Charlatan, a Zn-finger transcription factor, establishes a novel level of regulation of the proneural *achaete/scute* genes of *Drosophila*

Luis M. Escudero[1,*], Eva Caminero[1], Karen L. Schulze[2], Hugo J. Bellen[2] and Juan Modolell[1,†]

[1]Centro de Biología Molecular Severo Ochoa, CSIC and UAM, Cantoblanco, 28049 Madrid, Spain
[2]HHMI, Department of Molecular and Human Genetics, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA
*Present address: MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK
†Author for correspondence (e-mail: jmodol@cbm.uam.es)

Accepted 4 January 2005

Development 132, 1211-1222
Published by The Company of Biologists 2005
doi:10.1242/dev.01691

Summary

The proneural genes *achaete* (*ac*) and *scute* (*sc*) are necessary for the formation of the external sensory organs (SOs) of *Drosophila*. *ac* and *sc* are expressed in proneural clusters and impart their cells with neural potential. For this potential to be realized, and the SO precursor cell (SOP) to arise within a cluster, sufficient proneural protein must accumulate in the cluster. Here we describe a novel gene, *charlatan* (*chn*), which encodes a zinc finger transcription factor that facilitates this accumulation by forming a stimulatory loop with *ac/sc*. We find that loss of function of *chn* decreases the accumulation of Sc in proneural clusters and partially removes notum macrochaetae, while overexpression of *chn* enhances *ac/sc* expression and the formation of extra SOs. Moreover, *chn* is activated by *ac/sc* in proneural clusters. Chn apparently stimulates *ac/sc* by physically interacting with the proneural cluster-specific enhancers and increasing enhancer efficiency, thus acting as a stimulator of *ac/sc* expression in proneural clusters. *chn* is also required for the proper development of the embryonic peripheral nervous system, as its absence leads to loss of neurons and causes aberrant development of chordotonal organs.

Key words: *charlatan*, Zn-finger transcription factor, *achaete/scute*, Proneural genes, ASC (AS-C), Bristle development, *Drosophila*

Introduction

A classical example of two-dimensional pattern is that formed by the bristles and other types of sensory organs (SOs) in the epidermis of the adult *Drosophila* fly (Lindsley and Zimm, 1992). On the head and the dorsal mesothorax (notum), conspicuous large bristles (macrochaetae) arise in stereotyped positions, while smaller bristles (microchaetae) appear in density patterns. During the third instar larval and early pupal stages, the location of each macrochaeta is specified by the emergence of a precursor cell (SO precursor cell, SOP) at a stereotyped position of the imaginal disks, the larval epithelia that give rise to a large part of the adult epidermis (Cubas et al., 1991; Huang et al., 1991). This accurate positioning of SOPs in the imaginal disks is thought to be the culmination of a multistep process in which positional information is gradually refined (reviewed by Gómez-Skarmeta et al., 2003; Modolell and Campuzano, 1998).

A key step of this process is the expression of the proneural genes *achaete* (*ac*) and *scute* (*sc*) in groups of cells, the proneural clusters, that prefigure the sites of the future macrochaetae (Cubas et al., 1991; Romani et al., 1989; Skeath and Carroll, 1991). These genes, members of the *achaete-scute* complex (ASC) (reviewed by Campuzano and Modolell, 1992; Ghysen and Dambly-Chaudière, 1988; Ghysen and Dambly-Chaudière, 1989), encode transcriptional factors of the basic helix-loop-helix (bHLH) family. These factors confer to cells the potential to become SOPs, presumably by implementing neural differentiation programs. From each proneural cluster, a fixed number of SOPs are born, usually one or two. The proneural clusters of the wing imaginal disks (the precursors of each heminotum, wing and mesothoracic pleura) not only appear in constant positions, but each of them has a characteristic size, shape and time of appearance and disappearance (Cubas et al., 1991; Skeath and Carroll, 1991). Moreover, a typical cluster that gives rise to one bristle may consist of 20 to 30 cells, but the SOP is selected from a smaller subgroup of cells that accumulate higher levels of Ac-Sc proteins than their neighbors, which constitute the proneural field (Cubas et al., 1991; Cubas and Modolell, 1992; Skeath and Carroll, 1991). This subgroup and the SOP, which accumulates the highest levels of Ac-Sc, always occupy the same position within the cluster. Hence, the expression of *ac/sc* in proneural clusters is exquisitely regulated.

The regulation of *ac/sc* is effected by means of two classes of *cis*-regulatory sequences, namely, cluster-specific and SOP-specific enhancers. The first type normally directs expression of both *ac* and *sc* in one specific proneural cluster and defines many of its characteristics, such as position, size and shape. These cluster-specific enhancers appear to be controlled by local combinations of transcription factors that together form a prepattern (reviewed by Ghysen and Dambly-Chaudière, 1988; Gómez-Skarmeta et al., 2003). Expression occurs only at sites with the appropriate combinations of factors. Although in a few cases some of the prepattern

factors have been identified, most of them remain unknown. Moreover, we still lack a clear understanding of how the inputs of the prepatterning factors are integrated into the patterns of proneural gene expression characteristic of each cluster.

