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Fasciclin II controls proneural gene expression in *Drosophila*

(sensory organ precursor/cell adhesion molecule/pattern formation/neurogenesis/achaete–scute complex)

LUIS GARCÍA-ALONSO*, MARK F. A. VANBERKUM†, GABRIELE GRENNINGLOH‡, CHRISTOPH SCHUSTER, AND COREY S. GOODMAN§

Howard Hughes Medical Institute, Division of Neurobiology, Department of Molecular and Cell Biology, Life Science Addition, Room 519, University of California, Berkeley, CA 94720

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ABSTRACT **Fasciclin II (Fas II), an NCAM-like cell adhesion molecule in *Drosophila*, is expressed on a subset of embryonic axons and controls selective axon fasciculation. Fas II is also expressed in imaginal discs. Here we use genetic analysis to show that Fas II is required for the control of proneural gene expression. Clusters of cells in the eye-antennal imaginal disc express the achaete proneural gene and give rise to mechanosensory neurons; other clusters of cells express the atonal gene and give rise to ocellar photoreceptor neurons. In *fasII* loss-of-function mutants, the expression of both proneural genes is absent in certain locations, and, as a result, the corresponding sensory precursors fail to develop. In *fasII* gain-of-function conditions, extra sensory structures arise from this same region of the imaginal disc. Mutations in the Abelson tyrosine kinase gene show dominant interactions with *fasII* mutations, suggesting that Abl and Fas II function in a signaling pathway that controls proneural gene expression.**

Proneural genes of the achaete–scute complex such as achaete (*ac*) and scute (*sc*) and the atonal (*ato*) gene control development of neural precursor cells in *Drosophila* (e.g., for reviews see refs. 1–4). The proneural genes are typically expressed in clusters of cells (proneural clusters), which become neuralized—that is, they acquire the capacity to generate neurons. A lateral inhibition mechanism controlled by neurogenic genes such as Notch assures that typically only one cell from within each proneural cluster becomes a neural precursor [a neuroblast in the central nervous system or a sensory organ precursor (SOP) in the peripheral nervous system] (e.g., for reviews see refs. 5–7).

Much is known about the mechanisms leading from proneural gene activity to formation of individual neural precursor cells and their subsequent differentiation. However, less is known about the mechanisms controlling the patterned expression of proneural genes, which underlie the formation of proneural clusters. Axis-patterning genes can control the expression of proneural genes in the embryo and possibly in imaginal discs. But it is likely that other kinds of regional or local cell interaction mechanisms are involved or might be interposed downstream of axis-patterning genes to regulate the induction of proneural gene expression.

In the present study, we show that fasciclin II (Fas II) regulates proneural gene expression. Fas II is a member of the immunoglobulin superfamily, is related to vertebrate neural cell-adhesion molecule (NCAM) (8) in both structure and sequence, and can mediate homophilic cell aggregation *in vitro* (9–11). Analysis in *Drosophila* of both loss-of-function and gain-of-function conditions (11–13) shows that Fas II functions as a neuronal recognition molecule to control selective patterns of axon fasciculation.

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Here we use genetic analysis to define another function for Fas II in the regulation of proneural gene expression. In *fasII* loss-of-function mutants, the expression of two proneural genes—achaete and atonal—is absent in certain regions in the eye-antennal imaginal disc, and, as a result, the corresponding sensory precursors fail to develop and the sensory structures they normally generate are missing on the head. In *fasII* gain-of-function conditions, extra sensory structures are seen in this same region of the head. These complementary loss-of-function and gain-of-function phenotypes show that Fas II is required for induction of proneural gene expression in certain locations of the eye-antennal imaginal disc.

