintained via *cut* auto-regulation (Blochlinger et al., 1991). This suggests that proneural genes influence neuronal type by regulating neuronal type selector genes.

The *pox-neuro* (*poxn*) gene was originally isolated based on its sequence homology to two paired box-containing segmentation genes. In the PNS, *poxn* serves as another binary switch to differentiate the poly-innervated (chemosensory) es organs, where it is expressed, from the mono-innervated (mechanosensory) es organs, where it is absent (Dambly-Chaudiere et al., 1992). Mutations in *poxn* transform poly-innervated organs to mono-innervated ones, whereas *poxn* ectopic expression results in the reciprocal transformation. Interestingly, *poxn* is capable of inducing the expression of *cut*, raising the possibility that *cut* acts in multiple stages during AS-C-dependent es organ differentiation (Vervoort et al., 1995). Elucidating the molecular mechanisms by which

cut and *poxn* function will enlighten our understanding of how neuronal subtype identity is established.

Proneural genes also influence neuronal subtype identity

When expressed ectopically in the same location, the AS-C genes promote es organ formation exclusively, whereas *ato* promotes ch organ formation predominantly. The conclusion drawn from these gain-of-function experiments is that, in addition to neuronal feature, proneural genes also influence neuronal subtype identity (Chien et al., 1996; Jarman et al., 1993). However, it is not clear whether this conclusion is generally applicable to other situations such as the es organ versus photoreceptor decision, which is determined by the AS-C genes and the *ato* gene, respectively. I attempt to address this issue in studies described in Chapter 3. The results lend further support to the conclusion that proneural genes not only determine the neuronal fate but also influence neuronal subtype identities.

The *ato* gene

Isolation and Characterization of the ato gene

Several observations argue for the presence of other AS-C gene-like bHLH proneural genes responsible for ch organ and photoreceptor development. First, the AS-C is not required for ch organ development, since embryos with all the AS-C genes removed produce normal ch organs. In contrast, mutations in Da, the heterodimerizing partner for the AS-C products, eliminate the entire PNS including both es and ch organs. As a result of an effort looking for such missing proneural genes, *ato* was isolated by Jarman et al. (1993) based on the sequence homology between its bHLH domain and those of the AS-C genes. It appears to fulfill all the expectations for such a proneural gene as reviewed below.

ato encodes a protein of 312 amino acids with a bHLH domain at its C-terminus. The bHLH domain of *ato* shares around 40% sequence identity with those of the AS-C genes (as compared to about 70% among members of the AS-C). No significant homology was found outside the bHLH domain. An acidic region preceding the bHLH domain may function as a transcriptional activation domain (Ptashne, 1988). Like the AS-C proteins, Ato can form a heterodimer with Da that binds to various E boxes (Jarman et al., 1993) and activates transcription (Rosay et al., 1995).

Function of ato in sensory organ development

In embryos and in imaginal discs, *ato* is expressed in the proneural clusters and SOPs that will give rise to larval and adult ch organs (Jarman et al., 1993). Removal of *ato* activity either by point mutations or by using overlapping deficiencies eliminate all adult and almost all larval ch organs, leaving es organs unaffected (Jarman et al., 1993; Jarman et al., 1995). Conversely, ectopic expression of *ato* results in ectopic ch organ formation (Chien et al., 1996; Jarman et al., 1993). These experiments firmly establish *ato* as a proneural gene for both larval and adult ch organs.

One somewhat surprising yet exciting phenotype in *ato* mutant flies is the complete lack of ommatidia in the eye (Jarman et al., 1994). The subsequent analysis in mutant as well as mosaic eye discs demonstrate that R8 photoreceptors, the founder photoreceptors for ommatidial assembly, fail to form in the absence of *ato* function. Consistent with being a proneural gene for R8 formation, *ato* is initially expressed in eye discs as a continuous strip immediately anterior to the morphogenetic furrow (MF), around which major patterning events of eye morphogenesis are thought to occur. This early expression is then progressively restricted first into one row of regularly spaced intermediate groups and finally into three rows of R8 cells. Moreover, generalized expression of *ato* is

sufficient to rescue some ommatidia in *ato* mutant flies. Thus, *ato* is also a proneural gene for photoreceptor development.

A similar set of experiments (i.e. analyzing expression pattern and loss-of-function as well as overexpression phenotypes) reveal that *ato* also acts as a proneural gene for a subset of olfactory sensilla on the antenna and all the olfactory sensilla on the maxillary palp (Gupta and Rodrigues, 1997). These sensory organs employ a somewhat different developmental strategy as compared to es organs or ch organs. For instance, the precursor cells go through an additional round of cell division before terminal differentiation and programmed cell death appears to play a role in setting up the final pattern of the olfactory sensory organs (Reddy et al., 1997). Therefore, it seems likely that a new mode of *ato* action is involved in development of these olfactory sensory organs.

Interesting questions to be addressed

As described above, *ato* instructs formation of diverse types of sensory organs in different developmental contexts. This raises a few intriguing questions. For example, how are the dynamic patterns of *ato* expression in different development settings achieved? How does the same proneural gene *ato* direct formation of distinct types of sensory organs?

(1) Transcriptional regulation of *ato*.

In the light of progressive determination, it becomes apparent that the spatiotemporal expression patterns of proneural genes determine primarily where and when sensory organs will form and possibly the type of sensory organs formed. Little was known about transcriptional regulation of the *ato* gene at the time I joined the lab in the summer of 1993 shortly after Andy Jarman in the lab published the original identification and

functional analysis of *ato*. I decided to tackle this issue because of its obvious importance.

There are two basic components that constitute the picture of transcriptional regulation of *ato*: transcription factors that regulate *ato* expression and the enhancer elements in the regulatory region of *ato* that interact with the upstream regulators. Since *ato* is responsible for several superficially distinct developmental processes, it was postulated that a complex spectrum of transcription factors may be involved in regulating *ato* expression in response to diverse signaling cascades. Instead of limiting myself to a certain pathway, I decided to take a "bottom up" approach: I began by searching for tissue-specific enhancer elements in the *ato* regulatory regions and then moved "upwards" to identify the corresponding transcription factors. Ideally, I hoped this would lead me to a more systematic and comprehensive view of transcriptional regulation of *ato*. In Chapter 2 of this thesis, I report the identification of distinct regulatory domains of *ato*, which collectively may account for *ato* expression in every tissue and essentially in every stage of its expression during ch organ and photoreceptor development. As this project came along in the past few years, several genes have been shown to influence *ato* expression during photoreceptor development (Baker et al., 1996; Baker and Yu, 1997; Borod and Heberlein, 1998; Brown et al., 1995; Dokucu et al., 1996; Heberlein et al., 1995) (summarized in Table 1). Incorporating this information and the data I obtained, I present models describing how *N* and *hh* influence *ato* expression in the eye.

(2) Function of proneural genes in specifying sensory organ identity.

Ectopic expression along with rescue experiments indicate that the AS-C genes specify the es neuron fate, whereas *ato* specifies the ch neuron fate. It is therefore believed that, in addition to neuronal feature, proneural genes also determine neuronal subtype identity

(Chien et al., 1996; Jarman et al., 1993). Whether this conclusion is generally applicable still remains to be determined. The fact that, when ectopically expressed, *ato* also promotes es organ formation, although to a lesser extend, already implied that the picture is not black and white.

Besides ch organs, *ato* is also a proneural gene for photoreceptor cells. This provides us with another chance to examine roles of proneural genes in specifying sensory organ identity. The experiments described in Chapter 3 focus on comparing the ability of *ato* and *sc* in directing photoreceptor formation. The results contrast with previous findings in that *sc*, like *ato*, is able to promote photoreceptor formation, while still possessing the competence to drive es organ development even in the eye. Taken together, it appears that proneural genes indeed specify certain neuronal subtype identity, not exclusively, but preferentially.

Previous studies showed that the difference between *ato* and *sc* in specifying ch organ fate versus es organ fate results from divergent sequences in their bHLH domains, especially the basic domains. Distinct co-factors interacting with the basic domains could be the direct explanation for this difference (Chien et al., 1996). Also, it is formally possible that Ato itself may bind to different co-factors in directing ch organ or photoreceptor development. In Chapter 4, I describe an attempt at looking for gene products that directly interact with Ato.

Despite all the effort described in this thesis and that made by other researchers, our understanding of *ato* is still fragmentary. In Chapter 5, I discuss a few issues that need to be pursued in the future.

Neurogenesis in *Drosophila*

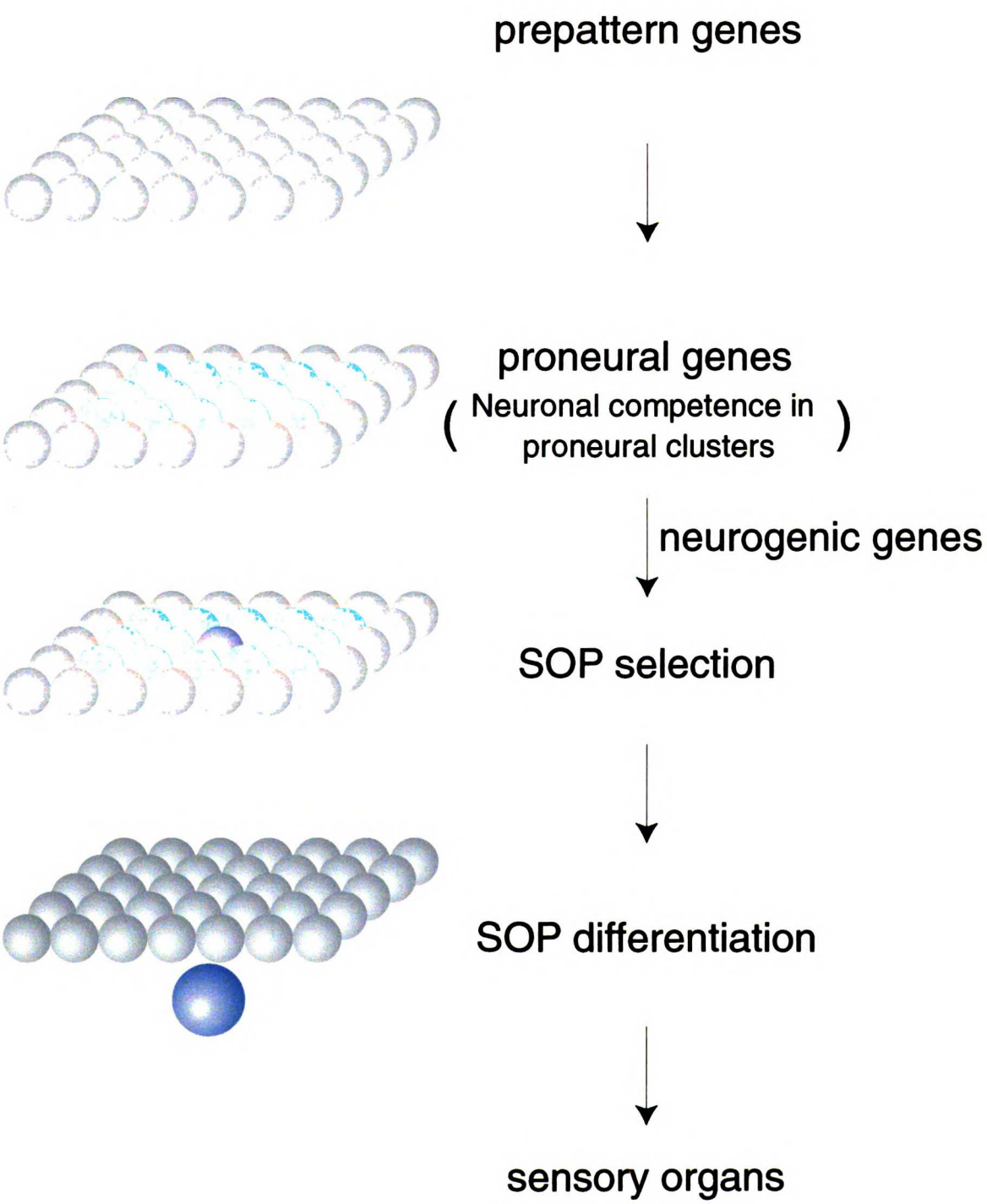

Fig. 1

Table 1 Genes affecting *ato* expression

gene name	gene product	effect on *ato* expression	reference
hedgehog (*hh*)	secreted, signaling molecule	necessary and sufficient to induce *ato* expression	Borod and Heberlein, 1998; Heberlein et al., 1995
Notch (*N*)	transmembrane receptor	promote *ato* expression anterior to the MF; limit *ato* expression to R8s posterior to the MF	Baker and Yu, 1997
rough (*ro*)	homeobox-containing transcription factor	inhibit *ato* expression	Dokucu et al., 1996
daughterless (*da*)	bHLH transcription factor	required for *ato* expression in the MF	Brown et al., 1996
hairy (*h*)	bHLH transcription repressor	inhibit *ato* expression anterior to the MF	Brown et al., 1995
extramacrochaetae (*emc*)	HLH protein	inhibit *ato* expression anterior to the MF	Brown et al., 1995

CHAPTER 2

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

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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* PNS have provided useful insights into this question.

Sensory organs that constitute the larval 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 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 (Campuzano and Modolell, 1992; Cubas et al., 1991; Ghysen and Dambly-Chaudiere, 1989; Romani et al., 1989). Hence, regulation of proneural gene expression plays a key role in setting up the stereotyped pattern of the *Drosophila* PNS.

The *achaete* (*ac*) and *scute* (*sc*) genes, two members of the *achaete-scute* complex (AS-C), 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 (Gomez-Skarmeta et al., 1995; Martinez and Modolell, 1991; Ruiz-Gomez and Ghysen, 1993). Some patterning genes have been found to directly or indirectly regulate *ac* and *sc* expression in regions where specific es organs will form (Cubadda et al., 1997; Gomez-Skarmeta et al., 1996; Ramain et al., 1993; Skeath, 1992). 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*) (Martinez et al., 1993; Van Doren et al., 1992) or repressing their expression in cells outside proneural clusters (in the case of *h*) (Ohsako et al., 1994; Skeath, 1991; 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; Heitzler et al., 1996; Lecourtois and Schweisguth, 1995). 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*), acts as the proneural gene for chordotonal (ch) organs and photoreceptors (Jarman et al., 1994; Jarman et al., 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 dorso-ventral 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 indicate that the neurogenic gene *Notch* is involved in setting up this initial pattern. Finally,

Materials and Methods

DNA constructs

5' *ato-lacZ* reporter constructs

A 4.2 kb BamHI-BamHI 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 BamHI 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 BglII, end blunted with Klenow enzyme and then redigested with SacI to give the 2.6 kb SacI-BglII fragment. This fragment was ligated to a XbaI-SacI adapter (CTAGACGCTGACTACGAGC / TGCGACTGATGC) first and the resulting insert was cloned into the XbaI site and the blunted BamHI site of the pCaSpeR-βgal transformation vector to generate 5'F:2.6. For constructs with larger fragments of the 5' regulatory region, a 7.2 kb XbaI-BglII fragment from phage λgt 6 was transferred into BglII and SpeI double digested pBS.Bm4.2 to generate pBS.XbBm in which the 5' BamHI-BglII fragment in pBS.Bm4.2 was replaced by the XbaI-BglII fragment. pBS.XbBm was digested with SmaI and BglII followed by filling in to give a 5.1 kb SmaI-BglII fragment. This fragment was inserted into filled BamHI site of pCaSpeR-βgal to generate 5'F:5.1. For 5':F7.2, pBS.XbBm was cut with BglII, filled in and digested with XbaI. The resulting 7.2 kb insert was transferred into XbaI and filled BamHI site of pCaSpeR-βgal. Additional 5' sequence included in 5'F:9.3 was excised from λgt 6 as a 5.0 kb SalI-XhoI fragment (SalI site was filled in.), subcloned into SmaI and XhoI sites of pBluescript, recovered as a XbaI-XhoI fragment and inserted into XbaI and XhoI double digested 5'F:7.2 to give 5'F:9.3.

3'*ato-lacZ* reporter construct

A 7.2 kb SalI-HindIII fragment including *ato* open reading frame and 5.8 kb downstream sequence was excised from λgt 6 and subcloned into SalI and HindIII sites of

pBluescript. The insert containing 3' enhancer region for 3'F:5.8 was recovered as a ScaI-SacII fragment and religated to the vector derived from 5'F:2.6 which was digested with XbaI, end filled followed by SacII 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 SacII and BsiWI double digestion. After filling in the BsiWI end, a 2.1 kb SacII-ScaI 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 BglII-XbaI 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 BamHI and XbaI sites of pBluescript, reexcised as a BglII-SacII fragment with BglII end filled and then ligated with both the 2.1 kb SacII-ScaI genomic fragment and XbaI, BsiWI digested pCaSpeR-βgal in which XbaI 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. Rat anti-Ci antibody (Mab 2A1) was generated by Tabata et al. (1994) and was obtained from Pedro Aza-Blanc.

Whole mount in situ hybridization

A 3.4 kb EcoRI-KpnI fragment from the *lacZ* coding region excised from plasmid pBS.*khc*:*lacZ* (Giniger et al., 1993) was used as a template for digoxigenin (Bohringer manheim) 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 (pH7.2) overnight, dehydrated with a graded ethanol series and critical-point dried in CO2. 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.

Gel retardation assay

His-CiZn protein and the positive control probe from the *patched* promoter region (Aza-Blanc et al., 1997) used in the gel retardation assay was provided by Pedro Aza-Blanc.