The second type of enhancer mediates the strong expression of proneural genes in SOPs (Culí and Modolell, 1998) by allowing self-stimulatory loops of expression of *ac*, *sc* and *asense* (*ase*), another bHLH member of the ASC (Brand et al., 1993; Domínguez and Campuzano, 1993; Jarman et al., 1993a). The activation of these loops in one of the cells of the proneural field is an early and essential step of SOP commitment. This loop is also dependent on the presence of the Senseless (Sens) protein (Jafar-Nejad et al., 2003). The SOP-specific enhancers are also the targets of the inhibitory interactions that occur within the cells of the proneural cluster mediated by the Notch signaling pathway via E(spl) proteins (Culí and Modolell, 1998; Giagtzoglou et al., 2003). By antagonizing these enhancers, N signaling, activated by Ac/Sc in the cells of the cluster, maintains them in a non-SOP state (mutual inhibition) (reviewed by Artavanis-Tsakonas et al., 1995). However, in a little-understood process, one cell of the proneural field escapes this inhibition, starts the proneural self-stimulatory loop and becomes an SOP. The developing SOP then signals via Notch in order to impede the remaining cells of the field from becoming SOPs (lateral inhibition) (Heitzler and Simpson, 1991; Simpson, 1990; Simpson, 1997). These SOP-specific enhancers are also the targets of positive interactions between the cells of proneural clusters mediated by the EGFR, which is necessary for the emergence of the SOPs of the notum macrochaetae (lateral cooperation) (Culí et al., 2001). To prevent the determination of excess SOPs from a proneural cluster, the levels of EGFR signaling must be regulated. This event seems to be accomplished in part by a negative effect on EGFR signaling of the N-mediated interactions that occur among cells of the proneural cluster.

The *ac*, *sc* and *ase* genes are also necessary for the formation of the external SOs of embryos and larvae (Dambly-Chaudière and Ghysen, 1987). The process is similar to that in the imaginal disks (Ruiz-Gómez and Ghysen, 1993). Other proneural genes are responsible for the development of the internal chordotonal organs (*atonal*) (Jarman et al., 1993b) and other neurons of the larval peripheral nervous system (PNS) (*amos*) (Huang et al., 2000; Villa-Cuesta et al., 2003).

Here we report the identification of a novel gene, *charlatan* (*chn*), which is involved in the development of the adult pattern of macrochaetae. *chn* defines a new level of control of *ac/sc* that is intermediate between the prepattern genes and the *ac/sc* self-stimulation mediated by the SOP-specific enhancers. Thus, *chn*, which encodes a zinc finger transcription factor, is activated by *ac/sc* in the proneural clusters of the wing disk. In turn, *chn* stimulates the expression of *ac/sc* in these clusters. This enhanced expression facilitates the formation of SOPs. Our data indicate that the Chn protein reinforces the expression of *ac/sc* by acting, probably directly, on the proneural cluster-specific enhancers of the ASC. *chn* is also required for correct development of the embryonic/larval PNS, as its absence removes neurons and causes malformations of chordotonal organs.

Materials and methods

Drosophila stocks

The *Drosophila* stocks used were: $ln(1)sc^{10.1}$ and $Dp(1,2)sc^{19}$ (Lindsley and Zimm, 1992), Ax^{M1} (Díaz-Benjumea and García-Bellido, 1990), pnr^{VX6} and pnr^{V1} (Heitzler et al., 1996), *y w FLP122*; *act-FRT* y^+ *FRT-Gal4 UAS-GFP/SM6a-TM6b Tb* (Ito et al., 1997), *y w FLP122*; *P[ubiGFP] FRT42D/CyO*, *y w FLP122* f^{36A}; *ck Pf[+]* FRT42D/CyO, *y w FLP122;* y^+ FRT42D/CyO (FlyBase). *C765-Gal4* (Gómez-Skarmeta et al., 1996), *MS1096-Gal4* (Lunde et al., 1998), *eyg-Gal4* (Aldaz et al., 2003), *MS248-Gal4* (Sánchez et al., 1997), *en-Gal4 y ap-Gal4* (Calleja et al., 1996), *pnr-Gal4* (Heitzler et al., 1996), *dpp-Gal4* (Staehling-Hampton et al., 1994), *sca-Gal4* (Hinz et al., 1994), *69-B-Gal4* (Brand and Perrimon, 1993), *NP1212* (GETDB, Gal4 Enhancer Trap Insertion Database), *UAS-sc* and *UAS-ato* (Parras et al., 1996), *AS1.4DC-lacZ* (García-García et al., 1999), *ANP-A-lacZ* (M. J. García-García, PhD thesis, Universidad Autónoma de Madrid, 1999), *3.7-lacZ* (Gómez-Skarmeta et al., 1995), *2.3-lacZ* (Culí, 1998), *SRV-lacZ* (Culí and Modolell, 1998), $chn^{42/18}$ (Kania et al., 1995), and chn^{ECJ1}, *UAS-chn*, *UAS-chni*, *UAS-bda*, this study.