MATERIALS AND METHODS

Genetics. The generation and characterization of *fasII* mutant alleles have been described (11, 13). In brief, protein null mutations in the *fasII* gene (e.g., $fasII^{eB112}$) are lethal, while one viable hypomorphic (partial loss-of-function) allele ($fasII^{e76}$) produces ≈10% of normal Fas II protein (11). Generation of the different GAL4 enhancer trap lines and the upstream activating sequence (UAS)-*fasII* reporter construct used here have been described (13). Other mutations and chromosomes have been described (14). Gynander individuals were obtained from the cross $y\ fasII^{eB112}/FM7c \times R(1)2/y^{+Y}$ raised at 25°C. Gynander individuals showing mosaic territories in the head and/or thorax were fixed in 70% ethanol/glycerol (3:1) for several days. Soft tissue was removed by boiling the head and thorax in 10% KOH for 10 min, and the resulting cuticle was washed and dehydrated in an ethanol/xylene series before mounting in Permount.

For antibody staining, *fasII* hypomorphic females were generated in the following crosses raised at 17°C: $fasII^{eB112}/FM7c \times fasII^{e76}$ for anti-Atonal, $fasII^{eB112}/A31 \times fasII^{e76}$ for anti-Achaete, and $fasII^{eB112}/FM4;\ A101/TM6B \times fasII^{e76}$ for anti-β-galactosidase. We avoided a first chromosome balancer that carries $In(1)sc^{8}$ to prevent perturbation of Achaete expression in control individuals carrying this balancer. The *A31* enhancer trap line contains a *P*-element insertion in the *fasII* gene, which does not cause a mutant phenotype (11); the *A101* enhancer trap line contains a *P*-element insertion in the neuralized gene (15, 16). *A101/+* females were distinguished by the absence of the Tb marker of TM6B.

Bristle and ocellar (OC) phenotypes in adult heads (15–20 per genotype) were scored under the dissecting microscope.

Abbreviations: SOP, sensory organ precursor; NCAM, neural cell-adhesion molecule; PVT, postvertical; OC, ocellar; VT, vertical; OR, orbital; UAS, upstream activating sequence.

*Present address: Centro de Biología Molecular Severo Ochoa, Consejo Superior de Investigaciones Cientificas, Universidad Autónoma de Madrid, Madrid, Spain.
†Present address: Department of Biological Sciences, Wayne State University, Detroit, MI.
‡Present address: Glaxo Institute for Molecular Biology, Geneva, Switzerland.
§To whom reprint requests should be addressed.

The penetrance of missing bristles is expressed as a percentage of hemiheads since bristle precursor cells arise independently from the left and right eye-antennal imaginal discs. However, since the median ocellus arises from both the left and right imaginal discs, we present the missing ocelli data as a percentage of heads.

Immunohistochemistry. Third-instar crawling female larvae were selected from the appropriate crosses. Eye-antennal imaginal discs were dissected, fixed in 4% paraformaldehide (or PEM-formaldehyde when using anti-Atonal antibody) for 15 min, and washed in several changes of PBT (phosphate-buffered saline with 0.3% Triton X-100; ref. 17). Nonspecific binding was blocked for 30 min in PBT plus 5% normal goat serum (PBTN) and the following antibodies were used at the indicated dilution: anti-Achaete monoclonal antibody (mAb) 984A11, 1:20; anti-Atonal rabbit antiserum, 1:5000; and anti-β-galactosidase mAb, 1:50 (Promega), to reveal *A101* enhancer trap expression. After several washes with PBT, discs stained with mAb 984A11 or anti-β-galactosidase were detected with a horseradish peroxidase-conjugated goat anti-mouse antibody (1:200; The Jackson Laboratory). Anti-Atonal staining was detected with a biotin-conjugated donkey anti-rabbit secondary antibody (1:100; Amersham) and the Vectastain ABC-Elite kit (Vector Laboratories). After several washes, discs were incubated for 10 min in 200 ml of PBT/diaminobenzidine (DAB) plus 3 ml of 8% $NiCl_2$ and 3 ml of 10% hydrogen peroxide to produce a black reaction product. Double staining with anti-Fas II mAb 1D4 (G. Helt, personal communication) was performed in an identical fashion except that $NiCl_2$ was not added to the DAB reaction mixture in order to maintain the red-brown reaction product. Discs were mounted in PBS/glycerol and photographed.