Probes for Ci-1 and Ci-2 sites are synthesized by PCR using the following oligos: Ci-1.fw (5'-ATG CGA CTC TCT GAC CAC CAA CAT AAA AT-3') and Ci-1.cp (5'-ATG CAT TTT ATG TTG GTG GTC AGA GAG TC-3') for the Ci-1 site; Ci-2.fw (5'-ATG CAT TTT AGT TGG GTG GAA TCA CAC GG-3') and Ci-2.cp (5'-ATG CCC GTG TGA TTC CAC CCA ACT AAA AT-3') for the Ci-2 site.

The following oligos were used to replace the Ci-1 and Ci-2 sites by randomly chosen sequences: Ci-1.mtfw (5'-ATG CGA CTC TCT ACA CGA ACC CAT AAA AT-3') and Ci-1.mtcp ((5'-ATG CAT TTT ATG GGT TCG TGT AGA GAG TC-3') for the Ci-1.mt site; Ci-2.mtfw (5'-ATG CAT TTT AGT GTA GAG TGG TCA CAC GG-3') and Ci-2.mtcp (5'-ATG CCC GTG TGA CCA CTC TAC ACT AAA AT-3') for the Ci-2.mt site.

Binding conditions were the same as for Gli protein binding assay described by Kinzler and Vogelstein (1990). For each reaction, 20 ng of the His-CiZn protein and 2 ng of the labeled probe were used. For competition assays, 200 ng unlabeled probe or mutant probe were incorporated.

Mutating the Ci-1 site in the 3' regulatory region of *ato*

For mutating the Ci-1 site in the *ato* 3' regulatory region, four primers were synthesized: ymtGli.PCR1 (5'-GCC GAC AAG GAT GCA CAG ACA TTC G-3'), ymtGli.PCR2 (5'-TGT CAA CAT TTT ATG GGT TCG TGT AGA GAG TCA AAT TGT-3'), ymtGli.PCR3 (5'-ACA ATT TGA CTC TCT ACA CGA ACC CAT AAA ATG TTG ACA-3'), ymtGli.PCR4 (5'-CAT GTG TGT TAT CCC AAC CTC CAA T-3'). ymtGli.PCR2 and ymtGli.PCR3 contain the same mutated Ci-1 site as used in the binding competition assay. Two PCR reactions were carried out using plasmid containing the *ato* 3' regulatory region as template and ymtGli.PCR1/ymtGli.PCR2 or ymtGli.PCR3/ymtGli.PCR4 as primer pairs. 10 ng of each of the two amplified fragments were denatured then annealed. The resulting hybrid was used as the template for another round of PCR reaction with ymtGli.PCR1 and ymtGli.PCR4 as primers. The amplified fragment was digested with BglII followed by partial digestion with ClaI. The 1.3 kb BglII-ClaI fragment bearing mutant Ci-1 site was recovered and used to replace

the wild type BglII-ClaI region in pBS.3'SalI-HindIII to generate pBS.3'SalI-HindIII*Ci. The cloning strategy for 3'F:5.8*Ci-1 is essentially the same as for 3'F:5.8 described previously except that pBS.3'SalI-HindIII*Ci was used instead of pBS.3'SalI-HindIII.

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. 2.1). The constructs were introduced into flies via germ-line transformation and the expression pattern of *lacZ* was studied by double immunofluorescence using anti-β-galactosidase and anti-Ato antibodies (Fig. 2.2).

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. 2.2B). The expression pattern is very similar but not identical to the expression of endogenous *ato* in these cells (Fig. 2.2A,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 was fused to *lacZ* (5'F:5.1), the embryonic *lacZ* expression pattern was identical to that observed with 5'F:2.6 (data not shown), but additional expression was detected in leg (Fig. 2.2E) and wing imaginal discs (data not shown). Here, *lacZ* expression closely mirrored the endogenous *ato* expression (Fig. 2.2D,F). Strong *lacZ* expression was also seen in a large crescent and a few small groups of cells in the brain lobes (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. 2.2G-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; Jarman et al., 1995) (Fig. 2.3A). While the 5'F:7.2 fusion gene is not expressed in eye discs (Fig.

2.2H), eye expression is detected when 9.3 kb of upstream region are fused to *lacZ* (Fig. 2.3B). 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. 2.3C), similar to that of endogenous *ato*. Both 5' and 3' enhancers also direct expression in ocelli precursors (Fig. 2.3B, 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. 2.4B. 1.1 kb of genomic sequence immediately preceding the coding region were included in both transgenes to provide basic promoter elements (Fig. 2.1). 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. 2.4A) 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. 2.4C). 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. 2.4D-F).

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, 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; Jarman et al., 1995). While the 3' enhancer directed *lacZ* gene expression anterior to the MF in the ato^1 mutant (Fig. 2.5A),

the 5' enhancer failed to drive *lacZ* expression posterior to the MF in this mutant (Fig. 2.5B). 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. 2.5C), while expression from the 5' enhancer was completely eliminated (Fig. 2.5D). 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. 2.5B 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* (Baker et al., 1996; Baker and Yu, 1997))-expressing intermediate groups covering roughly the anterior half of the MF, the earliest known patterning event in eye morphogenesis (Baker et al., 1996; Baker and Yu, 1997; Jarman et al., 1995). The initial expression of *ato* anterior to the MF was thought to occur in a homogeneous dorso-ventral stripe (Jarman et al., 1994; Jarman et al., 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. 2.6): 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. 2.6A). These initial clusters can only be observed in lightly stained samples. More intense staining causes these clusters to be concealed by *ato* 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. 2.6B) and immunocytochemical staining with an anti-Ato antibody (Fig. 2.6C). 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, 1992).

When larvae carrying the N^{ts1} allele and the 3' enhancer-*lacZ* fusion gene were shifted to the restrictive temperature for two 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. 2.7A,B). The endogenous *ato* gene responded to *N* inactivation similarly in the initial stripe (Fig. 2.7C,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 which prefigure the future ommatidia.

Role of Cubitus Interruptus (Ci) in regulating *ato* expression in the eye

Genetic and molecular analysis have revealed that *hh* signaling pathway regulates *ato* expression in the eye (Borod and Heberlein, 1998; Heberlein et al., 1995 and María Domínguez, personal communication). Among all identified components of this pathway, *cubitus interruptus* (*ci*) is the only gene that encodes a transcription factor. It contains multiple zinc-finger motifs (Aza-Blanc et al., 1997; Eaton and Kornberg, 1990;

Orenic et al., 1990). Ci mediates *hh* signaling in regulating *hh* downstream target genes in all the cases tested so far. In eye discs, *ci* is expressed all over in the anterior compartment with increased level of expression in a stripe right adjacent to the MF (Fig. 2.9B), roughly corresponding to the location of the *ato* initial stripe (Motzny and Holmgren, 1995; Strutt and Mlodzik, 1997: María Domínguez, personal communication and Fig. 2.9 C). This spatial correlation leads to the speculation that *ci* directly mediates the inductive function of *hh* on *ato* expression. Targeted expression of full length *ci* under the control of the *hairy* promoter does not cause discernible change with regard to the endogenous *ato* expression (Fig. 2.9D). When expressed in the same pattern, Ci75, a repressive form derived from proteolysis of full length Ci (Aza-Blanc et al., 1997), affects *ato* expression significantly (Fig. 2.9E). The initial stripe appears broader but the protein level is somewhat lower. No initial clusters and intermediate groups were observed. The expression posterior to the MF (i.e., in R8 cells) is more or less normal, albeit slightly irregular. This finding is consistent with Ci75 being a repressor of the *hh* target genes (Aza-Blanc et al., 1997).

Perhaps the most decisive way of exploring the role of *ci* on *ato* expression is to investigate whether the regulatory region of *ato* contains functional Ci binding site. We therefore performed a systematic sequencing of the *ato* 3' regulatory region and found two putative sites (refer to as Ci-1 and Ci-2 sites respectively) in good agreement with the identified Gli/Ci consensus binding site (Fig. 2.8A). in vitro binding assay with the zinc-finger DNA binding domain of Ci revealed that one of the sites binds to Ci in a sequence specific manner (Fig. 2.8B). To assess the potential *in vivo* function of this site, we generated 3'*ato-lacZ* reporter construct 3'F:5.8*Ci-1 in which the Ci-1 site was replaced by the same random sequence Ci-1mt used in the gel retardation assay. When compared to the 3'F:5.8 lines (Fig. 2.9F), transgenic lines carrying the 3'F:5.8*Ci-1 transgene show no change in the *lacZ* expression pattern in the initial stripe and the initial clusters

anterior to the MF in the eye disc, as judged by whole mount in situ hybridization. However, posterior to the initial stripe, *lacZ* starts to be expressed in roughly three rows of cells with certain periodicity (Fig. 2.9G). The spatial relationship between these *lacZ*-expressing cells and the endogenous *ato*-expressing intermediate groups and R8 cells has not been determined yet. Nevertheless, these results indicate that Ci inhibits *ato* expression in some cells posterior to the MF via the Ci-1 site. This inhibition is possibly involved in setting up the regular spacing of intermediate clusters and R8 cells.

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, leg disc, 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 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*. (4) During photoreceptor development, Cubitus Interruptus appears to directly regulate *ato* expression posterior to the MF. This event may be involved in setting up the proper spacing of developing ommatidia.

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 (Gomez-Skarmeta et al., 1995; Martinez and

Modolell, 1991; Ruiz-Gomez and Ghysen, 1993). 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, leg discs, 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 (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 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 take 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 dorso-ventral 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 (Baker et al., 1996; Cagan and Ready, 1989), 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 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 (Heberlein and Moses, 1995; Heberlein et al., 1995; Ma et al., 1993). The initial stripe of *ato* expression co-localizes with this competence zone. Given that enhanced proneural gene expression in SOP cells which 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.

_ci_ may mediate _hh_ signaling in regulating _ato_ expression in the eye

So far, most studies regarding *ato* regulation have been focused on eye development. It was demonstrated that ectopic expression of *hh* anterior to the MF in eye discs is sufficient for activating *ato* expression (Heberlein et al., 1995). Extensive genetic and molecular investigations on *hh* signaling pathway have revealed that the putative zinc-finger transcription factor Ci mediates *hh* signaling. A recent study (Aza-Blanc et al.,

1997) showed that Ci can be cleaved, resulting in a repressive form (Ci75) which then translocates into nucleus to repress expression of *hh* and its target genes. Hh functions by inhibiting proteolysis of Ci and thereby releases *hh* target genes from Ci repression. Based on these observations, it was proposed that Hh synthesized by differentiated photoreceptors posterior to the MF inhibits production of Ci75 in cells anteriorly thus derepresses *hh* and its target genes from Ci. In turn, newly synthesized Hh acts on more anterior cells repeatedly in the same manner, resulting in continuous MF propagation anteriorly. Through a yet unknown mechanism which is likely to involve *ci*, this regulatory loop also induces neuronal differentiation via *ato*. Consistant with this model, ectopic expression of Ci75 in anterior area where its formation is normally provented by *hh* leads to inhibition of endogenous *ato* expression. Recent experiments by María Domínguez (personal communication) illustrated that *hh* has dual function on *ato* expression. It induces *ato* expression anterior to the MF through Ci. Within and posterior to the MF, it inhibits *ato* expression in cells between intermediate clusters possibly through *rough* (*ro*), thus allowing precise ommatidial spacing to occur. From her study, it is unclear whether Ci also participate in the posterior event. Our in vitro binding assay as well as mutagenesis studies show that *ci* indeed is directly involved in regulating *ato* expression posteriorly. Whether *ci* activity is controlled by *hh* signaling pathway, and what role this regulatory event plays in ommatidial development remain to be determined.

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

Fig. 2.1 *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* which 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.

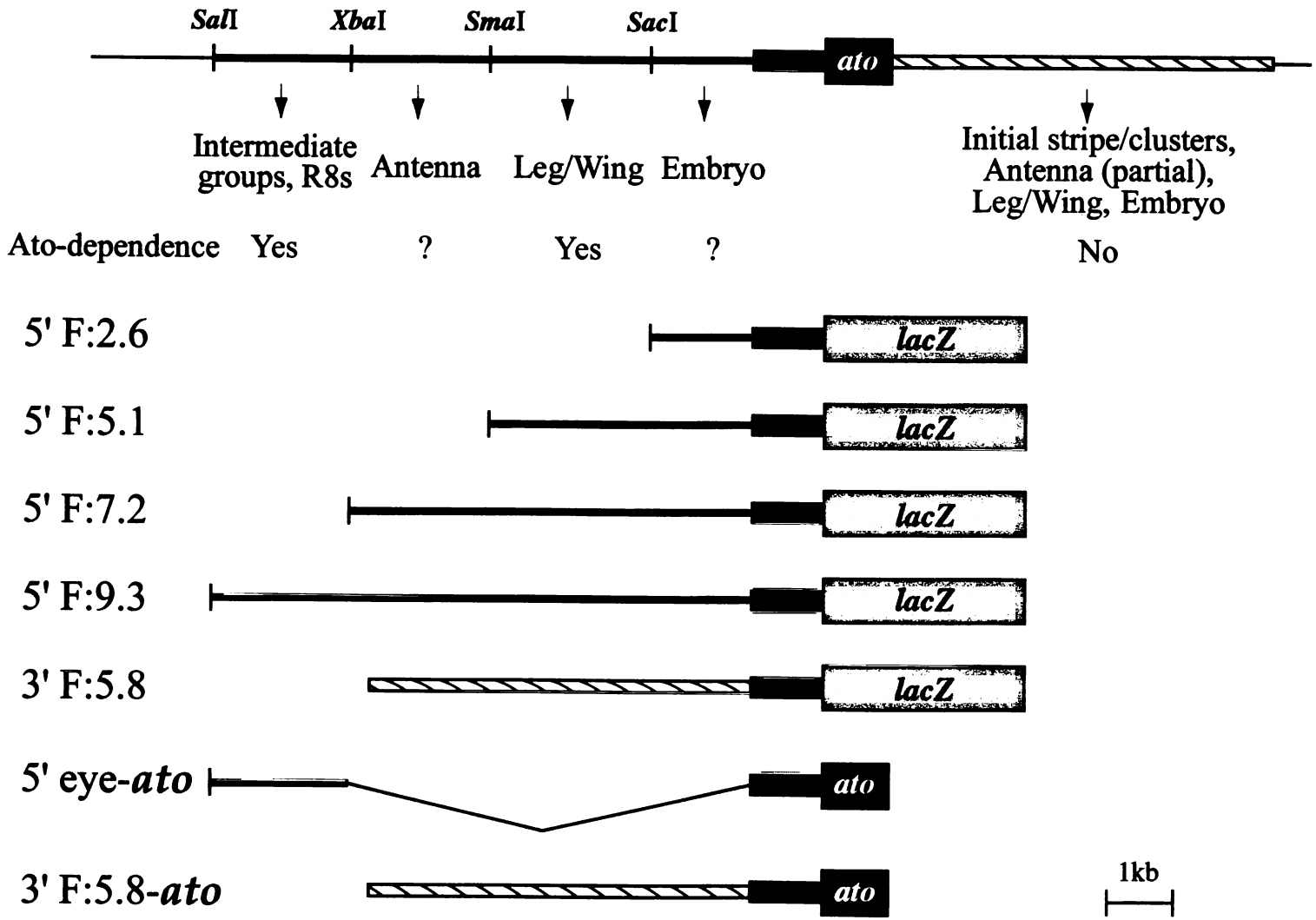
SalI
XbaI
SmaI
SacI
ato
Intermediate groups, R8s
Antenna
Leg/Wing
Embryo
Initial stripe/clusters, Antenna (partial), Leg/Wing, Embryo
Ato-dependence
Yes
?
Yes
?
No
5' F:2.6
5' F:5.1
5' F:7.2
5' F:9.3
3' F:5.8
5' eye-ato
3' F:5.8-ato
lacZ
ato
1kb

Fig. 2.1

Fig. 2.2 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 b-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). b-galactosidase appears later and persists longer compared to endogenous Ato. Therefore, in this case, b-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. b-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).