Molecular biology

UAS-chn was prepared by subcloning the cDNA of *CG11798* from clone SD05496 (BDGP) into the pUASt vector (Brand and Perrimon, 1993). *UAS-bda* was prepared by subcloning a PCR product containing the entire ORF of *bda* that was obtained using genomic Oregon R DNA as template. The PCR product was sequenced to confirm the fidelity of amplification. To prepare *UAS-chni*, a 400 bp fragment of *chn* cDNA was amplified by PCR using 5′-GGGATCCCAAGCGGCTGCAGCTGC-3′ upper primer and 5′-TGGAAGCTTCAACTCGTGCACGCC-3′ lower primer. The PCR product was cloned as a *Bam*HI-*Kpn*I fragment in the pHIBS vector (Nagel et al., 2002), to make the pHIBS-chn construct. A *Bam*HI-*Sac*I fragment from pHIBS-chn was subcloned in the pBluescript vector to generate pBS-chn. A *Kpn*I-*Sal*I fragment from pHIBS-chn and a *Kpn*I-*Sal*I fragment from pBS-chn were cloned in opposite directions in the pUASt vector, thus forming the final RNAi construct. All the UAS constructs were injected into *y w* embryos to obtain transgenic *Drosophila* lines by standard procedures (Rubin and Spradling, 1982).

All overexpression experiments were carried out at 25°C, except for the flies shown in Fig. 1D,E,F, which were raised at 18°C, and those shown in Fig. 1G,H,I, which were cultured at 18°C, shifted to 25°C at 72 hours after egg laying, and returned to 18°C at puparium formation.

P-element mutagenesis

Males carrying the P element *l(2)42/18* ($chn^{42/18}$*/CyO*) were crossed to females carrying the Δ2-3 transposase (Cooley et al., 1988). Excisions of the *l(2)42/18* transposon were selected by the loss of the w^+ eye marker in the F1 progeny. Individuals were crossed to *w; If/CyO* flies and balanced. One hundred lines that failed to complement the original mutation were selected. Lines with the strongest defects in the embryo PNS, as detected by staining with mAb 22c10, were selected and the presence of *chn* mRNA was examined. chn^{ECJ1} mutant embryos lacked this mRNA. To identify its molecular lesion, genomic DNA, prepared from homozygous chn^{ECJ1} mutant larvae, was used as a template in PCR reactions to amplify the genomic region near the insertion point of the P element *l(2)42/18*.

Mosaic analyses

To generate clones of cells mutant for *chn*, either *y w hs-FLP122,* f^{36a}*; ck Pf[+] FRT42D/CyO* or *y w hs-FLP122; P[ubi-GFP], FRT42D/CyO* females (stocks described in FlyBase) were crossed with *w;* chn^{ECJ1}*, FRT42D/CyO* males. Recombination was induced by heat treatment at 37°C for 30 minutes (Xu and Rubin, 1993). To generate clones of cells overexpressing *UAS-chni*, males carrying this transgene were crossed with *y w FLP122*; *act-FRT* y^+ *FRT-Gal4 UAS-*

GFP / SM6a-TM6b Tb females and recombination was induced by incubation at 37°C for 10 minutes.

Histochemistry

Antibody staining was performed as described (Cubas et al., 1991). Primary antibodies were: anti-Sens (Nolo et al., 2000), anti-Sc (Skeath and Carroll, 1991), anti-Ato (Jarman et al., 1995), mAb 22c10 (Zipursky et al., 1984) and anti-β-galactosidase (Cappel). A guinea pig anti-Chn antibody was prepared against a His-tagged Chn fragment corresponding to amino acids 213-417 cloned into the pET28a vector. However, it only allowed clear visualization of the Chn protein under overexpression conditions (Fig. 5N,O). Secondary antibodies were from Jackson and Amersham. In-situ hybridizations to detect *chn* mRNA were performed as described (González-Crespo and Levine, 1993) using an antisense DIG-labeled RNA probe.