Electron Microscopy. Selected adults were fixed overnight at 4°C in 1% gluteraldehyde/1% paraformaldehyde/1 M cacodylate, pH 7.2, and dehydrated in an ethanol series (25%, 50%, 75%, and 100%; 10 min each). While in fresh 100% ethanol, samples were transferred to microporous specimen capsules for critical point drying. Dried samples were mounted onto aluminum supports (10 × 15 cm) with colloidal silver paste and sputter coated with 60:40 gold/palladium alloy to a thickness of 25–28 nm (1.5 kV; 1200–1400 mA). Mounted specimens were viewed and images were recorded by scanning electron microscopy (ISI model DS-130; working distance, 40 mm; accelerating voltage, 10 kV).

***fasII* Rescue Construct.** A *fasII* minigene construct was created by combining appropriate portions of the *fasII* cDNA and genomic clones. An 8.5-kb *Sal* I/*Pst* I fragment of the 5′ flanking region of the *fasII* gene was obtained by partial digest of genomic DNA. This fragment was ligated to the 2.6-kb *Pst* I/*Hin*dIII fragment of the *fasII* cDNA encoding the transmembrane form (*fasIITM*; see ref. 11), and then both were ligated into a *P*-element transformation vector. This *fasIITM* minigene contains ≈5 kb of 5′ flanking DNA, the 2.5-kb first intron, and 2.6 kb of cDNA. Transformants carrying this minigene rescue the lethality of $fasII^{eB112}$ and exhibit 5–10% of wild-type Fas II expression during embryonic development.

RESULTS

Analysis of *fasII* Loss-of-Function Mutants. In addition to their pair of large compound eyes, adult flies have three simple eyes, called ocelli, located near the midline on the dorsal surface of their heads (Fig. 1*A*). Ocelli are generated from the eye-antennal imaginal discs, which give rise to the dorsal and lateral adult head epidermis and sensory structures. Adult flies also have bristles, each of which contains a mechanosensory neuron, on their heads. The bristles of the dorsal head, including postvertical (PVT), OC, vertical (VT), and orbital (OR) bristles, also arise from the eye imaginal disc from a region close to where the ocelli arise. In the late third-instar eye-antennal disc, Fas II is expressed throughout the disc with peaks at the morphogenetic furrow, on differentiating photoreceptors, and in the prospective area where the ocelli and associated bristles develop (data not shown).

The heads of adult flies homozygous for the hypomorphic *fasII* allele ($fasII^{e76}/fasII^{e76}$) often appear wild type, although one or both PVT bristles can be missing (Table 1; Fig. 1*B*). The stronger allelic combination $fasII^{e76}/fasII^{eB112}$ shows stronger phenotypes in which the PVT, OC, and VT bristles are usually missing; the ocelli are sometimes missing, and the compound eyes have a rough appearance. Raising these mutants at 17°C rather than at 25°C strongly enhances the penetrance and expressivity of these phenotypes: $fasII^{e76}/fasII^{e76}$ individuals now lack both PVT bristles, while $fasII^{e76}/fasII^{eB112}$ individuals show a much more penetrant phenotype of missing ocelli (Fig. 1*C*). At both temperatures, the OR bristles develop normally (Table 1). A series of these same phenotypes (missing bristles, missing ocelli, and rough eyes) ranging from wild type to severe are observed in *fasII* null mutants ($fasII^{eB112}$) that have been rescued from lethality either by a *fasII* transgene (*fasIITm31.2*) or by combining certain GAL4 enhancer trap lines (B119, E35, G13) with a UAS-*fasII* reporter (13) (Fig. 1*D*).