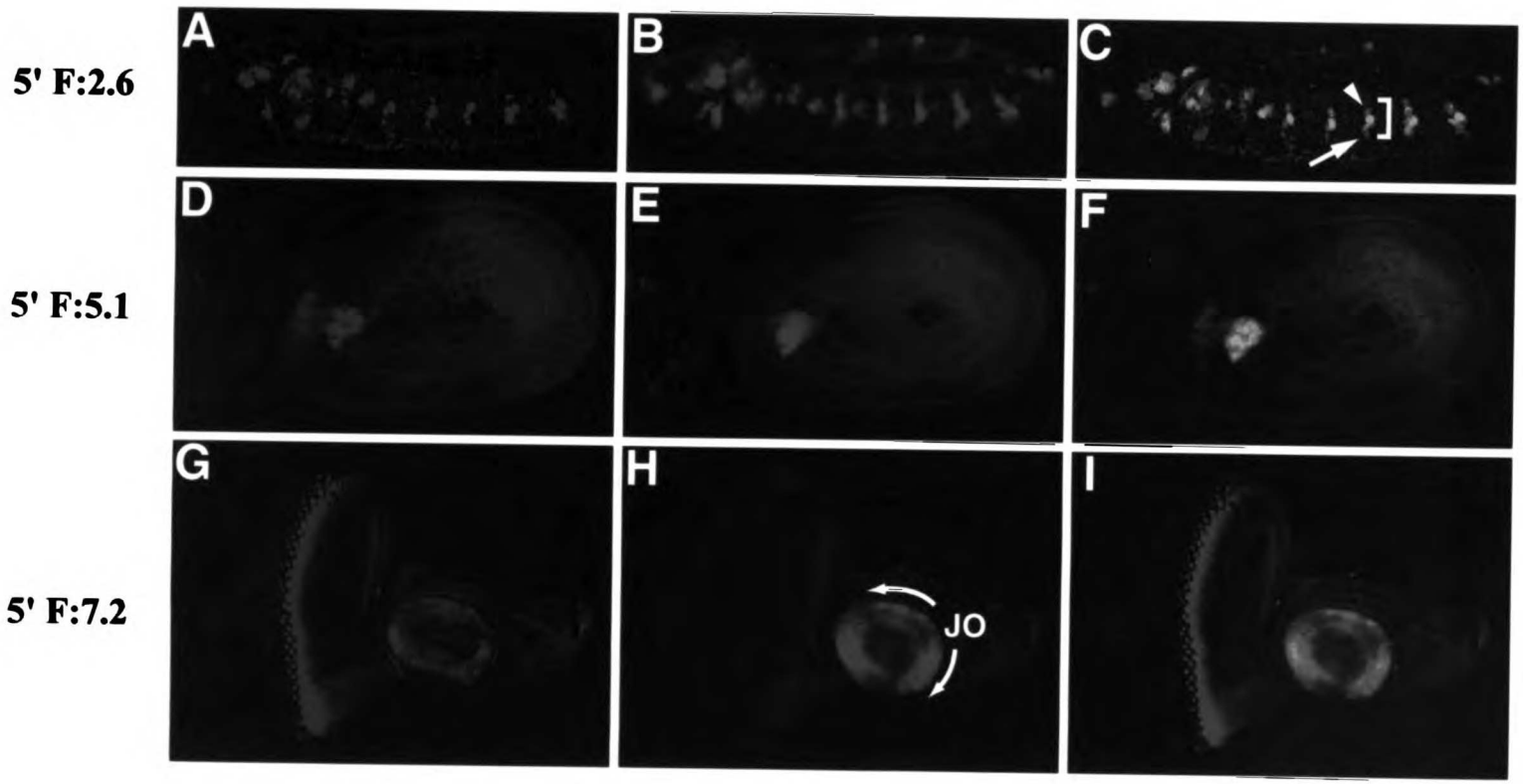

Fig. 2.2

Fig. 2.3 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.

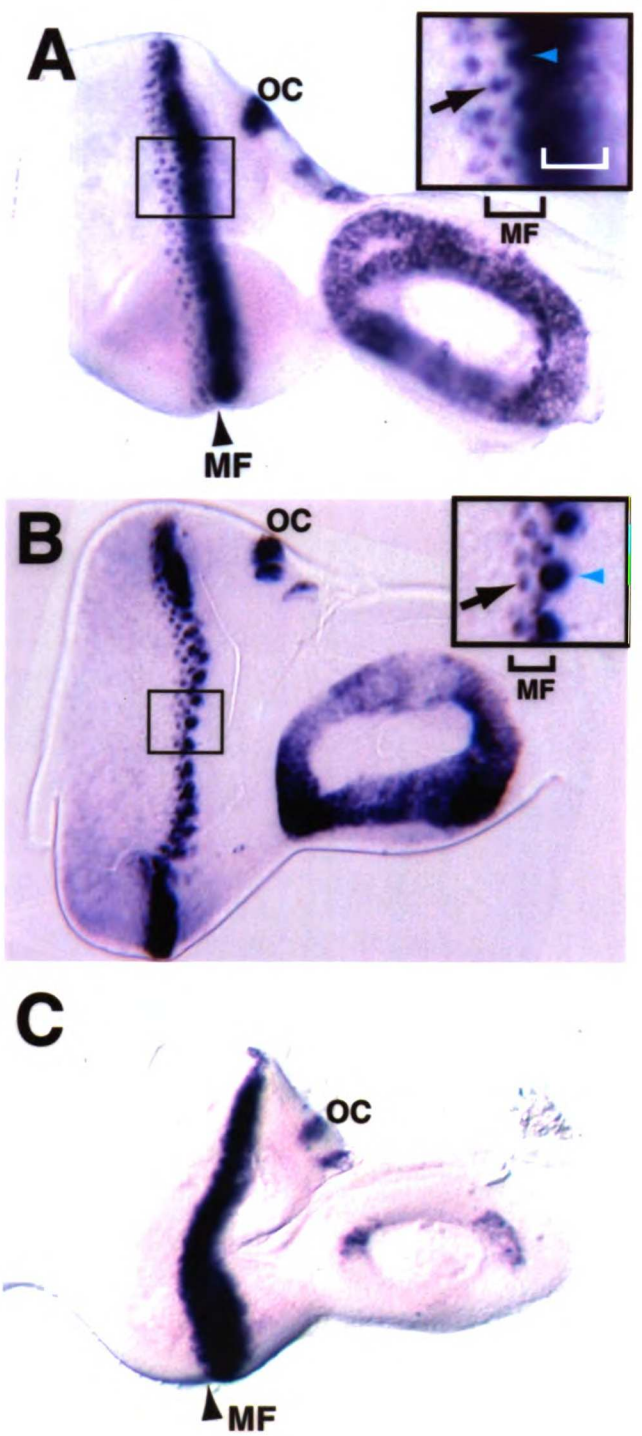

Fig. 2.3

Fig. 2.4 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.

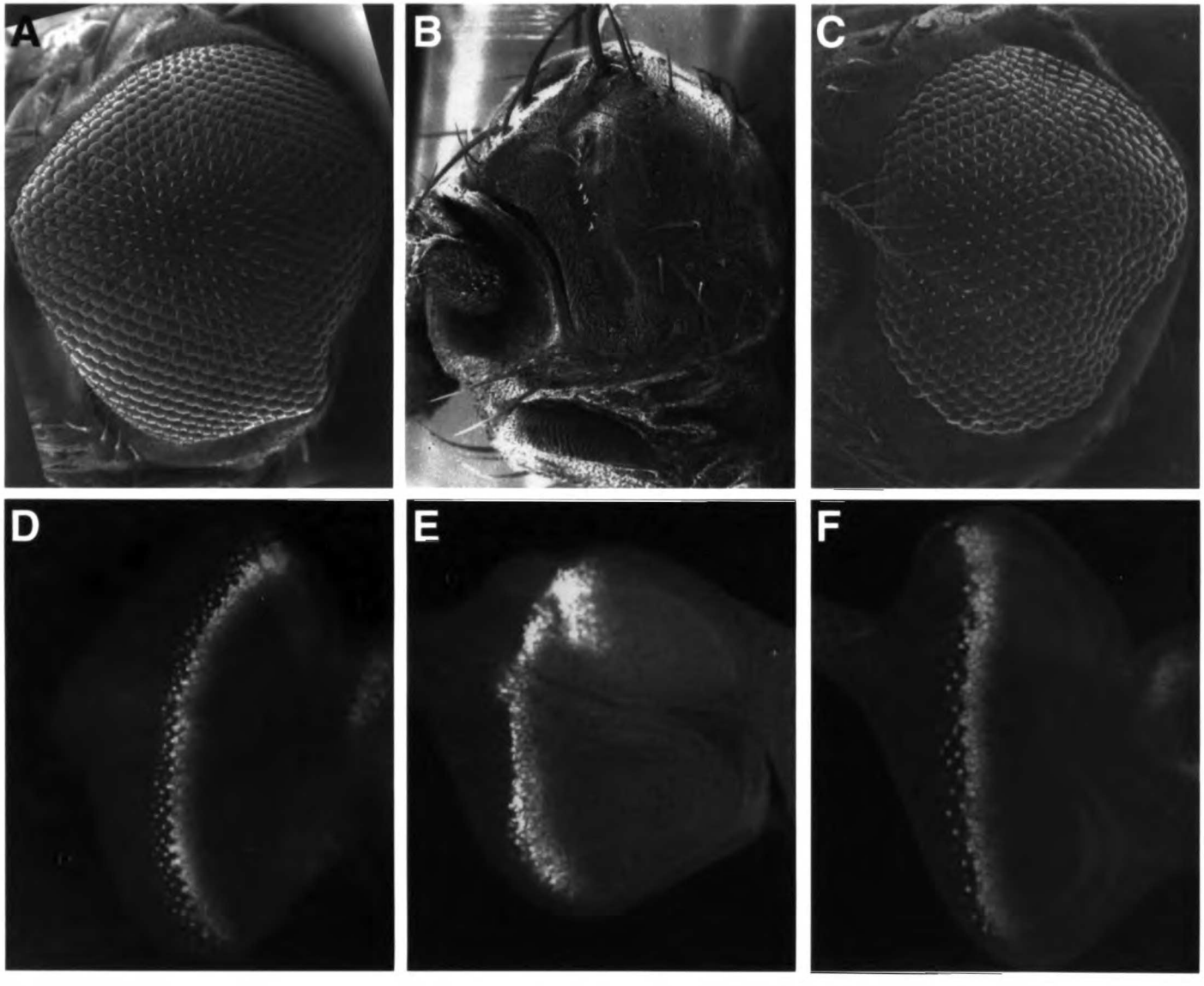

Fig. 2.4

Fig. 2.5 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.

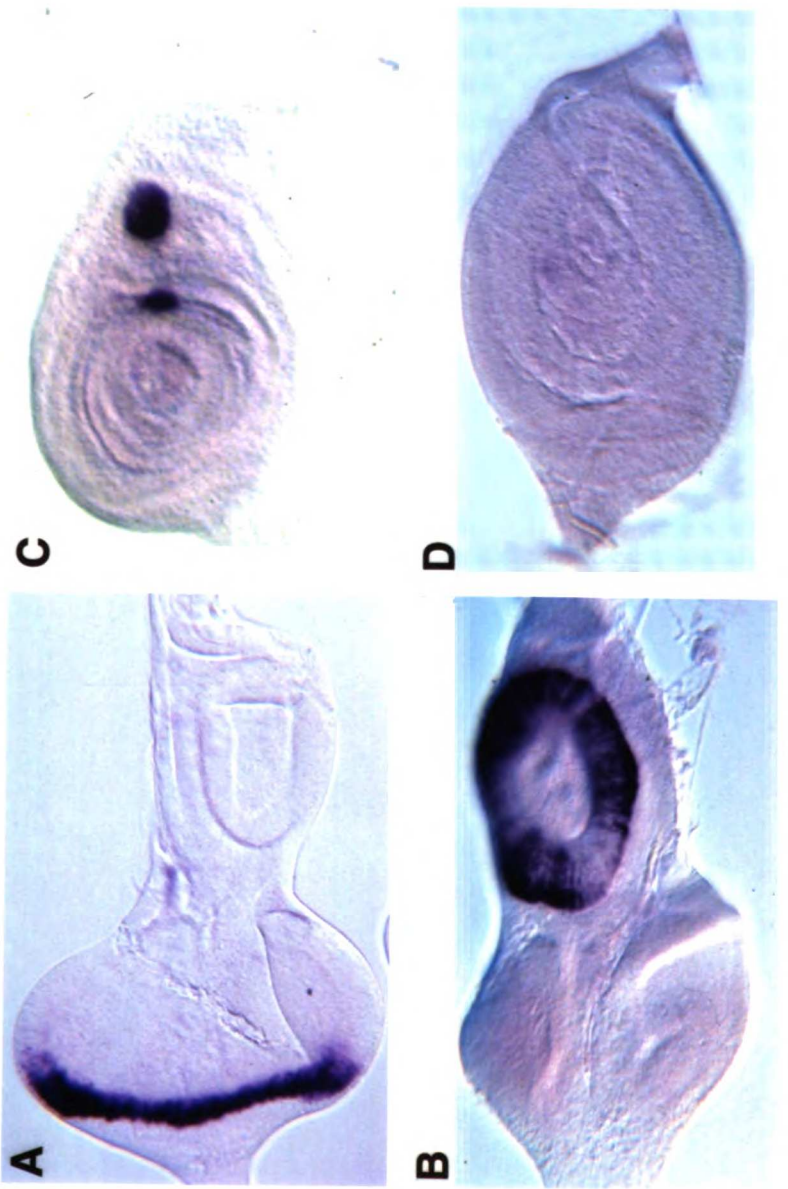

Fig. 2.5

Fig. 2.6 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.

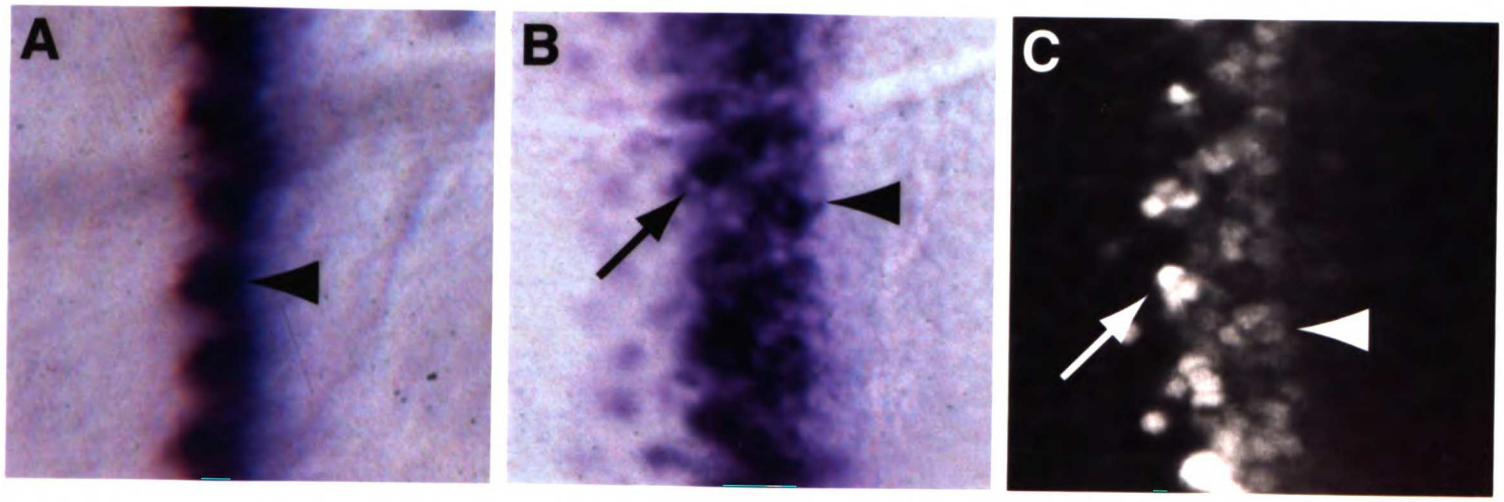

Fig. 2.6

Fig. 2.7 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).

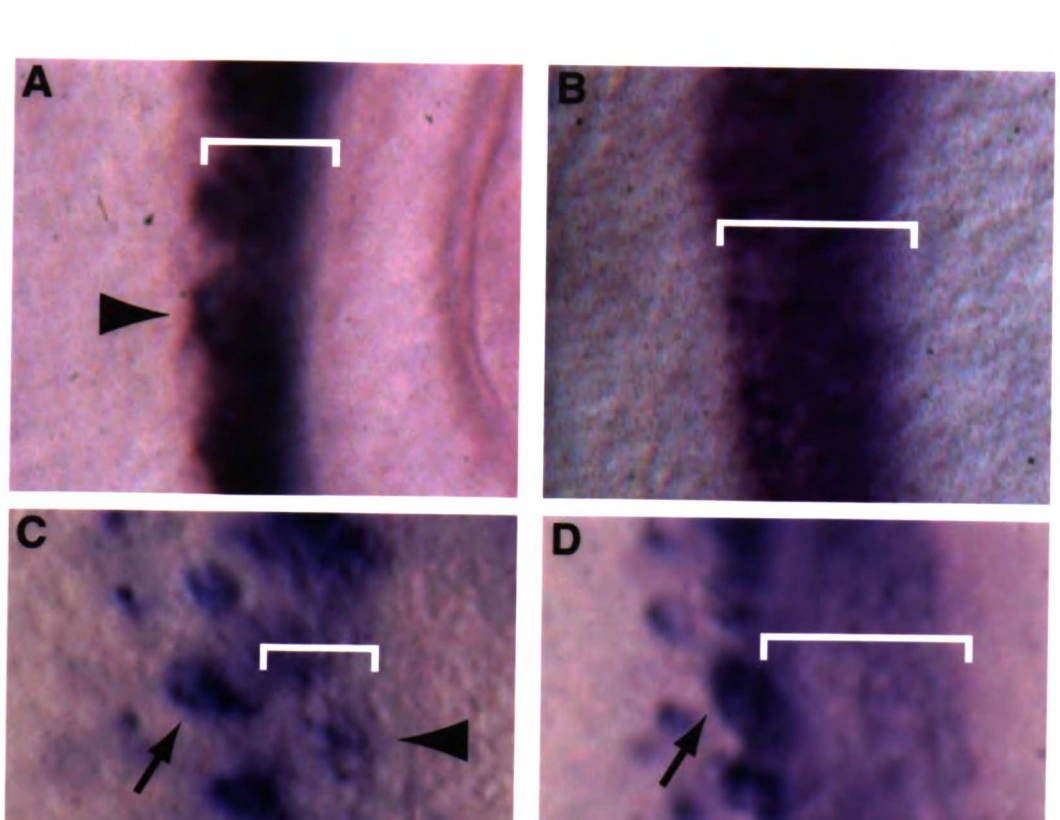

Fig. 2.7

Fig. 2.8 in vitro binding of Cubitus interruptus to putative Ci binding sites in the 3' regulatory region of *ato*

(A) Two potential Ci binding sites (Ci-1 and Ci-2) found in the 3' regulatory region of *ato* share high degree of sequence identities (8/9 for Ci-1 and 7/9 for Ci-2) to the consensus Gli/Ci binding site. Mismatched nucleotides are boxed. The mutant forms (Ci-1mt and Ci-2mt) were generated by scrambling the sequences while keeping the same nucleotide composition. (B) His-tagged zinc finger domain of Ci (His-CiZn, see Aza-Blanc et al., 1997) show sequence-specific binding to the Ci-1 site (lane 2-5). This binding was competed by sequences containing the Gli/Ci consensus binding site from the *patched* promoter (lane 3) or the Ci-1 site (lane 4) but not by sequence with Ci-1mt (lane 5). Ci-2 site does not bind to His-CiZn (lane 6-9). PC: DNA sequence from the *patched* promoter as positive control (Aza-Blanc et al., 1997).