Electrophoretic mobility-shift assays

A Chn protein fragment (Chn5ZF) that contained the five zinc finger motifs (Asn 297-His 604) was produced in *Escherichia coli*. This protein was purified with the NTA-system (Qiagen). Six partially overlapping probes of approximately 300 nucleotides each were synthesized, which together covered the entire sequence of the 1.4 kb DNA fragment containing the DC enhancer (García-García et al., 1999). The primers used were: DC1 probe, 5′-GAGGAACAAAGAGCAGG-3′ and 5′-TTATAGTCCCCACTG-3′; DC2 probe, 5′-AAACCGCAGCAGTTC-3′ and 5′-GGAATGAGATTGCGG-3′; DC3 probe, 5′-AAAAAACCGCCGCTG-3′ and 5′-AACTTTCCCTGCACC-3′; DC4 probe, 5′-ACATATTTCCGGCGC-3′ and 5′-TGTAC-GACTACAGGC-3′; DC5 probe, 5′-TG-GTAGGGTAGGATC-3′ and 5′-GACT-TATCGTCACGG-3′; DC6 probe, 5′-TTCATTCATCCGGCG-3′ and 5′-GTC-GACTTTCGGTTTTTCG-3′. Probes were labeled with [α-^{32}P]dCTP (3000Ci/mmol) by PCR. The composition of the binding buffer was 50 mmol/l Tris-HCl (pH 7.5), 50 mmol/l KCl, 10% glycerol, 1 mmol/l DTT, 100 µmol/l $ZnSO_4$, 7.5 µg/ml poly(d(C-I)). The amount of Chn5ZF used was 2 µg for each binding reaction.

Results

chn encodes a Zn-finger transcription factor

The *chn* locus was first identified in a screen for lethal P elements that affected embryonic development (Kania et al., 1995). The *chn* insertion (*chn*$^{42/18}$; Fig. 1A), located at chromosomal subdivision 51EF, caused an abnormal morphology of the larval PNS neurons, some of which appeared enlarged, while others, such as those of the lateral chordotonal organs, appeared bunched. In an independent experiment, we mobilized an EP element from a nearby locus and obtained the insertion *EPIL6* located near *chn*$^{42/18}$ at the 5′ end of *CG11798* (Fig. 1A). The EP elements carry several Gal4 UAS binding sites and can therefore be crossed with Gal4 drivers to ectopically express adjacent genes (Rørth, 1996). Expression of drivers that direct expression of Gal4 in the wing imaginal disk induced formation of additional bristles, mostly macrochaetae (Fig. 1B,C), suggesting that *CG11798* is involved in bristle formation. We obtained cDNAs for *CG11798* and aligned their