To study the phenotype of the null condition of *fasII* in the adult, we generated mosaic individuals by the loss of a ring X

Table 1. Percentages of adult heads with missing ocelli or bristles in *fasII* mutant conditions

Genotype (*t*)	Ocelli	VT bristles	OC bristles	PVT bristles	OR bristles
WT (25°C or 17°C)	0	0	0	0	0
$fasII^{e76}/fasII^{e76}$	0	2	0	27	0
$fasII^{e76}/fasII^{e76}$ (17°C)	0	69	20	97	0
$fasII^{e76}/fasII^{eB112}$	5	90	50	97	0
$fasII^{e76}/fasII^{eB112}$ (17°C)	90	100	87	92	2
$fasII^{eB112}$ (mosaic)	100	100	100	100	90
$fasII^{e76}/fasII^{e76}$; $alb^4/+$	0	15	0	77	7
$fasII^{e76}/fasII^{eB112}$; $alb^4/+$	35	92	77	80	5
$fasII^{e76}/fasII^{e76}$; $abl^2/+$	0	10	2	85	0
$fasII^{e76}/fasII^{eB112}$; $abl^2/+$	27	87	75	94	20
$fasII^{e76}/fasII^{e76}$; $Df(3L)st^{E36}/+$	0	10	0	50	7
$fasII^{e76}/fasII^{eB112}$; $Df(3L)st^{E36}/+$	25	100	55	100	5

Results are based on a minimum of 15 individual animals (i.e., 30 hemiheads) per genotype. VT, OC, PVT, and OR refer to bristles on the head. Percentage of heads (ocelli) or hemiheads (bristles) is given with phenotype (1, 2, or 3 ocelli missing or any bristle of a given type missing). All crosses were raised at 25°C unless indicated otherwise. WT (wild type) is CantonS. $fasII^{e76}$ and $fasII^{eB112}$ are mutant hypomorphic and null alleles, respectively, of *fasII*. The $fasII^{eB112}$ mosaic is from genetic gynanders (see text for details). abl^2 and abl^4 are mutant alleles of *abl*; $Df(3L)st^{E36}$ is a deficiency that removes *abl*.