A

consensus Gli/Ci site	GACCACCCA
Ci-1	GACCACC**A**A
Ci-1mt	**ACA**C**GAA**C**C**
Ci-2	**TT**CCACCCA
Ci-2mt	**CCA**C**T**C**TAC**

B

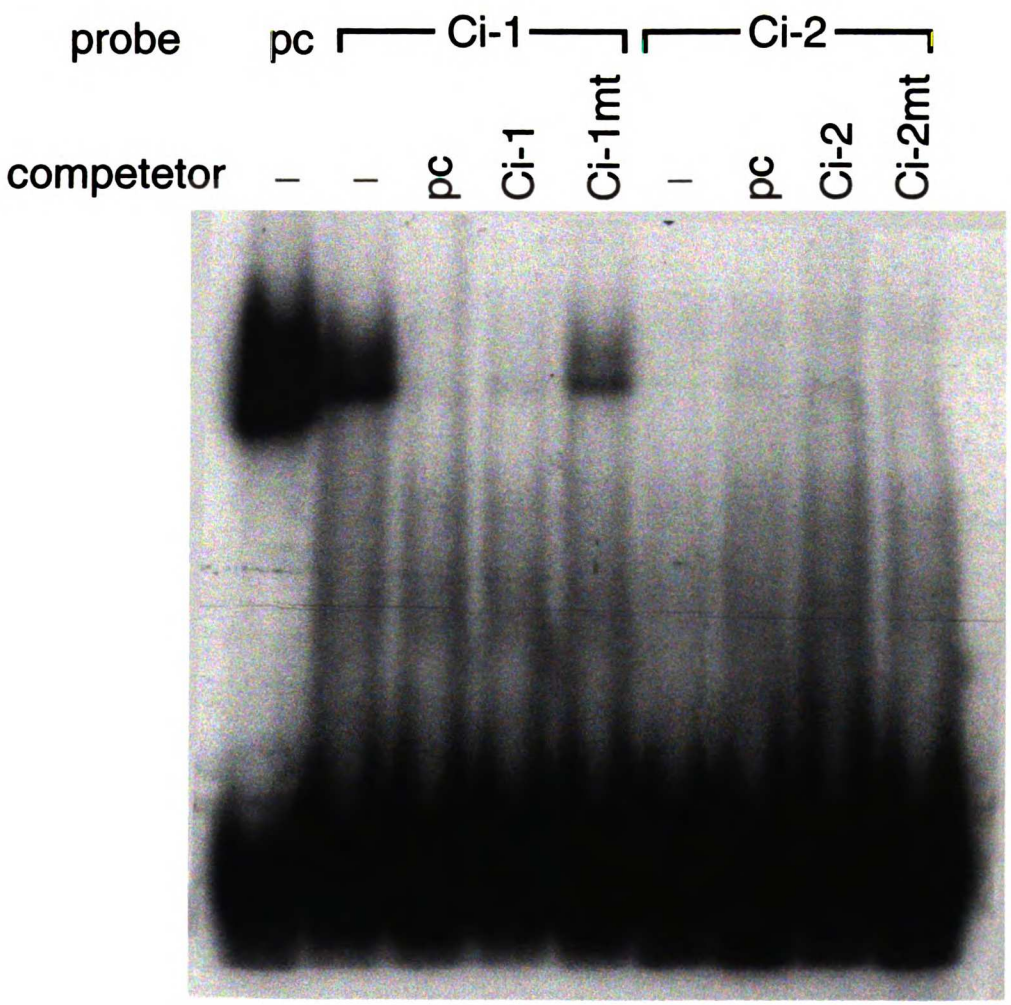

Fig. 2.8

Fig. 2.9 *cubitus interruptus* affects *ato* expression through Ci-1 site

(A-C) Eye-antennal imaginal disc from wild-type third instar larva double stained with antibodies against Ato (green, A,C) and Ci (Mab 2A1, red, B,C). Full length Ci protein is detected ubiquitously at a low level in the anterior field. The highest expression is seen in a band immediately anterior to the MF (B) which co-localizes with the initial stripe of *ato* (C). (D,E) When ectopically expressed under the control of the *hairy*-gal 4 driver, UAS-*ci* (full length) does not affect endogenous *ato* expression significantly (D), whereas UAS-*ci*76 reduces *ato* expression greatly in the initial stripe, most of the initial clusters and intermediate groups. Expression in R8s within and posterior to the MF appears normal (E). (F,G) Mutation of the Ci-1 site in the 3' regulatory region results in ectopic activation of the 3'F:5.8 reporter gene expression in cells (arrow in G) within and posterior to the MF. Initial clusters are less apparent as compared to that with the wild-type Ci-1 site (arrowhead in F).

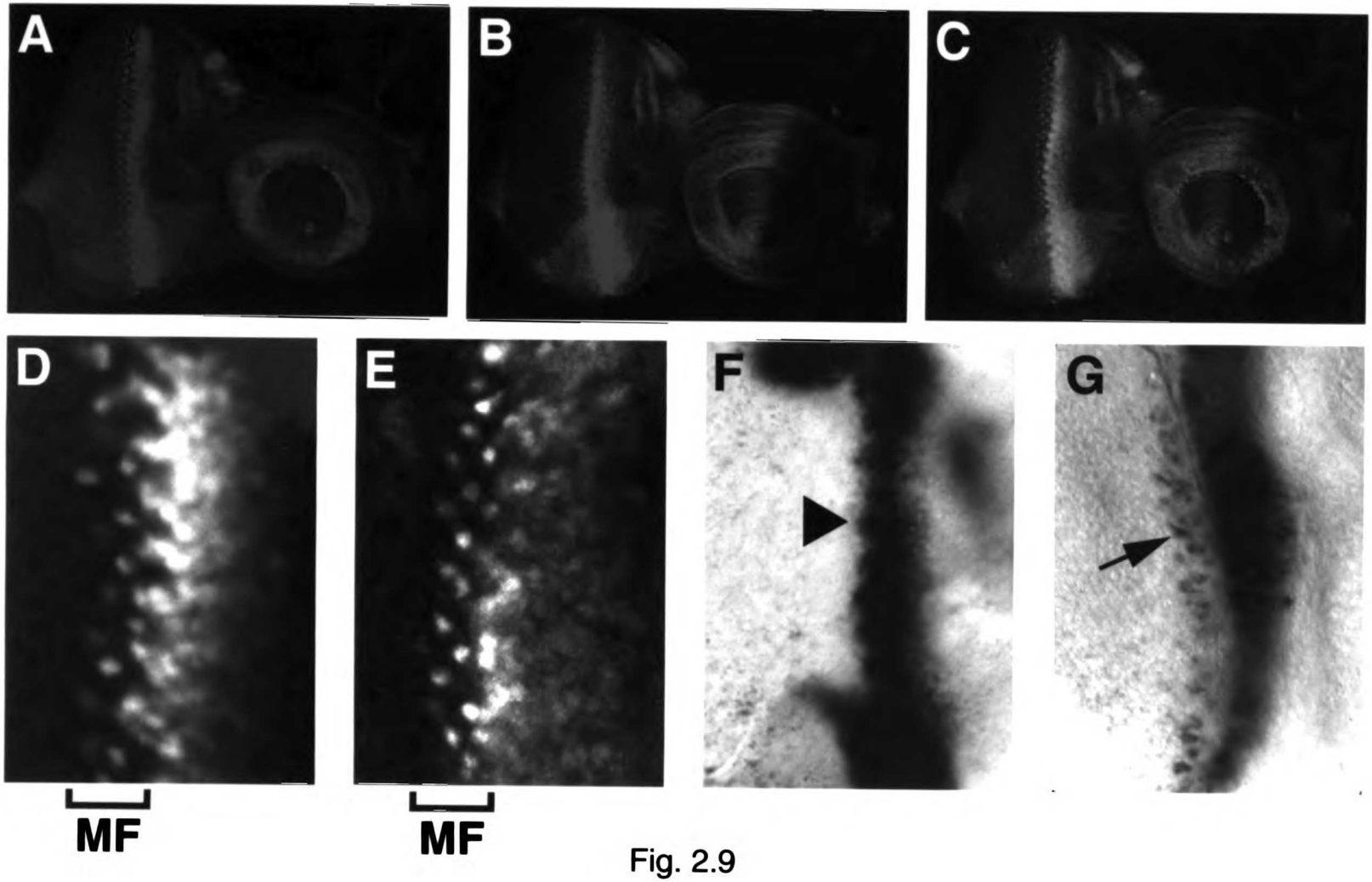

Fig. 2.9

CHAPTER 3

ato, *scute* and photoreceptor formation:
Role of proneural genes in determination of
neuronal subtype identity

Introduction

The Drosophila PNS is composed of four morphologically and functionally different types of sensory organs: external sensory (es) organs, internal chordotonal (ch) organs, photoreceptor cells and multiple dendritic (md) neurons (For review, see Jan and Jan, 1993). How are these distinct neuronal identities specified?

All the cells of a sensory organ in the Drosophila PNS are derived from a single SOP cell, whose formation is determined by the expression of proneural genes. All the identified proneural genes-*achaete* (*ac*), *scute* (*sc*) and *lethal of scute* (*l'sc*) of the *achaete-scute* complex (AS-C) (Alonso and Cabrera, 1988; Romani et al., 1989; Villares and Cabrera, 1987), *daughterless* (*da*) (Caudy et al., 1988) and *ato* (Jarman et al., 1993) encode transcription factors of the basic helix-loop-helix (bHLH) class. The sequence homology in their bHLH domains is about 70% among members of the AS-C and around 40% between *ato* and AS-C genes. During neurogenesis, expression of proneural genes in proneural clusters endows these cells with the competence to assume a neuronal fate. Subsequently, through lateral inhibition mediated by *Notch* signaling pathway, this initial expression is resolved and enhanced only in one or a few SOPs within each proneural cluster therefore fully committing these cells to a neuronal fate as opposed to an epidermal fate (Jan and Jan, 1990). It has been well established that *ac* and *sc* genes carry the proneural function for es organ development (Romani et al., 1989), and *ato* acts as a proneural gene for development of both ch organs and the photoreceptor cells in the eye (Jarman et al., 1993; Jarman et al., 1995). Although expression of different proneural genes in their distinct spatio-temporal patterns ultimately result in stereotyped sensory organs, it is not completely clear what roles proneural genes play in specifying neuronal precursor identities.

The neuronal cell type is acquired progressively and involves groups of genes, each regulating specific aspects of neuronal development. First, proneural genes provide cells in proneural clusters with the competence to become neuronal precursors. This competence is further restricted and also enhanced only in the SOP cells (Ghysen and Dambly-Chaudiere, 1989; Jan and Jan, 1990), a process that involves the *N* signaling pathway. The singled out SOP cells acquire distinct precursor identities later on. Whether proneural genes exert any influence on neuronal subtype specification was not addressed at the time when this model of "progressive determination" was proposed. There are two formal possibilities regarding this issue. First, proneural genes only assign a generic neuronal potential to the SOPs. They do not provide direct constraint about the type of sensory organs the SOPs will form. Second, proneural genes not only determine the SOP formation, but also contain some neuronal subtype information. Identification of the *ato* gene allows us to distinguish the above possibilities. It has been demonstrated that *sc* failed to rescue ch organ defect in *ato* mutant (Chien et al., 1996). Moreover, ectopic expression of the AS-C genes produces es organs exclusively (Jarman et al., 1993; Rodriguez et al., 1990). Only *ato* is capable of promoting ectopic ch organ formation (Chien et al., 1996; Jarman et al., 1993). These observations led to the idea that in addition to providing neuronal competence, proneural genes also influence the neuronal precursor identity. One support comes from the finding that in mutants lacking most of the MP2 precursors, a subset of well characterized neuronal precursors in the CNS, the correct identity of the MP2 precursors can only be restored by ectopic expression of *ac* and *sc*, but not that of *l'sc* and *ato*, even though the latter are sufficient to convey neuronal identity and some features of the MP2 precursors (Parras et al., 1996; Skeath and Doe, 1996).

Es organs and ch organs are closely related, so are MP2 neurons and the neuroblasts surrounding them. For example, both es organs and ch organs are composed of four cells

derived from a single SOP. Both es neurons and ch neurons are single dendritic neurons. Moreover, SOPs for es organs and for ch organs are bipotential, that is, they can development into es organs when Cut is present, or ch organs when Cut is absent. It is therefore necessary to examine whether proneural genes also specify other types of sensory neurons besides the es and ch fates, before any generalized conclusion can be reached.

In this chapter, I compare the ability of *ato* and *sc* in directing formation of photoreceptors which are more distantly related to other PNS neurons in terms of their morphology, function and developmental process. I demonstrate that when ectopically expressed, *sc* is capable of rescuing some ommatidia in an *ato* mutant background that otherwise lacks ommatidia. Often, the rescued ommatidia only have outer photoreceptor cells. The exact nature of these photoreceptors has not been determined. Nonetheless, these observations suggest that *sc* indeed is able to promote some photoreceptor formation. Whether it functions in exactly the same way as *ato* does, or it uses different mechanisms to drive different types of photoreceptor formation remains to be determined. Occasionally, well developed macrochaete-like es organs are found in the middle of a *sc*-rescued ommatidium, suggesting that *sc* still retains its proneural function for es organ even in the eye. Furthermore, I show that *Xath5*, an *ato* homolog from Xenopus (Kanekar et al., 1997), is also able to induce photoreceptor formation. It thus appears that different bHLH proneural genes in *Drosophila* and those from other species may share similar or overlapping proneural function.

Materials and Methods

The methods used for scanning electron micrography and whole mount in situ hybridization have been described previously (Sun et al., 1998).

DNA constructs

The *sc* coding sequence was obtained by PCR using pUC19-sc (a gift from Cheng-ting Chien) as the template and the following primers: 5'-CAG TCA TGA AAA ACA ATA AT-3' and 5'-GAC GGA TCC TTG GGG ATT AAG TCA which incorporate a BspHI or a BamHI site respectively. This fragment was then digested with BspHI and BamHI. A genomic fragment containing 1.1 kb basic promoter region of *ato* was obtained by SacII and BglII double digestion of plasmid pBS.Bm4.2 (a gift from Andy Jarman. See Sun et al., 1998 for description.) , followed by gel purification and AflIII digestion. The resulting SacII-AflIII fragment was ligated to the above BspHI-BamHI fragment and then inserted into SacII and BamHI sites of the pBluescript to generate pBS.SacII-*sc*. The nucleotide sequence of the *sc* coding region derived from PCR reaction was confirmed by DNA sequencing. The cloning strategies for both pCaSpeR.5'eye enhancer-*sc* (5'eye-*sc*) and pCaSpeR.3'enhancer-*sc* (3'F:5.8-*sc*) rescue constructs are essentially the same as for pCaSpeR.5'eye enhancer-*ato* (5'eye-*ato*) and pCaSpeR.3'enhancer-*ato* (3'F:5.8-*ato*) respectively (Sun et al., 1998), except that a 2.1 kb SacII-SmaI fragment excised from pBS.SacII-*sc* was used in both case to replace the 2.1 kb SacII-ScaI fragment used to make *ato* rescue constructs.

pUAST-*Xath5* was constructed by Monica Vetter. It contains the complete *Xath5* coding sequence under the control of the upstream activation sequence (UAS) for Gal4.

Histology

Fly heads were fixed with 4% formaldehyde in 0.1 M sodium phosphate buffer (pH7.2) overnight, dehydrated with a graded ethanol series, embedded in epoxy. Compound eyes were sectioned tangentially at 2 μm and stained with 1% Toluidine Blue and 1% Sodium Borax.

Isolation of the gal4-7 enhancer trap line

The gal4-7 enhancer trap line was isolated by Alice Turner and Cheng-ting Chien through mobilizing a known enhancer trap line 109C1 in an attempt to established gal4 lines that can rescue loss of ommatidia phenotype in *ato*^{1} mutant when UAS-*ato* chromosome is present. The P element is inserted on the third chromosome. This line is homozygous viable with no discernible phenotype by itself.

Immunohistochemistry

Imaginal discs from late third instar larvae and early pupae were fixed for 10 minutes with 4% Formaldehyde in 0.1 M sodium phosphate buffer (pH7.2), washed with PBT and blocked with 5% normal goat serum in PBT. Primary antibodies used are: rabbit anti-Ato (Jarman et al., 1995), mouse anti-Boss (from L. Zipursky) and rabbit anti-Prospero (Vaessin et al., 1991). DTAF or LRSC conjugated secondary antibodies were used. Discs were mounted in Slow Fade mounting medium (Molecular Probes) and then examined with a Bio-Rad MRC 600 confocal microscope.