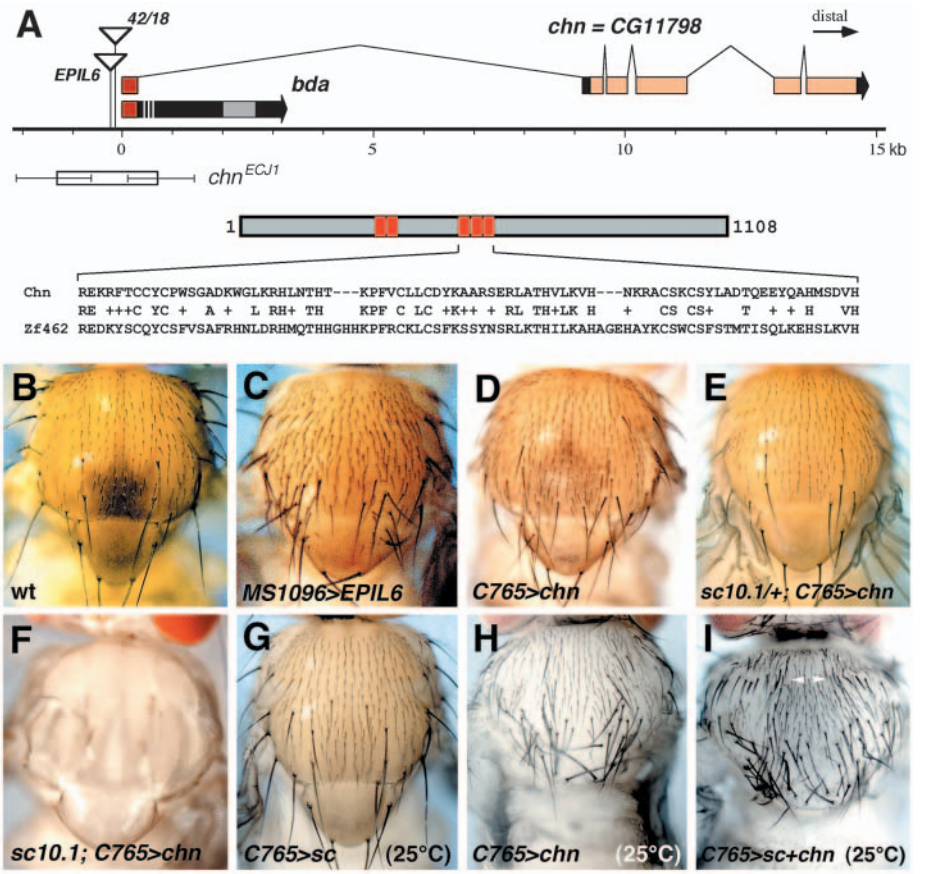

Fig. 1. Physical map of the *chn* locus and notum bristle phenotypes of the overexpression of *chn* and of its interaction with *ac/sc*. (A) The structure of the *chn* and *bda* transcripts, as deduced by sequence comparisons of cDNAs and the genomic DNA, are shown. Light rectangles indicate putative coding regions. The 5′ end of *chn* cDNA is taken as the origin of coordinates and corresponds to position 231192 of scaffold AE003812 of the *D. melanogaster* genome sequence (release 3.1). Available cDNAs suggest a common 5′ region in *chn* and *bda* transcripts (red). The possibility of a small intron (hatched region) in *bda* has not been ruled out. P elements (open triangles) are inserted at positions –230 (*EPIL6*) and –152 (*42/18*). Mutation *chn*^{ECJ1} is associated with a deletion (open rectangle with uncertainty lines for its end points). The structure of the Chn protein with the position of the C2H2 Zn-finger motifs (red rectangles) is indicated. The sequence of three of these motifs is compared with the similar region of the sequence of the human putative Zf462 protein. (B) Notum of a wild-type fly. (C) Expression of the EP line *EPIL6* with the *MS1096-Gal4* driver (25°C) generates extra bristles. (D) A similar phenotype is observed by overexpressing *UAS-chn* with the *C765-Gal4* driver at 18°C. At 25°C most individuals die before the pharate stage (see below). (E) Halving the genetic dose of *ac/sc* largely reduces the effect of overexpressing *chn* (*C765-Gal4* driver, 18°C), and removing *ac/sc* renders overexpression of *chn* inactive in bristle formation (F). (G-I) *UAS-sc* and *UAS-chn* interacted synergistically in the formation of extra bristles. Flies were cultured at 18°C except that, approximately from 48 to 0 hours before puparium formation, they were kept at 25°C. (G) *UAS-sc; C765-Gal4*. (H) *UAS-chn; C765-Gal4*. (I) *UAS-sc/UAS-chn; C765-Gal4*. Note in I, the large increase in macro- and mesochaetae on the anterior region of the notum (arrowheads).

sequences with that of the genomic *chn* DNA. As shown in Fig. 1A, there were two classes of cDNAs, which shared a common short (273 bp) 5′ region, indicating the presence of two types of transcripts resulting from alternative splicing. The first class of cDNAs, probably entirely colinear with the genomic DNA, contained an ORF (630 nt), that putatively encoded a polypeptide with no recognizable similarities to known motifs or proteins. We named this transcript '*belinda*' (*bda*), after the classical speechless film character. The second class of cDNAs resulted from spliced transcripts and coincided with the predicted gene *CG11798*. It encoded a putative 1108 amino acid protein with five C2H2 zinc finger motifs. The zinc finger region showed 35% identity and 55% similarity to the human 'Zinc finger protein 462' (Fig. 1A). As shown below, the transcripts encoding the *Drosophila* zinc finger protein were responsible for *chn* function in the developing PNS.

Overexpression of *chn* causes supernumerary bristles

When driven by *en-Gal4*, the *EPIL6* insertion induced the overexpression of both *chn* and *bda* transcripts in wing imaginal disks (not shown). Hence, we created flies carrying either a *UAS-chn* or a *UAS-bda* transgene. Overexpression of *UAS-bda* using several drivers (*MS1096-Gal4*, *C765-Gal4*, *ap-Gal4* and *MS248-Gal4*) did not cause noticeable phenotypic effects (data not shown). By contrast, overexpression of *UAS-chn* with these and other drivers gave rise to extra bristles. Ubiquitous expression with the *C765-Gal4* driver (Gómez-Skarmeta et al., 1996) caused the appearance of many macrochaetae near wild-type bristles, and it increased the density of microchaetae [141±10 microchaetae per female heminotum versus 103±7 in Oregon R controls (averages of 10 heminota) and Fig. 1D]. However, the bristles were always separated by epidermal cells, suggesting that Notch-mediated lateral inhibition (Artavanis-Tsakonas et al., 1999) was still active. With earlier-expressing drivers such as *MS248-Gal4* (Cavodeassi et al., 2002), overexpression gave rise to many bristles (see Fig. 4D). In addition, this early expression reduced the size of the heminota and interfered with their dorsal fusion. With the appropriate drivers many extra bristles appeared on other regions of the fly body, including wings (*C765-Gal4*, *MS1096-Gal4* and *nub-Gal4*), head (*MS248-Gal4*) and the metathorax (*MS1096-Gal4*) (data not shown).