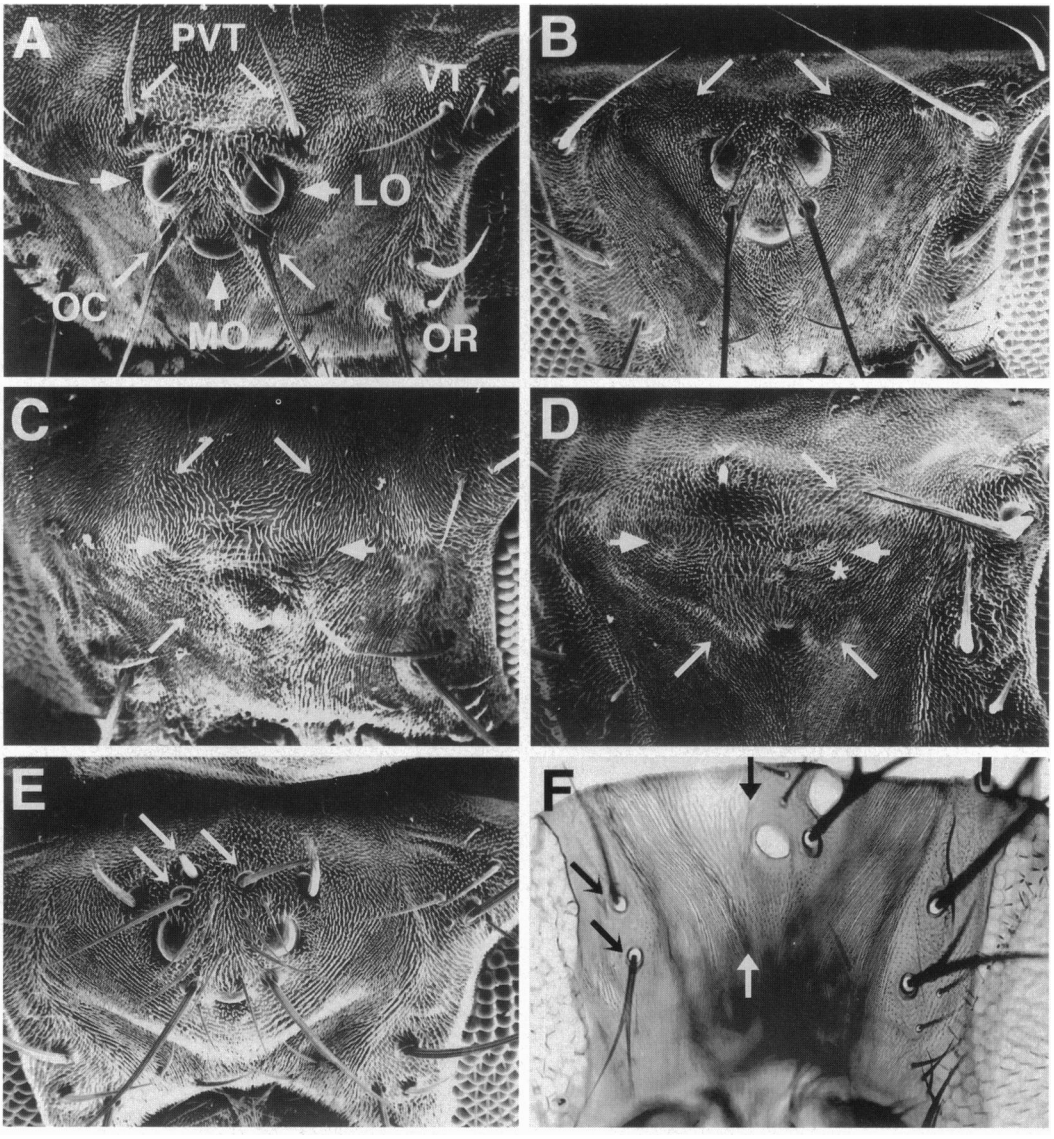

FIG. 1. Fas II controls sensory organ differentiation on the *Drosophila* head. Photomicrographs (*A–E*, scanning electron micrographs; *F*, bright-field micrograph) of the dorsal head region of adult flies from wild-type individuals (*A*) and partial loss-of-function (i.e., hypomorphic) (*B–D*), gain-of-function (*E*), and protein null loss-of-function mosaic (*F*) *fasII* mutants. (*A*) Wild type. Stereotypic array of sensory structures on the dorsal head are marked, including the bristles (long arrows) and the three simple eyes called ocelli (two lateral and one median; short arrows). A pair of PVT bristles lie just posterior to the lateral ocelli (LO) and a pair of OC bristles lie on each side of the median ocellus (MO). OR and VT bristles lie adjacent to the large compound eyes. (*B*) In a $fasII^{e76}$ (partial loss-of-function) mutant, the PVT bristles are missing (arrows indicate normal location of missing bristles). (*C*) In the more severe mutant allelic combination $fasII^{e76}/fasII^{eB112}$ (partial loss-of-function over null), all of the bristles are missing (long arrows) and the two lateral ocelli are also absent (short arrows). Only the MO is present. (*D*) Different GAL4 insertions are able to rescue the lethality of the null $fasII^{eB112}$ allele when driving the expression of a UAS-*fasIITm* (transmembrane form of Fas II) minigene (see text for details). In this example of a $fasII^{eB112}$; *E35-GAL4*; UAS-*fasIITm* mutant individual, all of the bristles (except for one PVT, chevron) are missing (long arrows) as well as most of the two lateral ocelli (short arrows). The MO and a small portion of the right lateral ocellus (asterisk) are present. In other rescued animals, the phenotypes are less severe, and in some cases the rescue results in a wild-type head. (*E*) On an otherwise wild-type background, overexpression of Fas II caused by expression of the UAS-*fasII* construct under control of the *D45-GAL4* insertion results in the appearance of extra bristles near the position of the PVT (three arrows mark the three ectopic bristles). Note that many trichomes (single epidermal cells) separate the new bristles, indicating that they have differentiated from independent SOP cells. (*F*) Cuticle of the dorsal head (frontal view) of a $fasII^{eB112}$, *y*/*R*(*1*)*2* gynander (mosaic) individual examined by light microscopy. Border between the *fasII* null mutant male territory (y^- at left) and wild-type *fasII* female territory (y^+ at right) is demarcated by black and white arrows. In the male $fasII^-$ territory, all bristles and ocelli on the dorsal surface are absent, but neighboring OR bristles (two black arrows) near the compound eye are present. Note that the epidermis in the male $fasII^-$ epidermal territory appears normal, as does the boundary between the mutant and wild-type territories.