Fly stocks

All Drosophila stocks were raised on standard cornmeal-yeast-agar medium at 25°C. *ato*^{1} is described by Jarman et al. (1994, 1995). UAS-*ato* and UAS-*sc* were established by Jarman et al. (1993) and Chien et al. (1996) respectively.

Results

Ommatidial rescue by *sc* expression under the control of *ato* regulatory regions in *ato* mutant

Eye morphogenesis begins at the posterior tip of eye imaginal discs in early third instar larvae and propagates anteriorly with its leading edge marked by the morphogenetic furrow (MF). *ato* acts as a proneural gene for photoreceptor development. Removal of *ato* function results in flies with no ommatidia in their eyes. *ato* is first expressed in a continuous stripe of cells immediately anterior to the MF followed by regularly spaced initial clusters. This early expression is driven by a regulatory region located 3' to the coding region. Within and posterior to the MF, this initial expression is further restricted into intermediate groups and finally into evenly distributed R8 photoreceptor cells (Jarman et al., 1994; Jarman et al., 1995; Sun et al., 1998) through the actions of a regulatory region located 5' to the coding sequences (Sun et al., 1998). Other photoreceptor cells within each ommatidium are recruited afterwards in a well-defined order, starting with R2/R5 and ending with R7 (Tomlinson and Ready, 1987).

To examine whether *sc* is able to induce photoreceptor formation when expressed in the endogenous *ato* pattern, we fused the 5' eye enhancer region or the 3' enhancer region and the basic promoter region of *ato* with the *sc* open reading frame and tested the ability of the resulting transgenes, 5'eye-*sc* and 3'F:5.8-*sc* respectively, in rescuing the ato^1 mutant eye phenotype (Fig. 3.1). Neither two copies of 5'eye-*sc* or 3'F:5.8-*sc* alone nor two copies of both induce ommatidia formation when introduced into an ato^1 mutant background (data not shown). Consistent with this, R8 photoreceptors are not formed in either case as judged by expression of an R8 marker, the Boss protein (data not shown). The latter contrasts dramatically with the rescue by 5'eye-*ato* and 3'F:5.8-*ato* together

(Fig. 3.1B). On the contrary, *ato*^{1} flies which also carry different combinations of *ato* and *sc* transgenes, i.e. two copies of 3'F:5.8-*ato* and 5'eye-*sc* or 3'F:5.8-*sc* and 5'eye-*ato*, develop eyes with significant numbers of ommatidia (Fig. 3.1C,D), although much less than that in flies with two copies of 5'eye-*ato* and 3'F:5.8-*ato* (Fig. 3.1B and Sun et al., 1998). In both circumstances, Boss protein is present in eye discs, but appears in fewer cells in the 3'F:5.8-*ato*,5'eye-*sc*;*ato*^{1} disc (Fig. 3.1G,H). The difference between the two cases became apparent when ommatidial structure was examined in sections of adult eyes (Fig. 3.1K,L). When *ato* is expressed posteriorly (and *sc* anteriorly), the rescued ommatidia display more or less normal asymmetric trapezoidal structure (Fig. 3.1 L) as that in wild type eyes (Fig. 3.1I) and 5'eye-*ato*,3'F:5.8-*ato*;*ato*^{1} eyes (Fig. 3.1J). When *sc* is expressed posteriorly (and *ato* anteriorly), however, most of the rescued ommatidia show altered structure: they often have less than 8 photoreceptors, most of which appear to be the outer photoreceptors as judged by the position and the size of the rhabdomeres (rhodopsin-bearing stack of microvilli).

Considering the fact that neither 3'F:5.8-*ato* nor 5'eye-*ato* alone rescues ommatidia while both together induce significant ommatidial formation (Sun et al., 1998), and assuming that the Sc protein is synthesized under the control of the *ato* regulatory regions, this set of experiments indicate that, in conjunction with *ato*, *sc* can function both anterior and posterior to the MF in promoting formation of some photoreceptors.

Expression of *sc* alone by a Gal4-UAS system is sufficient to induce photoreceptor formation in *ato* mutant

In the above experiments, *sc* seems capable of promoting some photoreceptor development. This raises the possibility that once expressed in the appropriate pattern, *sc*

alone may induce ommatidial formation. We tested this hypothesis by expressing *sc* in eye discs with a Gal 4-UAS system (Brand and Perrimon, 1993).

The gal4-7 enhancer trap line was isolated by mobilizing a known gal4 line in an attempt to establish enhancer trap lines capable of driving UAS-*ato* expression to rescue the loss of ommatidia phenotype in ato^1 mutant. This line directs gene expression in many more cells and presumably for longer time than the endogenous *ato* (Fig. 3.2A) in the posterior field of eye discs, as shown by *lacZ* in situ hybridization after crossed to a UAS-*lacZ* reporter line (Fig. 3.2B). *sc* expression from a UAS-*sc* transgene (Chien et al., 1996) under the control of the gal4-7 line is sufficient to restore some ommatidia in the ato^1 mutant background (Fig. 3.2D) but to a lesser extend as compared to that from a UAS-*ato* transgene (Jarman et al., 1993) (Fig. 3.2C). Since the ato^1 mutation is likely to be a hypomorphic allele and the Ato^1 protein may retain some residual activity (Jarman et al., 1995; Lage et al., 1997; Sun et al., 1998), it is possible that this rescue may result from ectopic activation of the endogenous ato^1 gene. We therefore examined the pattern of the Ato^1 protein by antibody staining in gal4-7, ato^1/UAS-*sc*, ato^1 discs (Fig. 2F) and observed no difference from the pattern in ato^1 discs (Fig. 2E and Jarman et al., 1995). This indicates that when expressed in the appropriate pattern in eye discs, *sc* alone can direct ommatidial formation and this action is independent of endogenous *ato* function. This result contrasts the previous observation that *sc* is unable to induce ch organ formation in the ato^1 mutant background in the ch organ rescue experiment (Chien et al., 1996).

_sc_-induced ommatidia have altered structure

Although compound eyes rescued by gal4-7/UAS-*sc* and gal4-7/UAS-*ato* in the ato^1 mutant exhibit quantitative differences with respect to the number of ommatidia formed,

their surface morphology revealed by scanning electron micrography appears similar (compare Fig. 3.2 C and D). We next examined the ommatidial structure in stained sections of the rescued adult eyes (Fig. 3.3A,B) and compared it with that in the wild type (Fig. 3.1I). In sections of the *ato* rescued eyes, ommatidia frequently contain multiple R8-like photoreceptors, since they locate in the center of the ommatidia and have smaller rhabdomeres (Fig. 3.3A). This is consistent with *ato* being a proneural gene for R8 formation (Jarman et al., 1994). Ommatidia rescued by *sc* expression, on the other hand, often lack center-located R8-like photoreceptor cells. Moreover, they only have 2 to 5 photoreceptors (Fig. 3.3B) as compared to 8 in wild type (Fig. 3.1I) and in eyes rescued by ato (Fig. 3.3A). This phenotype is reminiscent of the rescue in 3'F:5.8-*ato*,5'eye-*sc*;*ato*^{1} flies.

To further study the mechanisms responsible for defects in the *sc* rescued eyes, we stained eye discs with markers that label different subsets of photoreceptors. We failed to detect Boss protein in eye discs from gal4-7, *ato*^{1}/UAS-*sc*, *ato*^{1} third instar larvae (Fig. 3.3D); whereas discs of the same stage from UAS-*ato*/+; gal4-7,*ato*^{1}/*ato*^{1} larvae show apparent Boss expression (Fig. 3.3C). However, a clearly advanced MF is reproducibly seen in the gal4-7, *ato*^{1}/UAS-*sc*, *ato*^{1} discs, suggesting that photoreceptor differentiation has occurred posteriorly, albeit lacking Boss staining. Later, in the early pupal stage we detected Boss protein in a few well spaced cells in the posterior region of the disc (Fig. 3.3F) as opposed to many rows of Boss expressing cells seen in the discs misexpressing *ato* (Fig. 3.3E). One obvious explanation for the above finding is that R8 differentiation in the gal4-7, *ato*^{1}/UAS-*sc*, *ato*^{1} discs is simply delayed as compared to that observed in the UAS-*ato*/+; gal4-7,*ato*^{1}/*ato*^{1} discs. If this holds true, given that R8 is the founder cell which recruits other photoreceptors into each ommatidium, one would expect that differentiation of other photoreceptors, especially R7 cells whose formation strictly rely on R8 cells, should also be delayed. However, we detect Prospero protein, a marker

which is normally expressed in differentiated R7 cells and cone cells, in the third instar discs of the gal4-7, *ato*^{1}/UAS-*sc*, *ato*^{1} animal (Fig. 3.3J), suggesting that R7 cells, possibly other photoreceptors as well, may have already been specified before the R8 cells acquire their identity as judged by *boss* expression. This abnormality may also account for the structural defects we observed in the *sc*-rescued ommatidia.

sc specify ectopic es organs in the eye

sc normally specifies es organs: ectopic expression of *sc* exclusively promotes ectopic es organ formation in embryos and in imaginal discs (Balcells et al., 1988; Campuzano et al., 1986; Rodriguez et al., 1990). In the eye, *sc* is also a proneural gene for interommatidial bristles. These bristles develop during the early pupal stage after most photoreceptor cells have already formed. In gal4-7, *ato*^{1}/UAS-*sc*, *ato*^{1} flies, we occasionally observed ectopic macrochaete-like bristles in various positions in the eye (Fig. 3.2D and Fig. 3.4A). Some are formed in the middle of a well-developed ommatidium (Fig. 3.4A, B). These bristles resemble wild type macrochaetes and are morphologically distinct from the surrounding interommatidial bristles (Fig. 3.4B). Although the very low frequency of this event makes it difficult to determine the molecular mechanism responsible for this phenomenon, it appears that cells in the eye disc retain the potential to develop into macrochaete-like es organs which usually are not produced in the eye. More importantly, *sc* specifies ectopic es organs even in the eye.

Xath5 induces photoreceptor formation in *ato* mutant

To address whether the function of proneural genes in specifying distinct types of sensory organs is evolutionarily conserved, we tested the ability of vertebrate *ato* homologs to induce photoreceptor differentiation in *Drosophila*. Among all identified *ato*-related

genes in vertebrates, *Xath5* from Xenopus appears to be the closest homolog of *Drosophila ato* in terms of its sequence similarity to *ato*, its expression pattern, and function (Kanekar et al., 1997). When expressed under the control of the gal4-7 enhancer trap line, *Xath5* induces photoreceptor formation and ommatidia assembly in the ato^{1} mutant to an extent comparable to that caused by *Drosophila ato* (Fig. 3.4D), indicating *Xath5* being a true homolog of *ato*. This result suggests that function of the *ato*-related genes in promoting photoreceptor formation is conserved during evolution, and this activity is carried most likely by the bHLH domains, since *Xath5* exhibits no significant sequence homology outside its bHLH domain to *Drosophila ato*.

Discussion

During development of the *Drosophila* PNS, *ato* is a proneural gene for ch organs and photoreceptor cells in the eye, whereas *sc* is a proneural gene for the es organ fate. By expressing *sc* under the control of the *ato* regulatory regions or a gal 4 enhancer trap line, we demonstrate that *sc* is capable of inducing ommatidial formation in the ato^1 mutant background that otherwise lacks ommatidia while it still retains its ability to direct complete es organ development even in the eye. Moreover, we present data indicating that ommatidia rescued by *sc* differ in structure and possibly their developmental process from those rescued by *ato*. Finally, we illustrate that a Xenopus homolog of *ato* maintains the ability to direct photoreceptor formation in *Drosophila*.

Role of proneural genes in specifying neuronal subtype identity

Extensive genetic studies followed by molecular characterization of genes involved in PNS development led to the model of progressive determination of the PNS (Ghysen and Dambly-Chaudiere, 1989; Jan and Jan, 1990). On top of the hierarchy lies the proneural genes which assign neuronal competence to the cells of proneural clusters that express them. Only one or a few SOP cells from each proneural cluster fully establish neuronal identity through enhanced proneural gene expression. Neuronal subtype identity is specified later on. In recent years, several studies regarding the function of *ato* and *sc* in ch organ and es organ development have demonstrated that SOP cells specified by different proneural genes are indeed already distinct in their developmental potential (Chien et al., 1996; Jarman et al., 1993). In addition, we show here that when ectopically expressed in the eye, *sc* can induce es organ formation as well. It thus appears that proneural genes also contribute to neuronal subtype identity. However, the predominant phenotype of expressing *sc* in the ato^1 mutant eye is the rescue of ommatidia.

Reciprocally, *ato* is also capable of promoting es organ differentiation (Chien et al., 1996; Jarman et al., 1993). One emerging picture is that in addition to the common role of providing neuronal identity, proneural genes also influence, but not completely specify, neuronal subtype identity to the SOP cells. In this scenario, besides the neuronal nature, SOP cells have already been programmed to some extend by proneural genes towards certain neuronal subtypes. Establishment of their final identity, however, may still rely on the function of additional genes such as neuronal type selector genes like *cut*. An alternative model is that proneural genes not only determine the neuronal competence but also specify neuronal subtype identity. Formation of ectopic es organs by ectopic *ato* expression may simply result from ectopic activation of the endogenous AS-C genes by *ato*. Examining whether *ato* induced es organ formation in the absence of the AS-C will address this issue.

How do proneural genes affect neuronal subtype identity? There are two possibilities. First, as transcription factors, products of different proneural genes may regulate distinct set of downstream target genes by binding to different co-factors therefore instruct the cells to different developmental pathways. In fact, based on domain swapping experiment between *sc* and *ato*, Chien and colleagues (Chien et al., 1996) have demonstrated that the basic domain within each protein determines its functional specificity. By computer modeling, they further presented data suggesting that different DNA binding property of *ato* and *sc* may result from dimerizing with different co-factors through their basic domains. Identifying such co-factors will be of importance. Alternatively, the availability of the downstream target genes are pre-restricted by other transcription factors so that *ato* or *sc* are instructed to activate different organ-specific genes. These two models are not mutually exclusive. Characterizing direct downstream target genes of proneural genes will help to understand how proneural genes encode neuronal type information.

Is R8 absolutely required for differentiation of other photoreceptors?

During ommatidium assembly, R8 is the first of the eight photoreceptors to differentiate, which is directed by the proneural gene *ato* (Jarman et al., 1994; Jarman et al., 1995). Other photoreceptors within each ommatidium are successively recruited through local induction by R8. Their determination and recruitment into developing ommatidia do not require *ato* function but may involve other yet unidentified bHLH genes.

How does *sc* function to induce photoreceptor formation in our rescue experiments? There are two possible mechanisms. First, *sc* may mimic *ato* function to specify R8 photoreceptors, but is less potent than *ato*. Consistent with this, we observed *boss* expression in the eye disc and R8-like photoreceptor cells in some rescued ommatidia of adult eyes. Alternatively, *sc* may functionally substitute for other unknown bHLH genes and direct differentiation of non-R8 photoreceptor cells, bypassing requirement for R8 cells. Two observations seem to support this possibility. First, in gal4-7,ato^1/UAS-*sc*,ato^1 discs, *boss* expression is delayed and appears to start after the onset of expression of an R7 marker. Second, in adult eyes, ommatidia without R8-like photoreceptors are often seen. The major difference between these two possibilities is whether R8 cells are formed. One way to address this issue is to repeat the *sc* rescue experiment in an *ato* mutant background which is also defective in EGF signaling, since the EGF pathway is required for development of all photoreceptors except R8 cells. If the first possibility is true, then *sc* should still rescue R8 formation in this mutant. If *sc* uses the second mechanism, no photoreceptor should be formed. It remains possible that *sc* employs both mechanisms in rescuing photoreceptor formation.

Fig. 3.1 *sc* expression driven by the *ato* regulatory regions promotes photoreceptor formation

(A-D) Scanning electron micrographs of compound eyes. (E-H) Eye discs from third instar larvae stained with an antibody against Boss protein. (I-L) Tangential sections of compound eyes showing structure of ommatidia. The genotypes of the samples are as follow. (A,E,I) wild-type; (B,F,J) 3'*ato* 5'*ato,ato*^{1}; (C,G,K) 3'*ato* 5'*sc,ato*^{1} and (D,H,L) 3'*sc* 5'*ato,ato*^{1}. (3': 3' regulatory region of *ato*. 5': 5' regulatory region for *ato* expression in the eye.)

Compound eyes rescued by 3'*ato* 5'*ato* display similar morphology (B) to the wild-type (A), albeit slight roughness. *boss* expression in the eye disc (F) and the photoreceptor arrangement in ommatidia (J) also resemble those in the wild-type (E,I). Both 3'*ato* 5'*sc* and 3'*sc* 5'*ato* induce ommatidial formation to much lesser extend (C and D respectively). Limited *boss* expression was detected in both cases (G,H). Majority of the ommatidia in the 3'*sc* 5'*ato,ato*^{1} eye (L) exhibit normal photoreceptor organization. Some have less photoreceptors (arrowhead). All ommatidia in the 3'*ato* 5'*sc,ato*^{1} eye contain less than normal number of photoreceptors, almost all of which are outer photoreceptor cells.