Formation of additional bristles by overexpression of *chn* depended on the presence of the proneural genes *ac/sc*. Halving the dose of these genes sharply reduced the number of extra macrochaetae induced by the overexpression of *chn* (compare Fig. 1E with 1D). Removal of both *ac* and *sc* completely suppressed bristle formation (Fig. 1F). The genetic interaction between *sc* and *chn* was also manifested by the synergism of their overexpression in bristle formation (Fig. 1G-I), which gave rise to many macro- and mesochaetae in ectopic positions, such as the anterior notum (Fig. 1I).

chn is expressed in the PNS and the CNS

We examined the patterns of expression of *chn* in embryos and imaginal disks using in-situ hybridization. In early blastoderm stages, the expression of *chn* was ubiquitous, but before stage 5, *chn* mRNA disappeared from the poles of the embryo and faint stripes became visible (data not shown). At stage 5, *chn* mRNA also accumulated in the dorsal region, cephalic furrow and in the presumptive mesoderm (Fig. 2A,B). At stage 11, *chn* mRNA was found mostly in the mesoderm (Fig. 2C), and in

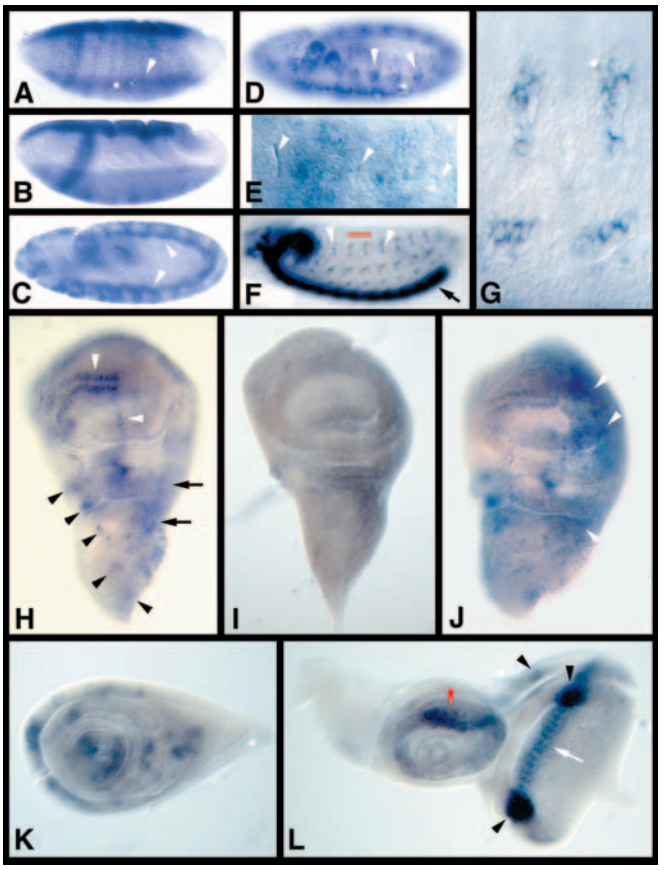

Fig. 2. Expression of *chn* in embryos and imaginal disks. Expression was detected by in-situ hybridization with an antisense RNA *chn* probe labeled with DIG. (A,B) Views of a stage-5 embryo focusing at the lateral surface or internally at the invaginating mesoderm (arrowhead), respectively. Note the weak segmental pattern in A at the level of the ectoderm. (C,D) Lateral views of a stage-11 embryo showing (arrowheads) expression in the mesoderm and in patches of the lateral ectoderm located between the tracheal pits, respectively. (E) High magnification view of a similar embryo showing the position of the tracheal pits (arrowheads) and the hybridization in between them. (F) At stage 15, expression is almost exclusively found in the developing PNS (arrowheads) and in the CNS (arrow). (G) A high magnification view of the region under the red line in F shows that expression occurs in cells of the clusters of sensory neurons (compare with Fig. 3A). The image is centered on the dorsal and lateral clusters. (H) In third instar wing imaginal disks, *chn* expression occurs in proneural clusters (arrowheads) and in the posterior notum and wing hinge (arrows). (I) Expression of *chn* in proneural clusters depends on *ac/sc*, as it is absent in $In(1)sc^{10.1}$ disks. (J) Overexpression of *UAS-sc* in the posterior compartment of the wing disk (*en-Gal4* driver) causes ectopic expression of *chn* (arrowheads). (K) *chn* is expressed in a number of patches of the leg disks, which correspond to proneural clusters since they are absent in a $In(1)sc^{10.1}$ mutant background (not shown), excepting for the femoral clusters, which express *ato* (Jarman et al., 1995). (L) Expression of *chn* in a third instar eye/antenna disk. Expression occurs ahead and/or at the morphogenetic furrow (arrow), at the presumptive head capsule (arrowheads) and at the second antennal segment (red arrowhead). This pattern is strongly reminiscent of that of the proneural gene *atonal* (Jarman et al., 1995).