chromosome [*R*(*1*)*2*]. Gynander individuals ($n = 31$) showed that mutant male territories (hemizygous for $fasII^{eB112}$) fail to differentiate the PVT, OC, and VT bristles, although they may still have some OR bristles (Table 1). In addition, the ocelli are missing, and the compound eyes have a rough phenotype (Fig. 1*F*). The epidermis of the regions where the ocelli and bristles should have developed is present and has a normal appearance as judged by the structure and pattern of trichomes. This is true even at the borders between Fas II^+ and Fas II^- territories. Thus, we observe no overt cell adhesion phenotype either within the mutant epidermal territory or at the borders between mutant and wild-type territories. In these gynanders, in addition to the missing sensory structures in the head, some macrochaetes (bristles) are missing in the thorax. Moreover, in the thorax, microchaetes have a lower density.

The absence of sensory organs in *fasII* loss-of-function mutants could result from the absence of the SOP cells; alternatively, the SOP might appear but fail to generate the appropriate progeny. To distinguish between these alternatives, we used the *lacZ* enhancer trap line *A101* (in the neuralized gene), which labels all SOP cells (15, 16). In wild-type flies carrying *A101*, β-galactosidase is expressed in the SOPs for all of the bristles and the differentiating ocelli and retina soon before and after puparium formation (Fig. 2*A*). However, in $fasII^{e76}/fasII^{eB112}$ flies carrying *A101*, we do not detect β-galactosidase expression in SOP cells in the prospective dorsal head epithelium (Fig. 2*B*), consistent with a failure of these SOP cells to be specified in *fasII* mutants. β-Galactosidase expression is also absent from the prospective ocelli, and there is a general reduction in its levels in the retina compared to wild type.

To investigate why SOPs and OC precursor cells are missing in *fasII* mutants, we examined the expression of both the Achaete and Atonal proteins. In *Drosophila*, achaete is one of the proneural genes for bristles, whereas atonal is the proneural gene for photoreceptors (18). In late third-instar larvae, the Achaete protein is expressed in the eye-antennal imaginal disc in three proneural clusters near the prospective OC region corresponding to the OR, OC, and PVT bristles (Fig. 2*C*; according to the fate map in ref. 19). The Atonal protein is expressed in two clusters of cells in the epithelial region from which the ocelli develop (one cluster for the lateral ocellus and the other for half of the median ocellus; Fig. 2*E*). $fasII^{e76}/fasII^{eB112}$ mutant eye-antennal imaginal discs at 17°C lack both Achaete-expressing OC and PVT proneural clusters (Fig. 2*D*) and Atonal-expressing OC proneural clusters (Fig. 2*F*); the