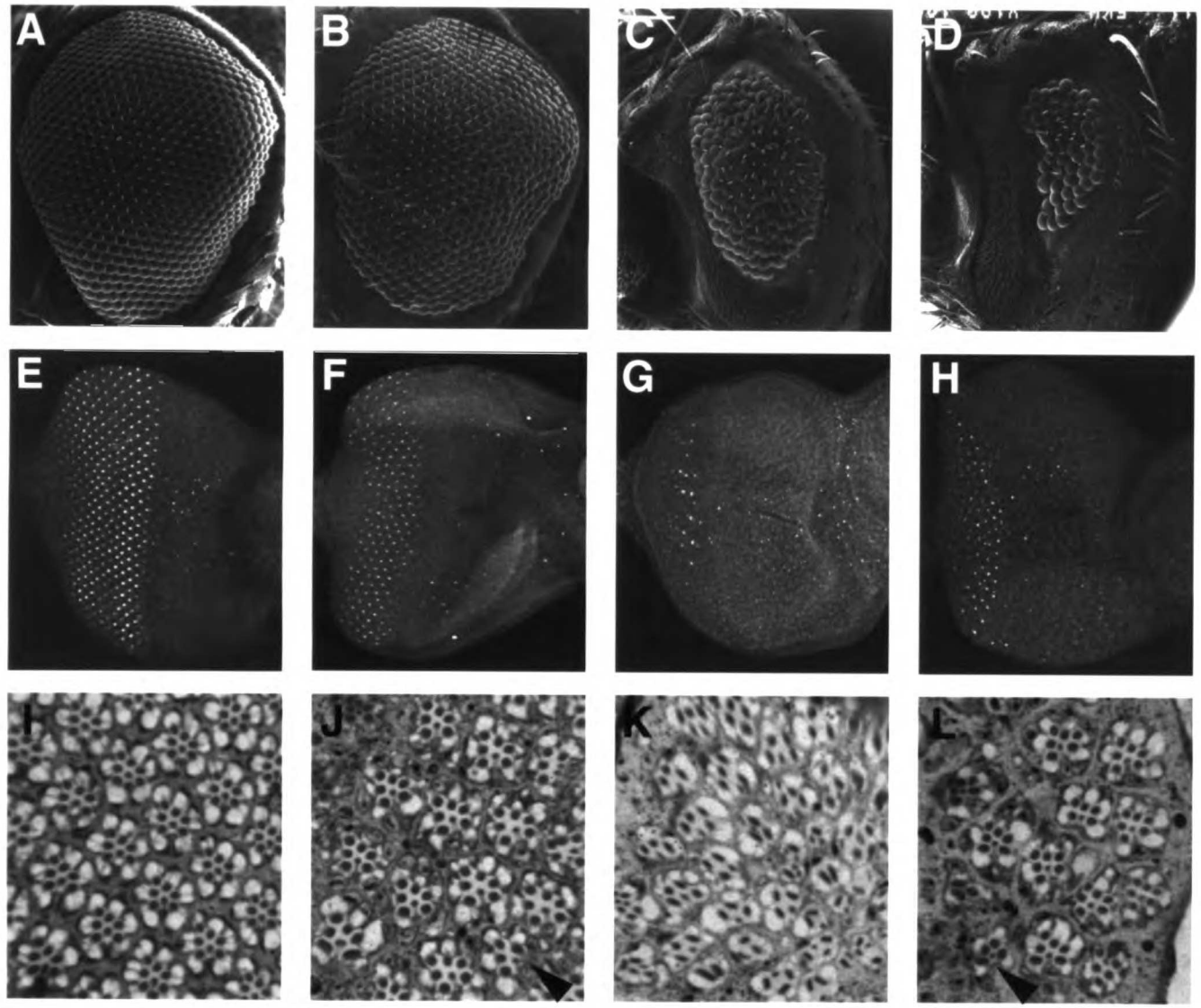

Fig. 3.1

Fig. 3.2 Ommatidial rescue by ectopic *sc* expression

(A) Eye disc from a wild-type third instar larva hybridized to a digoxigenin-labeled *ato* probe, showing the endogenous *ato* expression pattern. (B) Third instar larval eye disc of gal4-7/UAS-*lacZ*(nucl.) hybridized to a digoxigenin-labeled *lacZ* probe. gal4-7 drives reporter gene expression in many more cells particularly in the posterior field as compared to the wild type *ato* expression (A). (C, D) Scanning electron micrographs of UAS-*ato*/+; gal4-7,ato^1/ato^1 (C) and UAS-*sc*,ato^1/gal4-7,ato^1 (D). When ectopically expressed under the control of the gal4-7 line, *sc* alone induces ommatidial formation as *ato* does, but to lesser extend (compare D to C). Occasionally, fully developed macrochaete-like es organs (arrow in D and Fig. 3.4 A and B) are seen in different areas of the rescued eyes. (E) Third instar eye discs from ato^1 (E) and UAS-*sc*,ato^1/gal4-7,ato^1 (F) stained with an antibody against Ato. Ommatidial rescue by *sc* expression does not occur through ectopic activation of the endogenous ato^1, since the Ato^1 pattern in UAS-*sc*,ato^1/gal4-7,ato^1 shows no significant difference from that in ato^1.

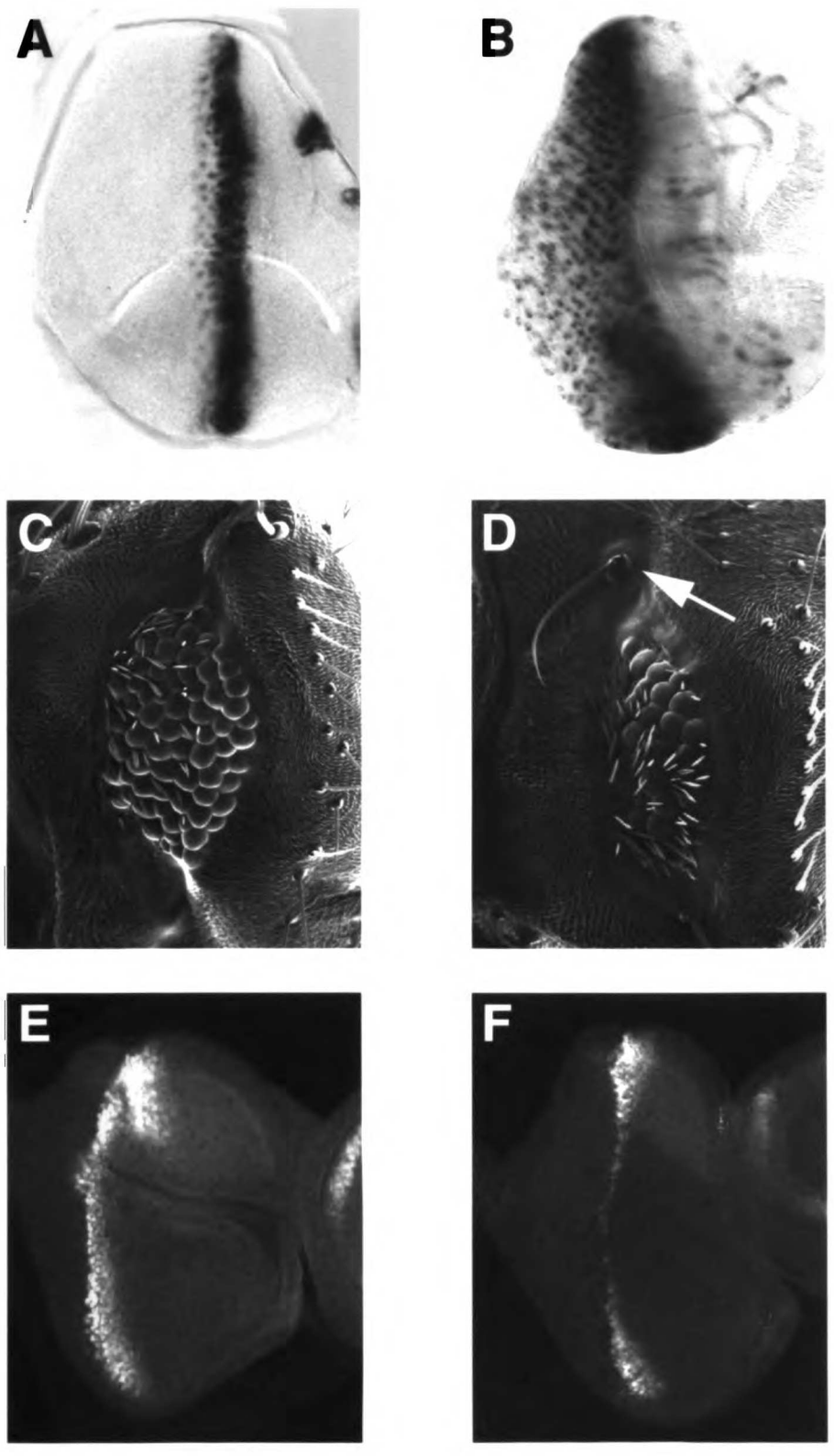

Fig. 3.2

Fig. 3.3 Different structure and developmental processes of ommatidia rescued by *ato* or *sc*

Genotypes of the samples are: UAS-*ato*/+; gal4-7,*ato*^{1}/*ato*^{1} (A,C,E,G) and UAS-*sc*,*ato*^{1}/gal4-7,*ato*^{1} (B,D,F,H). (A,B) Tangential sections of compound eyes showing structure of ommatidia. (A) An *ato*-rescued eye. Ommatidium often contains more photoreceptors than that in the wild type (compare with Fig. 3.1I). Multiple R8-like cells (arrow) in one ommatidium are frequently seen. (B) A *sc*-rescued eye. Most ommatidia only have outer photoreceptors, and the number is lower than normal. (C-F) Eye discs from third instar larva (C,D) or early pupae (E,F) stained with an anti-Boss antibody. *boss* expression is apparent in the third instar eye disc (C) and early pupal disc (E) of UAS-*ato*/+; gal4-7,*ato*^{1}/*ato*^{1}. In third instar discs of UAS-*sc*,*ato*^{1}/gal4-7,*ato*^{1} (D), *Boss* is not expressed, albeit clear advancement of the MF (arrowhead). Boss protein is only detected in early pupal discs (F) in less cells as compared to the *ato*-expressing discs of the same age (E). (G,H) Third instar larval discs stained with an antibody again Prospero. Expression is seen in both *ato*-expressing (G) and *sc*-expressing (H) discs.

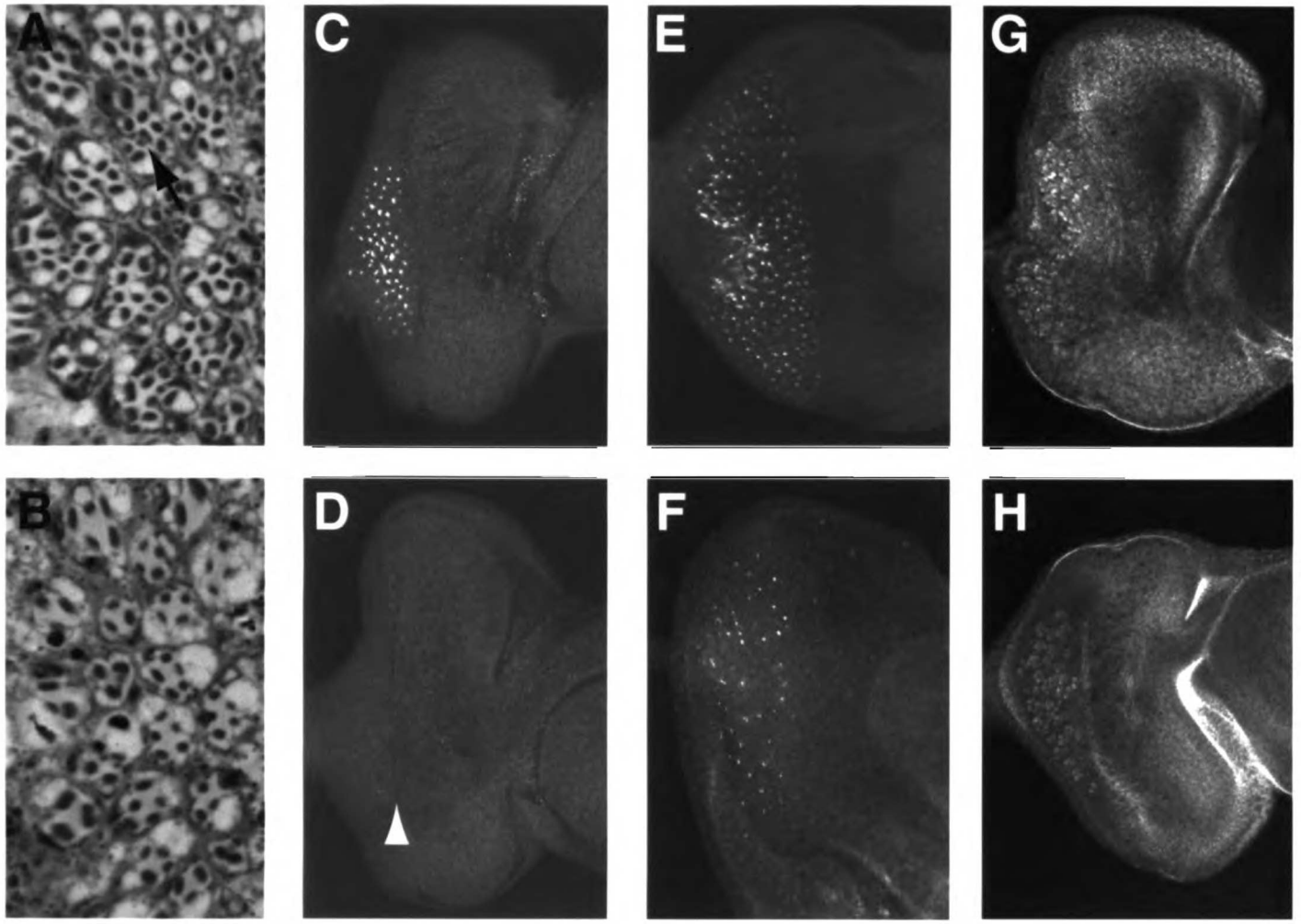

Fig. 3.3

Fig. 3.4 Sensory organ formation in the eye induced by ectopic expression of other bHLH genes

Scanning electron micrographs of compound eyes rescued by ectopic expression of *sc* and *Xato5* in ato^1 mutant background. (A,B) gal4-7, ato^1/UAS-*sc*, ato^1. In addition to photoreceptors, *sc* occasionally induces macrochaete-like es organ (arrowhead in A,B and arrow in Fig. 3.2 D) formation in different locations in the eye. These ectopic es organs are morphologically distinct from surrounding interommatidial bristles (arrow in B). (C) UAS-*Xato5*/+;gal4-7,ato^1/ato^1. Like *Drosophila ato*, *Xato5* promotes ommatidial development.

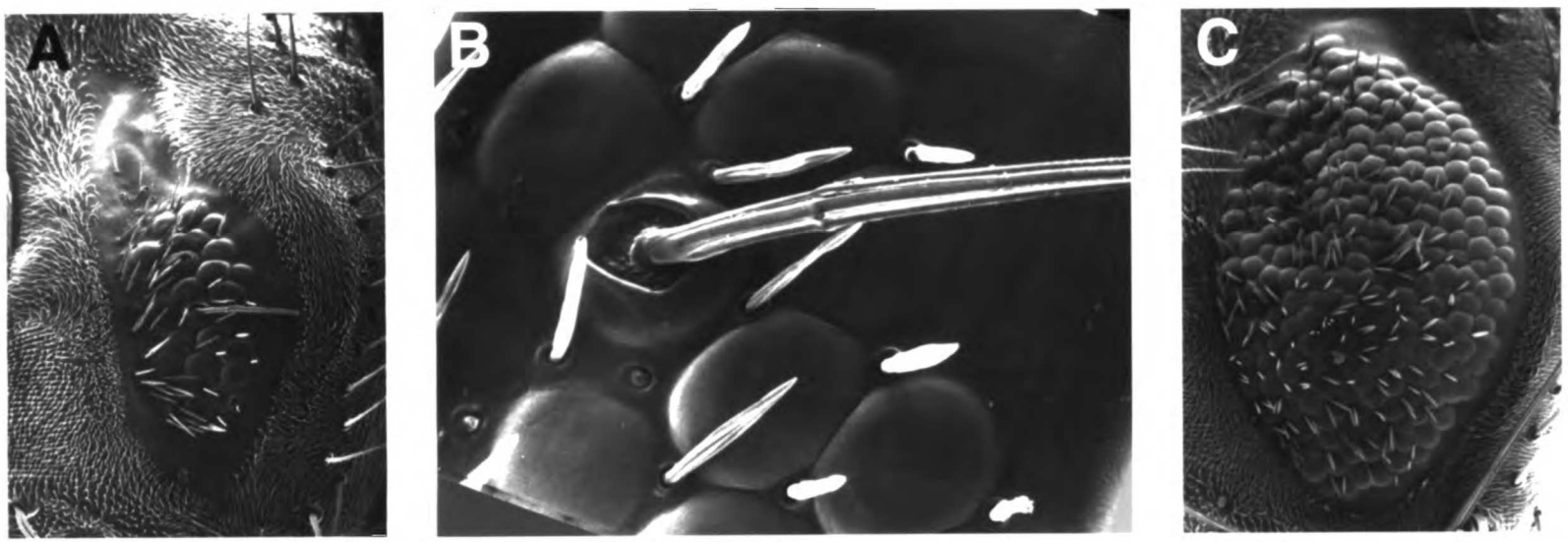

Fig. 3.4

CHAPTER 4

Ato interacting proteins: a yeast two-hybrid screen

Introduction

Biochemical and genetic experiments have suggested that proneural genes not only govern formation of neuronal precursors but also influence the specification of precursor identity in both CNS and PNS of *Drosophila*. Domain swapping experiment between Ato and Sc demonstrated that the bHLH domains of the two proteins, or the basic domains in particular, determine their specificity. The two basic domains differ in seven amino acid residues, all of which are indispensable and predicted not to contact DNA directly, but rather form a surface possibly involved in interaction with other co-factors (Chien et al., 1996). These findings suggest the possibility that organ-specific co-regulators may determine specificity of the resulting complexes and ultimately assign the identity to the sensory organ precursors. There is no direct data available to date to prove this model. However, the activities of some other bHLH proteins have been shown to be modulated by interacting with other classes of nuclear factors, such as MADS-domain proteins (Molkentin and Olson, 1996), leucine zipper proteins (Bengal et al., 1992) and LIM domain proteins (Johnson et al., 1997; Larson et al., 1996), and these types of interactions lead to initiation of specific developmental programs.