ectodermal patches between the tracheal pits (Fig. 2D,E), where neurons of the PNS appear (Ruiz-Gómez and Ghysen, 1993). Older embryos (stage 15, Fig. 2F) showed strong expression, which was mostly restricted to the central nervous system (CNS) and PNS. In the latter case, the pattern suggested that expression occurred in many of the neurons of the ventral, lateral and dorsal clusters of neurons (Fig. 2F,G; compare with Fig. 3A).

In third instar wing disks, expression of *chn* is observed in rows of cells on either side of the prospective anterior wing margin and in groups of cells that coincided with proneural clusters of *ac/sc* expression (Fig. 2H). This pattern suggests that *chn* may be positively regulated by *ac/sc*. Indeed, expression of *chn* in proneural clusters was abolished in disks null for *ac/sc* (*In(1)sc*$^{10.1}$) (Fig. 2I). Moreover, overexpression of a *UAS-sc* transgene in the posterior compartment of the disk (*en-Gal4* driver) induced ectopic expression of *chn* (Fig. 2J). Note that *chn* was also expressed independently of *ac/sc* in certain areas of the disk, such as the postnotum and posterior dorsal proximal wing (Fig. 2H,I, arrows). *chn* was also expressed in proneural clusters of the leg disks (Fig. 2K) and in the eye/antenna disk (Fig. 2L). In the latter case, the pattern seemed very similar to that of the proneural gene *atonal* (*ato*), and included the region of the morphogenetic furrow, and the presumptive cephalic capsule and second antennal segment.

Loss of *chn* causes loss of PNS elements

To examine the effects of the removal of *chn*, we obtained new LOF alleles by generating imprecise excisions of the *Pl(2)42/18* insertion. One of these, *chn*^{ECJ1}, is probably a null, as the excision removed at least part of the promoter region of *chn* (Fig. 1A), and homozygous embryos lacked the *chn* mRNA (as detected by in-situ hybridization, not shown) and died as embryos. In keeping with the expression of *chn* in the cells of the PNS, *chn*^{ECJ1} embryos displayed conspicuous anomalies in PNS cells. These included the absence of many neurons, especially in the dorsal and ventral clusters, and an abnormal morphology of chordotonal lateral neurons, which appeared bunched and lacked the typical apical dendrites (Fig. 3A,B, insets). Individually identifiable neurons such as the v'chn1 and the dbp were generally absent (Fig. 3B). Some of these defects were similar to the phenotype described for the original *Pl(2)42/18* insertion (Kania et al., 1995) but were more severe. Ubiquitous expression of *UAS-chn* in the epidermis (*69B-Gal4* driver) (Brand and Perrimon, 1993) largely rescued many of the missing neurons (Fig. 3C) and the morphological defects of the lateral chordotonal neurons (Fig. 3C, inset). However, overexpression of *UAS-chn* in a wild-type background (using *da-Gal4*, *69B-Gal4* and *1407-Gal4* drivers) did not appreciably affect the larval PNS (data not shown).

In the adult, the effects of *chn*^{ECJ1} were examined through clonal analysis. Clones of cells that lacked *chn* often failed to generate macrochaetae (Fig. 4A,B). These were observed to be missing in all notum positions, but scutellar bristles were the most sensitive. However, the penetrance was far from complete: clones including the dorsocentral (n=24) or the posterior postalar (n=8) positions lost approximately 25% of the bristles, while in scutellar clones (n=52) about 45% of the bristles were removed. We also observed, with low frequency, that the bristle shaft was missing, but not the tormogen cell,