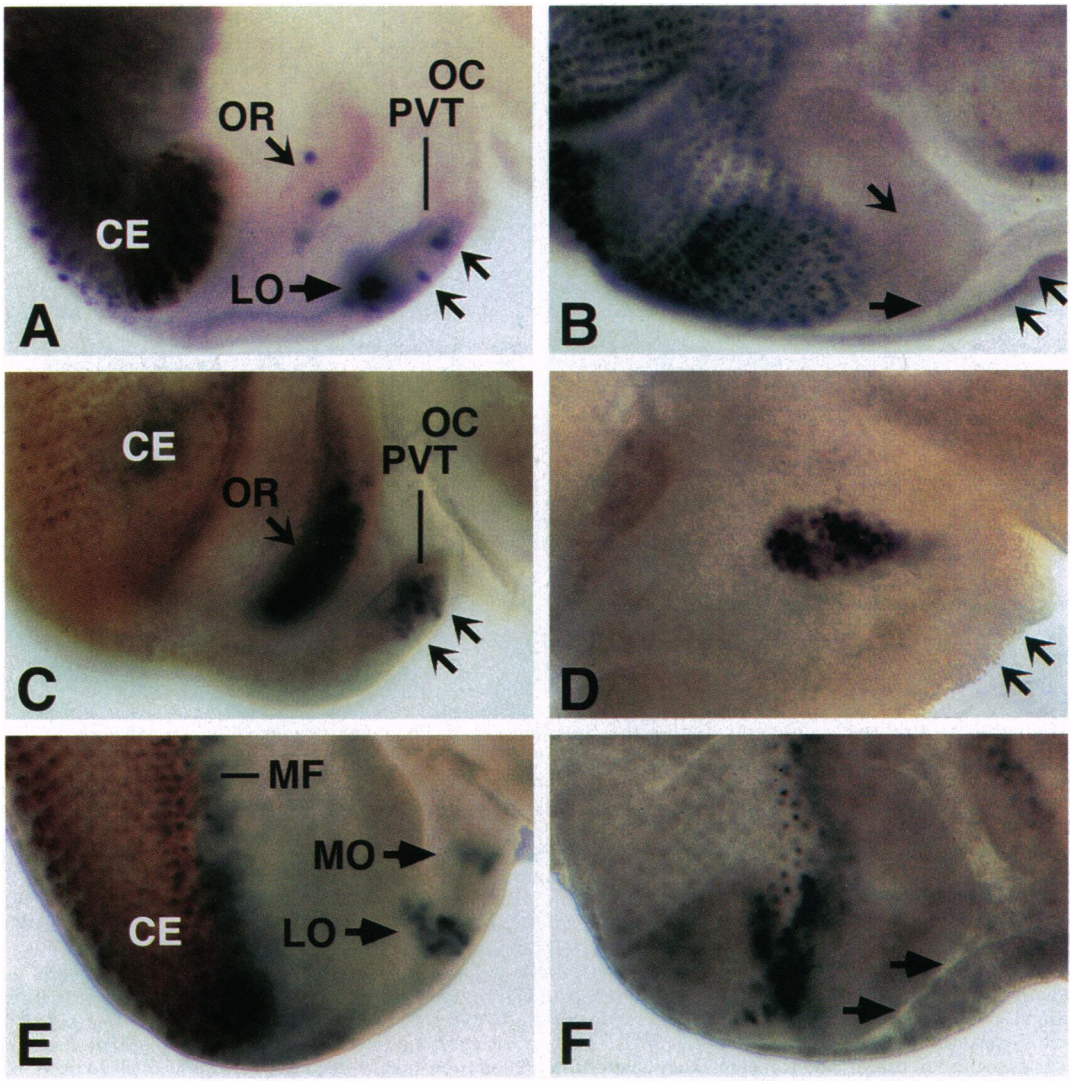

FIG. 2. Fas II regulates proneural gene expression in the region of the eye-antennal imaginal disc that gives rise to the *Drosophila* head. Third-instar larval eye-antennal imaginal discs from wild-type (*A*, *C*, and *E*) or $fasII^{e76}/fasII^{eB112}$ (*B*, *D*, and *F*) mutant individuals examined for expression of the A101 (neuralized) enhancer trap (*A* and *B*), Achaete protein (*C* and *D*), and Atonal protein (*E* and *F*). (*A*) Wild-type pattern of SOP cells is shown in the eye-antennal imaginal disc of a $fasII^{e76}/+$; *A101*/+ individual stained with anti-β-galactosidase (purple-black product). Different SOP cells for the lateral ocelli (LO; large arrow) and neighboring OC, PVT, and OR bristles (small arrows) can be seen, as well as staining in the developing compound eye (CE). (*B*) In a $fasII^{e76}/fasII^{eB112}$; *A101*/+ mutant, β-galactosidase staining is reduced throughout the disc (note reduced staining in the compound eye) but is absent in the presumptive region where the ocelli and neighboring bristles should form (arrows). Note that the OR bristle precursors are also not detected in this particular *fasII* mutant disc, although they usually are present in this mutant condition. (*C*) Eye-antennal disc of a $fasII^{e76}/+$ stained with an antibody against Achaete protein. Wild-type pattern of Achaete expression in the OR, OC, and PVT proneural clusters (arrows) is seen. (*D*) In a $fasII^{e76}/fasII^{eB112}$ mutant, Achaete expression is not detected in the OC and PVT proneural clusters (arrows), although the OR proneural cluster is clearly labeled. (*E*) Expression of the Atonal protein in a $fasII^{e76}/+$ eye-antennal imaginal disc is wild type. Atonal expression is detected in a large cluster representing the LO (arrow) and a smaller cluster corresponding to one-half of the median ocellus (MO) (arrow). Expression of Atonal is also seen in the morphogenetic furrow (MF) of the developing CE. (*F*) In a sibling $fasII^{e76}/fasII^{eB112}$ mutant imaginal disc, no expression of Atonal is detected in the OC regions (arrows).