The yeast two-hybrid screen (Bartel and Fields, 1995; Fields and Song, 1989; Fields and Sternglanz, 1994) is an efficient way to search for potential interacting proteins. In this system, interaction of two proteins within yeast cells brings together a DNA binding domain and a transcriptional activation domain fused to them respectively, therefore allowing transcriptional activation of the reporter genes to occur. Genes encoding the interacting proteins can be easily identified by isolating the corresponding yeast plasmids. Several proteins that interact with bHLH transcription factors have been identified successfully by this means (Gradwohl et al., 1996; Lee et al., 1995).

In the limited investigation described in this chapter, I attempt to identify Ato interacting proteins by a yeast two hybrid screen. From 3.1 million clones screened, I obtained 217 positive clones and further characterized about one half of them, among which 32 clones show specific interaction with Ato. I further examined the expression patterns of these clones by whole mount in situ hybridization in embryos and in imaginal discs and revealed a few genes with potentially interesting expression patterns.

Materials and Methods

Bait construct

To make the bait construct pBHA#1.C*ato*, plasmid pBS.Bg 4.0 (a gift from Andy Jarman) containing most of the *ato* coding region and the 3.0 kb downstream sequences was digested with BamHI. A 283 bp DNA fragment which contains the bHLH domain of *ato* was isolated by gel purification and inserted into BamHI site of the DNA binding domain vector pBHA#1, resulting in pBHA#1.C*ato*. The junction of the ligation and the orientation of the insert were confirmed by DNA sequencing using the following primer: 5'-TCA TCG GAA GAG AGT AG-3'.

Yeast transformation

Overnight culture of yeast strain L40 which carries two reporter genes *lacZ* and *his3* under the control of upstream Gal4 binding sites (i.e. the UAS sites) was diluted 1:50 to 1:100 with fresh YPD medium (20 g glucose, 20 g peptone (Difco) and 10 g yeast extract in 1 Liter) and incubated with shaking for additional 3 to 4 hours at 30°C. For each transformation reaction, cells in 10 ml yeast culture were harvested by spinning at 3k rpm for 5 minutes, washed once with ddH2O. The pellet was resuspended in 100 µl transformation buffer (100 mM LiAc/10 mM Tris(pH7.5)/1 mM EDTA). 500 ng to 1000ng transforming DNA and 100 µg carrier DNA (10 mg/ml, Clontech) were used for each transformation. 0.6 ml PEG solution (0.8 volume of 50% PEG 4000 solution, 0.1 volume of 1 M LiAc and 0.1 volume of 100mM Tris(pH7.5)/10 mM EDTA) was mixed with the yeast suspension and DNA by gentle vortexing and the reaction was incubated at 30°C for 30 minutes. After adding one tenth total volume of DMSO and brief vortexing, the reaction was immediately heat shocked at 42°C for 15 minutes, pelleted at 3k rpm for 5 minutes, resuspended in 100 µl TE buffer (10 mM Tris(pH7.5)/1 mM EDTA) and plated onto selection plates.

Transformation of the bait construct with the activation domain library was performed following essentially the same protocol except that more yeast culture (20 ml), DNA (5 μg each of the bait construct and the library plasmids, 200 μg carrier DNA), and solutions (200 μl transformation buffer, 1.2 ml PEG solution) were used. After heat shock and pelleting, the cells were resuspended in 3 ml YPD medium and incubated with shaking at 30°C for an additional 2 to 3 hours before plated on selection plates (Trp^- Leu His^-). 10 mM 3-aminotriazole (3-AT) was also included in the plate to inhibit basal *his3* expression so that the yeast cells can not grow on synthetic media lacking histidine unless *his3* gene is activated.

X-gal assay

A nitrocellulose membrane (Schleicher & Schuell) was placed onto a yeast transformation place to make membrane replica. This replica was then transferred to a aluminum foil boat with the colony side up, precooled above a pool of liquid nitrogen, and submerged in liquid nitrogen for at least 5 seconds to freeze the yeast cells. The membrane was transferred onto a Whatman paper presoaked with X-gal/Z buffer [1.5 ml X-gal solution (20 mg/ml 5-bromo-4-chloro-3-indolyl-b-D-galactoside in *N,N*-dimethylformamide) in 100 ml Z buffer (8.52 g Na2HPO4, 5.5 g NaH2PO4·H2O, 0.75 g KCl and 0.246 g MgSO4·7H2O in 1 liter, pH7.0)] in a Petri dish and kept at room temperature in dark for color to develop. β-galactosidase-producing colonies can be identified by their blue color.

Identification of activation domain plasmids encoding Gal4-Ato(C)-interacting fusion proteins

To further select for clones that contain the Gal4-Ato(C) interactors, individual positive clones (His^+X-gal^+) from the primary library screen were streaked onto the selection

plates ($Trp^-Leu^-His^-$ with 10 mM 3-AT) and the colonies that can grow on these plates were assayed for β-galactosidase production. The restreak/X-gal assay was repeated twice to establish His^+β-$galactosidase^+$ clones.

Yeast plasmid mini-prep

Yeast cells were resuspended in 50 µl TE buffer and lysed by adding 200 µl lysis buffer (10 mM Tris(pH8.0)/1 mM EDTA/100 mM NaCl/1% SDS/2% Triton X-100), 200 µl phenol/chloroform, about 100 µl total volume of glass beads (Sigma, 425-600 microns) and then vortex vigorously for 2 minutes. The mixture was spinned in microcentrifuge at the maximal speed for 5 minutes. Supernatant was transferred into a new eppendorf tube. Plasmid DNA was recovered by ethanol precipitation with 3X volume of ethanol, washed with 70% ethanol, speed-vacuum dried for 5 minutes and resuspended in 50 µl TE buffer.

Recovery of the activation domain plasmids encoding Ato(C) interacting peptides

To separate the activation domain plasmid from the bait construct, 0.5 µl of the yeast plasmid preparation from the library screen was used to transform bacterial strain MH4 by electroporation . Transformants that can grow on the M9 selection plate (Leu^-) contain only the activation domain plasmid which was then isolated by standard bacterial plasmid mini-prep.

Each activation domain plasmid recovered was co-transformed with the bait construct into yeast strain L40. Those that can restore the interaction (therefore, His^+β-$galactosidase^+$) were then co-transformed with control DNA binding domain construct pBHA-lamin to test the specificity of the interaction. Only the clones that did not interact with Lamin were considered as fusion proteins that interact specifically with the Gal4-Ato(C) fusion protein.

whole mount in situ hybridization

The activation domain plasmids recovered were digested with XhoI. Individual inserts were isolated by gel purification and used as templates for digoxigenin labeling. The labeling reaction was carried out according to the manufacturer (Bohringer Mannheim). Whole mount in situ hybridization in embryos and in imaginal discs using digoxigenin-labeled probes was described by Tautz and Pfeifle (1989).

Results

Screen the activation domain library for potential Gal4-Ato(C) interacting clones

The DNA-binding domain hybrid of Gal 4 and full length Ato activates transcription of the reporter genes in the absence of an activation domain hybrid. This conceivably results from transcriptional activity of the Ato protein. To circumvent this, only sequences for the C-terminal 92 amino acids including the bHLH domain were incorporated into the DNA binding domain plasmid pBHA#1·C*ato*, which does not activate expression of the reporter genes by itself. The activation domain library (a gift from Stephen J. Elledge) was constructed in activation domain vector pACT using cDNAs from third instar larvae of *Drosophila*. The pACT vector also contains the nutritional gene *leu2* which allows yeast or bacterial auxotrophs to grow on Leu^- selection plates. 3.1 million yeast colonies were screened after co-transformation of pBHA#1·C*ato* and the activation domain library, among which 238 colonies grew on His^- selection plates and turned blue in X-gal assays. After two more rounds of the same His selection/X-gal assay, 217 colonies remained $His^+\beta$-galactosidase$^+$. Given the difficulty of processing more than two hundred clones, I mainly focused on 95 clones that turned blue in less than 1 hour in the X-gal assay. Plasmids from each of these positive clones were isolated and the activation domain plasmids were separated from the DNA binding domain plasmid by introducing the isolated plasmid mixture into a Leu auxotroph, MH4, and selecting transformants that grow on the Leu^- M9 plate.

To exclude clones that interact with the Gal4-Ato(C) fusion protein non-specifically, I co-transformed individual activation domain plasmids together with a control activation domain plasmid, pBHA·lamin, which encodes a Gal4-Lamin fusion protein instead of Gal4-Ato(C), into the original yeast strain L40. Among 69 plasmids tested, 34 result in

His$^-$β-galactosidase$^-$ transformants, therefore the interaction between the corresponding activation domain hybrids and the Gal4-Ato(C) fusion protein detected in the library screen is likely to be specific. Furthermore, in most cases (32/34), I was able to restore this interaction by using isolated activation domain plasmids and pBHA#1·C*ato*. Thus I conclude that these 32 activation domain plasmids encode fusion proteins that specifically interact with Gal4-Ato(C) in yeast two-hybrid system.

Characterization of the clones interacting with the Gal4-Ato(C) fusion protein

As the first step to characterize the 32 activation domain plasmids isolated from the two-hybrid screen, I carried out nucleotide sequencing of the cDNA inserts in them. Sequence alignment revealed that several independent clones share overlapping sequences therefore are likely to be derived from the same coding sequence. In order to address their function during development, I first searched databases for potential homologous sequences, hoping that information about these sequences may shed light on molecular structure and function of the genes identified in the screen. In addition, I examined the expression patterns of the potential genes by whole mount in situ hybridization. For simplicity, only results from a few clones are presented below. More information is provided in Table 4.

Two independent clones (7-24, 9-6) map to different regions of the *Drosophila daughterless* (*da*) gene: one (9-6) is at the very N-terminus, the other is in the C-terminal portion which includes the bHLH motif. This finding indicates that in vivo and in vitro interaction between Ato and Da (Brand et al., 1993; Rosay et al., 1995) was recapitulated in the yeast two hybrid system, suggesting that the approach of searching for Ato interacting proteins by yeast two hybrid screen could be effective. In addition, interaction between Ato and Da seems to involve other domain(s) in the Da protein, for instance, the

N-terminal portion, besides the bHLH motif previously shown to be responsible for the interaction.

Three overlapping clones (6-22, 9-10, 11-17) share about 94% identity with part of a subclone (L43416) from a P1 clone (DS00004) that resides within the antennapedia complex. Several transcripts have been found in this P1 genomic region, but no correlation between the transcripts and subregions of the P1 clone has been established. Whole mount in situ hybridization reveals strong maternal contribution in early embryos (data not shown). Later embryos exhibit strong staining in the developing mesoderm (Fig. 4A). In a stage 16 embryo, the transcript is predominantly expressed in the gonad (Fig. 4B). In imaginal discs from third instar larva, ubiquitous expression is seen in leg discs, wing discs (data not shown) and eye-antennal discs (Fig. 4C) excluding the region around the MF which roughly corresponds to the area where *ato* is normally expressed. The significance of this distribution is unclear. Low or no larval CNS expression was observed.

Another group of three clones (1-10, 7-20 and 11-19) displays expression mainly in nervous system in the embryo (Fig. 4D,E). In the eye disc, expression in the anterior region is much stronger than that in the posterior region (Fig. 4F). No significant sequence homology was found between this group and the sequences in the database searched.

Clone 11-12 which completely spans two other clones (10-6, 10-7) exhibits an overall 69% identity to a yeast serine-rich RNA polymerase I suppressor. The expression pattern is strikingly similar to that from the above group (data not shown).

The expression pattern of stronger anterior to the MF and weaker posterior to the MF is best represented by another clone, 11-4 (Fig. 4I). In a stage 11 embryo, in addition to enhanced NS staining , a segmental pattern of tracheal pit is apparent. At stage 16, it is expressed in the CNS, trachea and hindgut (Fig. 4H). This clone shows 93% identity at the nucleotide level to *Drosophila hemomucin* gene which encodes a transmembrane protein thought to be involved in the induction of the immune response in *Drosophila* (Theopold et al., 1996).

Discussion

In Chapter 3, I presented data indicating that proneural genes affect the specification of sensory organ subtype identity during PNS development. However, the underlying molecular mechanism is largely unknown. Chien et al. (1996) suggested that the bHLH motifs, particularly the basic domains of different proneural genes may be responsible for specifying neuronal subtype identity through interacting with distinct co-factors. In this chapter, I described the effort of looking for such co-factors of the Ato protein by a yeast two-hybrid screen. The results are preliminary. In the interest of those who might pursue this study, I will discuss some potential problems about this project and then suggest a few lines of future experiments.

A major concern about this project is the lack of direct evidence for the existence of organ-specific co-factors for proneural gene products, despite the fact that some bHLH transcription factors do interact with other proteins to regulate transcription of specific target genes (Bengal et al., 1992; Johnson et al., 1997; Larson et al., 1996; Molkentin and Olson, 1996). Besides, the model that basic domains determine the specificity of proneural genes by binding to co-factors does not exclude the possibility that other domains outside the bHLH motif may also be involved. In an extreme case, the specificity could be assigned solely by non-bHLH sequences, most of which in the *ato* gene are not included in the bait construct. One such case has been reported for the bHLH/PAS class of transcription factors in that the PAS domain determines the specificity and the distinct recognition of target genes. All these impose certain risk onto this project.

Potential problems of yeast two hybrid screens are as apparent as its advantages. For instance, some actual in vivo interaction may escape detection for a variety of reasons.

For example, the activation domain hybrids could be toxic to the cells therefore the transformants can not grow. Moreover, fusion of the Gal4 DNA binding domain to Ato, or fusion of the activation domain to the target proteins may change their conformation which ultimately may lead to both false positive and false negative clones. In fact, many clones isolated in this screen are likely to be false positives: some seem to have open reading frames but in reversed orientation, some are not expressed at all in the tissues where *ato* is normally expressed. Unfortunately, all these problems can not be effectively prevented beforehand. Another caveat particular to this screen is that the co-factors may bind to Ato/DNA complexes rather than to Ato alone. This type of interaction can not be detected in this screen.

Despite all the risks, I have isolated some two hundred potential Ato(C) interacting clones. It should be noted that the majority of the clones characterized so far were represented more than once in the screen, strongly suggesting the screen being saturated. A crucial task at this point is to focus on promising clones. Several criteria can be employed in defining such clones. A counter screen using Gal4 DNA binding domain-*sc* bHLH region as the test construct may allow further elimination of clones interacting with Ato(C) non-specifically. Expression pattern determined by whole mount in situ hybridization may provide additional information to include or exclude certain clones. To assess whether the candidate genes exert any effect on PNS development, one could first map these genes onto chromosomes by in situ hybridization, then look for overlapping chromosomal deficiencies that would remove the corresponding genomic regions and hence the activity of these genes, and analyze for possible PNS defects. The availability of appropriate deficiencies, however, is sometimes limited. Moreover, lethality frequently caused by overlapping deficiencies makes the study of mutant phenotypes at later developmental stages (for instance, defects in photoreceptor development) difficult.

Once the interaction is confirmed, the role of the candidate gene in specifying neuronal subtype identity can be addressed by generating loss of function mutations in the gene and analyzing the resulting mutant phenotype. If this gene indeed determines the ch neuron and/or photoreceptor fates, their development in the mutant would be impaired. Conversely, misexpression of this gene may interrupt development of other sensory organs (for example, es organs), or more severely, lead to transformation of these organs into ch organs or photoreceptors.

Fig. 4 Expression patterns of clones interacting with Ato(C) in the yeast two hybrid system

Expression patterns in embryos and in imaginal discs are determined by whole mount in situ hybridization. Embryos are shown with anterior to the left and dorsal up unless otherwise mentioned. (A-C) Clone 6-22. (A) Lateral view of a stage 9 embryo. 6-22 is expressed in the developing mesoderm (arrow) and anterior midgut (arrowhead). (B) Dorsal view of a stage 16 embryo. Expression in the mesoderm is lost, but strong expression is seen in the developing gonad (arrow). (C) Eye-antennal disc. A band with lower expression level spans approximately the region around the MF where *ato* is expressed (bracket). (D-F) Clone 11-19. (D) Lateral view of a stage 11 embryo. 11-19 is expressed in the developing nervous system. (E) Ventral view of stage 15 embryo. Expression is specific to the CNS. (F) Eye disc. Expression in the anterior field (arrowhead) is higher than that in the posterior field. (G-I) Clone 11-4. (G) Lateral view of a stage 11 embryo showing expression in nervous system (arrowhead) and tracheal pits (arrow). (H) Stage 16 embryo. 11-4 is expressed in the CNS, trachea (arrow) and hindgut (arrowhead). (I) Eye disc. Expression anterior to the MF (arrowhead) is higher than posterior to the MF.

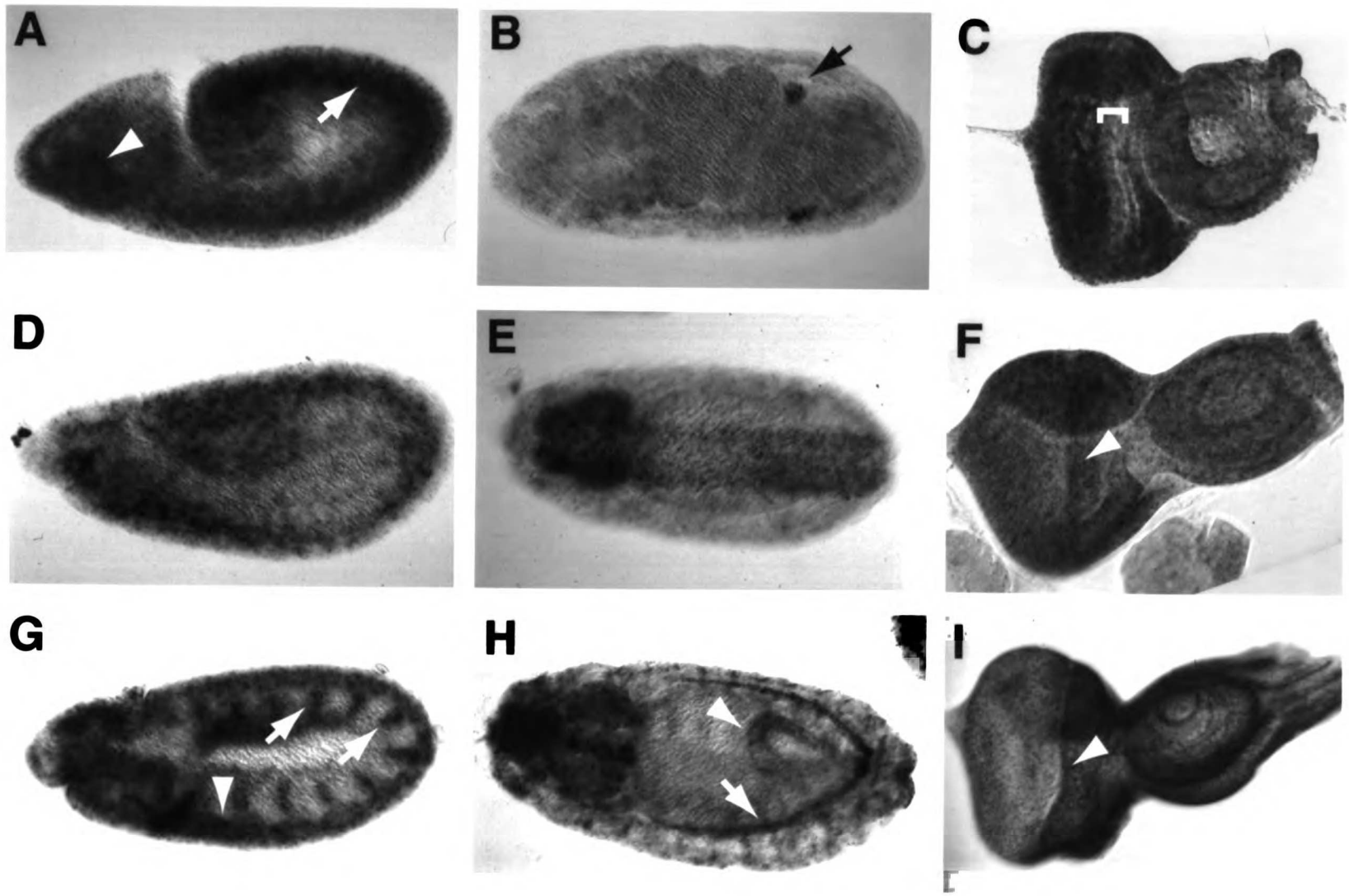

Fig. 4

Table 4 Clones specifically interacting with Ato(C) in the yeast two-hybrid system

Clone number	β-Gal assay	sequence	In situ pattern
1-10	30 min	≈ 7-20, 11-19	11-19 (Fig. 4.1D-F)
1-12	1 hr	Glue protein	?
2-9	30min	novel	only in salivary gland
2-10	30min	?	?
2-11	1 hr	≈ 4-11, 5-13	?
2-16	30min	?	?
3-1	30 min	?	?
3-6	30 min	?	?
4-7	30 min	?	?
4-12	30 min	2-9 group	2-9
5-1	30 min	Novel	?
5-3	30 min	?	?
5-13	1 hr	≈ 2-11,4-11	?
6-4	30 min	2-9 group	2-9
6-15	30 min	2-9 group	2-9
6-18	30 min	2-9 group	2-9
6-22	1 hr	≈ 9-10, 11-17	Fig. 4.1A-C
7-9	30 min	?	?
7-12	?	Novel	?
7-20	1 hr	≈ 1-10, 11-19	11-19 (Fig. 4.1D-F)
7-23	30 min	Novel	?
9-10	30 min	≈ 6-22, 11-17	6-22 (Fig. 4.1A-C)
10-1	1 hr	Novel	?

10-6	30 min	≈ 10-7,11-12	11-12
10-7	30 min	≈ 10-6,11-12	11-12
10-20	30 min	Yolk protein2	?
11-4	30 min	mucin?	Fig. 4.1G-I
11-12	30 min	≈ 10-6, 10-7	11-12
11-17	30 min	≈ 6-22, 9-10	6-22 (Fig. 4.1A-C)
11-19	30 min	≈ 1-10,7-20	Fig. 4.1D-F

* "β-Gal assay": minimal time before the colony turns blue in X-gal assay;
" ≈ ": colonies with overlapping sequences;
" ? ": not determined.

CHAPTER 5

CONCLUSION

Summary and prospectus:

Molecular and genetic studies have established *ato* as a proneural gene that directs development of embryonic and adult chordotonal organs (Jarman et al., 1993; Jarman et al., 1995), photoreceptor cells (Jarman et al., 1994; Jarman et al., 1995) and a subset of antennal olfactory organs (Gupta and Rodrigues, 1997). The Ato protein heterodimerizes with the ubiquitously expressed Daughterless protein and the resulting complex is capable of binding to the E boxes (Jarman et al., 1993), through which transcriptional activation of *ato* target genes may occur. This thesis has further explored the transcriptional regulation of *ato* during neurogenesis (Chapter 2). The most significant aspect of this work is the identification of the modularly arranged regulatory regions responsible for *ato* expression in all tissues where it is normally expressed. Moreover, in every tissue, expression in proneural clusters and subsequently in SOP cells appears to be controlled by separate regulatory regions. These findings provide a basis for further investigating issues such as how positional information encoded by upstream prepattern genes regulates *ato* expression in its spatiotemporal pattern.

It has been shown previously that ectopic *ato* expression yields ectopic ch organs and es organs, whereas *sc* function is restricted in promoting es organ formation only (Chien et al., 1996; Jarman et al., 1993). In Chapter 3, I demonstrated that *sc* is capable of directing some photoreceptor formation. Taken together, proneural genes differ in their ability to specify different types of sensory organs. This difference may result from binding of organ-specific co-regulators to the products of proneural genes. A yeast two hybrid screen was carried out in an effort to look for Ato-interacting proteins (Chapter 4).

In this chapter, I will discuss a few issues that need to be pursued in order to better understand *ato*'s function in directing sensory organ development.

How is the stereotyped expression pattern of *ato* achieved?

The *ato*-expressing proneural clusters are produced in invariant positions prefiguring the future ch organs and photoreceptor cells. The highly reproducible segmental pattern in the embryo implies a possible relation between *ato* and the anterior-posterior (A/P) and dorsal-ventral (D/V) patterning genes as was proved for regulation of the AS-C genes during embryonic CNS development (Skeath, 1992). That is, as a link, *ato* receives global positional information encoded by the upstream prepattern genes and transduces it to neuronal competence in cells of proneural clusters. So far, no prepattern genes have been identified that regulate *ato* expression. It is of importance to identity the patterning genes that constitute specific A/P and D/V coordinates from which *ato*-expressing proneural clusters arise. Comparing the relative positions of the proneural clusters and the domains expressing different patterning genes by double-labeling experiments may lead to prime candidate patterning genes. Their involvement in *ato* regulation could be determined by monitoring behavior of the *ato-lacZ* reporter genes in the corresponding mutant or overexpression background.

How does *ato* act as an organ-specific transcription factor?

Ato dimerizes with Da and binds to DNA in vitro in a sequence-specific manner (Jarman et al., 1993). Ato and Da also function together in vivo to activate transcription (Rosay et al., 1995). However, these findings do not rule out the involvement of other co-factors. In fact, it has been postulated that Ato and Sc may interact with different organ-specific co-factors through their basic domains, which may account for the difference between Ato and Sc in their ability to instruct development of distinct sensory organs (Chien et al., 1996). No such co-regulators have been identified.

Recent studies in vertebrates have demonstrated that members of the MEF2 (myocyte enhancer factor-2) family of MADS (MCM1, Agamous, Deficiens, Serum response factor) box transcription factors interact with Mash 1, the product of a vertebrate homolog of the AS-C genes, through bHLH domain of Mash 1 to synergistically activate transcription by using either MEF2 binding site or Mash 1 binding site (Black et al., 1996). A Drosophila *mef2* homolog (*d-mef2*) was isolated and has been implicated in myogenesis and neurogenesis (Lilly et al., 1994; Nguyen et al., 1994; Schulz et al., 1996; Taylor et al., 1995). It would be interesting to test whether D-Mef2 acts as a co-regulator for Achaete, Scute and Ato, or whether there exists a D-Mef2 equivalent for Ato. Identification of such co-factor of Ato, which could be achieved by conventional biochemical approaches (e.g. co-immunoprecipitation, screening expression library) and yeast two-hybrid system, may provide valuable insights into important issues such as how *ato* functions differently from *sc* and how the same gene *ato* specifies at least three distinct types of sensory organs.

How does *ato* instruct differentiation of three distinct types of sensory organs?

ato is a proneural gene for ch organs, photoreceptor cells and subsets of antennal olfactory organs. An important question is how action of one gene, *ato*, gives rise to three distinct types of sensory organs. This organ specificity could be achieved by four not mutually exclusive means as discussed below.

(1) Different forms of Ato may be responsible for different sensory organ identity. Northern analysis revealed only one mRNA species in embryos. Although this has not been tested with extracts from larvae, the lack of introns and alternative start codons in the *ato* gene makes it unlikely that alternatively spliced forms of *ato* exist.

Several cases of post-translational modification, mostly phosphorylation, of bHLH factors have been reported. For instance, Myogenin, a vertebrate bHLH myogenic factor, is phosphorylated at multiple threonine and serine residues within its basic domain by the FGF signaling pathway mediated by the protein kinase C. This phosphorylation prevents Myogenin from binding to DNA, which accounts for the inhibitory action of the FGF pathway on myogenesis (Li et al., 1992). *Drosophila* Achaete protein appears to be phosphorylated on a serine residue by the lateral inhibition pathway mediated by *N*. This may contribute to the anti-neurogenic effect of *N* in the non-SOP cells within a proneural cluster (Nakao et al., 1997). Recently studies of the *Xath-3* and *Math-3* genes, homologs of *ato* from Xenopus and mouse respectively, indicated that phosphorylation of the serine residue (in *Xath-3*) or the threonine residue (in *Math-3*) in their basic domains modulates the activity of the two proteins (Takebayashi et al., 1997). Although *Drosophila* Ato protein lacks threonine and serine residues in its basic domain, phosphorylation on residues outside the basic domain or modification of Ato by other means can not be ruled out.

(2) Ato may interact with distinct co-factors in different pathways.

The findings that Ato and Sc differ in their ability to promote sensory organ formation, and that this difference may result from binding to distinct co-factors (Chien et al., 1996) raise the possibility that Ato itself may interact with different co-regulators thus initiating different developmental programs. Obvious candidates are organ-specific transcription factors such as *glass* (*gl*), *eyes absent* (*eya*) and *sine oculis* (*so*) for photoreceptor development. So far, no co-factor other than Da has been identified.

(3) Ato (or its complex) may activate different downstream target genes to initiate different developmental programs.

Even if Ato only interacts with Da, the heterodimer may still act in concert with other organ-specific transcription factors to regulate expression of distinct downstream target genes in different developmental contexts. Identifying direct downstream target genes is of crucial importance in understanding not only how *ato* initiates a neuronal program, but also how it allocates sensory organ identity. Potential candidates may be provided by approaches such as yeast one-hybrid screen (Li and Herskowitz, 1993).

All direct downstream target genes of the AS-C identified thus far include the AS-C genes themselves and those involved in lateral inhibition such as members of the *Enhancer of split* complex, *scabrous* and *Bearded* (Singson et al., 1994). Similarly, refinement of *ato* expression as well as initiation of *sca* expression in the eye also require Ato function (see Jarman et al., 1995 and Chapter 2). These findings may help to understand how expression of proneural genes is restricted to and enhanced in SOP cells. It would not, however, explain how proneural genes direct neuronal differentiation and how *ato* specifies different sensory organ fates.

(4) Sensory organ identity may be specified by determinants acting downstream of *ato*. In this scenario, *ato* would exert the same function, possibly the proneural function, in all three different developmental pathways. The sensory organ identity is later assigned to SOP cells by other determinants further downstream of *ato* after the SOPs have acquired neuronal competence. A genetic screen looking for modifiers of *ato* may uncover genes involved in identity specification.

How does *ato* integrate information from multiple signaling pathways in the eye?

In the eye, *ato* serves as a link between patterning events and photoreceptor differentiation. Multiple signaling pathways regulate *ato* expression. *hh* is necessary and

sufficient to activate *ato* expression anterior to the MF (Borod and Heberlein, 1998; Heberlein et al., 1995). It appears to inhibit *ato* expression in cells between intermediate clusters and R8 cells within and posterior to the MF (María Domínguez, personal communication). *N* also exerts dual function on *ato* expression: it enhances *ato* expression in the initial stripe but inhibits it in non-R8 cells within intermediate groups in the MF (Baker et al., 1996; Baker and Yu, 1997). *so* and *eya* are required for initiating *ato* expression (Jarman et al., 1995). So, Eya and Dachshund (Dac) were recently shown to form a complex which is sufficient to induce ectopic compound eye formation (Chen et al., 1997; Pignoni et al., 1997). One direct target gene of this complex is likely to be *ato* (Francesca Pignoni, personal communication). *Drosophila* EGF receptor (DER) pathway also appears to affect *ato* expression and R8 specification (Ross Cagan, personal communication). Inhibitory bHLH or HLH factors Hairy and Emc act synergistically to prevent premature *ato* expression in the anterior field (Brown et al., 1995). Elucidating the molecular mechanism whereby all there signaling pathways converge to regulate *ato* expression may rely on a systematic analysis of the eye regulatory regions identified in this study (Chapter 2).

Post-transcriptional regulation of *ato*

N appears to have opposite effects on *ato* transcript and Ato protein anterior to the MF: loss-of-function mutation of *N* yields ubiquitous and higher transcript level (Chapter 2) but much lower protein level (Baker and Yu, 1997) compared to those in the wild type, strongly suggesting a post-transcriptional control of *ato* by *N* signaling. The molecular mechanism by which *N* exerts dual function on *ato* expression remains to be determined. *N* could modulate mRNA stability, translation efficiency and protein stability. This may involve the so-called Bearded box (Lai and Posakony, 1997) present in *ato*'s 3'UTR (Eric

Lai and James Posakony, personal communication), the nine nucleotide sequence shared by the *Bearded* gene and the m4 member of the *Enhancer of split* complex, since this consensus sequence has been shown to confer instability on transcripts that contain them and also affects protein level (Lai and Posakony, 1997; Leviten et al., 1997). How the Bearded box functions and whether the ones in *ato* 3'UTR mediate post-transcriptional regulation by *N*, however, are unclear.

Role of *ato* in CNS development

Most of the vertebrate *ato* homologs identified so far including *Math1* (Akazawa et al., 1995), *Math2/Mex1* (Shimizu et al., 1995), *Math3/Xath3/NeuroM* (Roztocil et al., 1997) (Takebayashi et al., 1997), *Math4a/ngn2* (Gradwohl et al., 1996), *Math4b/ngn3* (Sommer et al., 1996), *Math4c/ngn1* (Ma et al., 1996), *Xath5* (Kanekar et al., 1997), *Math5* (Nadean Brown, personal communication) and *NeuroD* (Lee et al., 1995) are expressed predominantly, if not exclusively, in the developing CNS. Moreover, mice lacking *Math1* have cerebellum defects, indicating a role of *Math1* in CNS development. These studies in vertebrates raise the possibility that *Drosophila ato* may also play a role in CNS development. In fact, *ato* is expressed in patches of cells in the embryonic CNS (Jarman et al., 1993). In the larval CNS, *ato* is seen in a large crescent and a few small groups of cells in each brain lobe. The identity of these *ato*-expressing cells has not been determined. Null mutation of *ato* (ato^3) causes lethality, most likely due to the CNS defect, because ch organs and photoreceptors are dispensable for animals to live. Taken together, it appears that *ato* may play a role in CNS development. Since the regulatory regions responsible for *ato* expression in both embryonic and larval CNS have been mapped (Chapter 2 and unpublished observation), function of *ato* and development fates of these CNS cells can now be analyzed by targeted expressing genes that would, for example, eliminate these cells specifically.

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