expression of Achaete in the OR proneural cluster is not affected (Fig. 2*D*). The larval imaginal disc epithelium looks normal. Moreover, Engrailed expression, which is normally present in the OC region, is present in *fasII* mutants, suggesting an overall correct specification of this region (data not shown).

Analysis of *fasII* Gain-of-Function Conditions. Fas II was overexpressed in the eye-antennal disc using a variety of GAL4 enhancer trap lines and a UAS-*fasII* reporter transgene (A111, C109, and D45; ref. 13). In these *fasII* gain-of-function conditions, we observed extra bristles in the vicinity of the PVT bristles (Fig. 1*E*). Several epidermal cells are present between these bristles, suggesting that the extra bristles did not arise from the same sensory organ precursor cell. In these experiments, we also observed extra bristles in other regions of the adult fly, including, for example, extra Scutellar bristles in the thorax. Thus, this gain-of-function phenotype suggests that Fas II is sufficient to increase proneural gene activity.

Genetic Interactions Between *fasII* and *abl*. The results described above are consistent with Fas II regulating the regional induction of proneural gene expression by a local cell signaling function. CAM-mediated signal transduction has been shown in the ability of NCAM to promote neurite outgrowth (20). In this model, in the absence of Fas II, epithelial cells at specific locations are unable to respond to positional information required to activate proneural gene expression. It is likely that some sort of signal transduction pathway is interposed between the membrane-associated Fas II and the nuclear proteins encoded by the proneural genes. To identify candidate proteins participating in a Fas II-mediated signal transduction pathway, we looked for loss-of-function mutations that can act as dominant enhancers of the partially penetrant phenotypes produced by the hypomorphic $fasII^{e76}$ mutant allele. All tested mutations in the Abelson tyrosine kinase gene (e.g., see refs. 21 and 22) consistently act as dominant enhancers of *fasII* mutant phenotypes (Table 1), while single copies of each of these *abl* mutant alleles on their own (i.e., *abl*/+) show no phenotype.

DISCUSSION

Fas II is expressed in a dynamic fashion throughout the third-instar eye-antennal disc, where it preferentially controls expression of the proneural genes achaete and atonal in certain regions. In *fasII* loss-of-function mutations, the expression of both proneural genes is absent in certain locations, and, as a result, the corresponding sensory precursors fail to develop, and the sensory structures they generate are absent from the adult head. In *fasII* gain-of-function conditions, extra sensory structures are seen in this same region of the adult head. Thus, Fas II appears to control induction of proneural gene expression in the eye-antennal imaginal disc. Moreover, our results suggest that Fas II controls proneural gene expression by functioning in a signal transduction pathway that includes the Abelson tyrosine kinase.

Different proneural clusters are more sensitive than others to the loss of Fas II expression. Although Fas II controls the induction of proneural gene expression in certain regions, it may be that other cell surface molecules, either alone or in combination with one another, might regulate induction of proneural gene expression in other regions.

Previous studies in *Xenopus* have shown that NCAM is an early marker of neural induction (e.g., see ref. 23) and that the putative proneural *XASH* gene can promote NCAM expression (24). In *Drosophila* embryos, Fas II expression is also downstream from proneural genes, being expressed by subsets of central nervous system neurons (11). However, here we have shown that Fas II is also required prior to the appearance of sensory neurons in the epithelium for induction of proneural gene expression. It is possible that early expression of NCAM or other CAMs in vertebrates might function in a similar fashion.

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