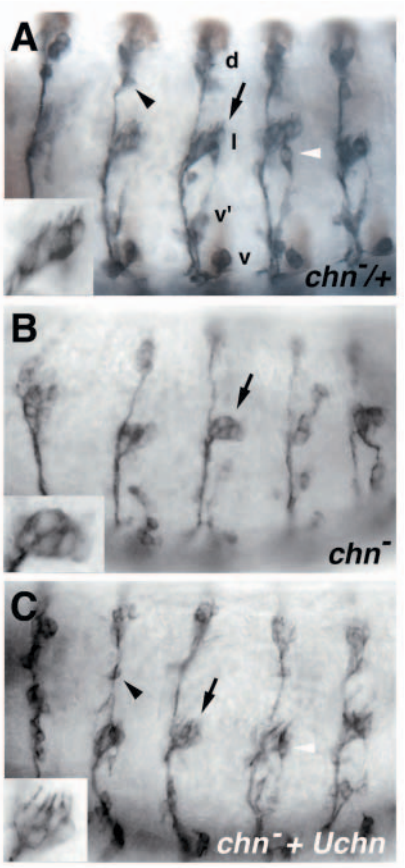

Fig. 3. *chn* is necessary for proper development of the embryonic PNS. Embryos were stained with 22c10 antibody (gray) and anti-β-galactosidase (brown, a marker for the balancer chromosome) antibodies. Lateral views of segments T1 (at left) to A4 are shown. Insets show higher magnification views of the lateral A2 group of chordotonal organs (arrowed). (A) *chn*^{ECJ1}/+ embryo. Its nervous system appears wild type. d, l, v' and v indicate the dorsal, lateral, ventral' and ventral neuronal clusters. Black and white arrowheads point to the dbp and the v'chn1 neurons. (B) *chn*^{ECJ1} homozygous embryo. Note the disorganized pattern, the reduced number of neurons compared with (A), the absence of dbp and v'chn1 neurons, and the altered morphology of the lateral group. (C) *chn*^{ECJ1} embryo in which *UAS-chn* is expressed with the *69B-Gal4* driver. Note the substantial recovery of the wild-type phenotype.

suggesting a role of *chn* in formation of the sensory organ. No defects were seen in the pattern of notum microchaetae.

We further examined the effects of the removal of *chn* in macrochaetae formation with the help of an RNA-interference construct (Sharp, 2001), *UAS-chni*. A strong (*UAS-chni*^{S}) and a weak (*UAS-chni*^{W}) expressing line were used. *UAS-chni*^{S} clearly antagonized *chn* function, because it largely rescued the extra bristle and heminota fusion phenotypes of *MS248-Gal4; UAS-chn* (Fig. 4D,E). No rescue was observed by replacing *UAS-chni*^{S} by *UAS-lacZ* or *UAS-GFP*, which indicated that the effect was not caused by reduced expression of *UAS-chn* in the presence of an additional UAS transgene. Moreover, overexpression of *UAS-chni*^{S} with *MS1096-Gal4* sharply decreased accumulation of the endogenous *chn* mRNA in the wing margin (not shown, see below). These experiments indicated that the *UAS-chni*^{S} acted as a LOF allele of *chn*. With

Table 1. Number of macrochaetae/heminotum under conditions of *UAS-chni* expression and in different genetic backgrounds

	+/+ *sca-G>chni^S*	+/+ *ap-G>chni^S*	*Ax^{M1}*/+ *ap-G*	*Ax^{M1}*/+ *ap-G>chni^S*	*sc$^{10.1}$*/+ *C765>chni^W*	+/+ *pnr-G>chni^W*	*sc$^{10.1}$*/+ *pnr-G>chni^W*	*pnr^{V1}*/ *pnr-G*	*pnr^{V1}*/ *pnr-G>chni^W*
ANP	0.48	1.00	1.00	1.00	0.85*	–	–	–	–
PNP	0.30	1.00	0.33	0.14	1.00	–	–	–	–
PS	1.00	1.00	0	0	1.00	–	–	–	–
ASA	1.00	0.78	0	0	1.00	–	–	–	–
PSA	0.93	0.68	0	0	0.04	–	–	–	–
APA	0.88	1.00	1.00	1.00	1.00	–	–	–	–
PPA	0.95	0.93	0.58	0	0.12	1.00	0.63	1.00	1.00
ADC+PDC	1.93	1.98	0.40	0.08	1.96	2.00	0.77	0.16	0
ASC+PSC	1.15	1.68	1.32	0.20	1.34	1.82	1.13	1.83	0.08

Results are averages of 60-80 heminota.
*, flies *C765>chni^W* and *sc$^{10.1}$*/+; *C765* had wild-type bristle patterns.

the drivers *ap-Gal4* or *sca-Gal4* (the latter promotes expression in proneural clusters), *UAS-chni^S* moderately removed notum macrochaetae (Table 1), while microchaetae were not affected. With the *MS248-Gal4* driver, the macrochaetae in the medial part of the notum, dorsocentrals and scutellars, were often missing (Fig. 4C), but, similar to the homozygous *chn^{ECJ1}* clones, in no case was the phenotype fully penetrant.

The effectiveness of both *UAS-chni^S* and *UAS-chni^W* in removing macrochaetae was increased when the accumulation of *ac/sc* in proneural clusters was compromised (Table 1). Thus, flies overexpressing *UAS-chni^W* with *C765-Gal4* or *pnr-Gal4* had, respectively, wild-type or almost wild-type phenotypes (Table 1), but a sizable number of macrochaetae were lost in the heterozygous *In(1)sc$^{10.1}$*/+ genetic background. The *N* allele *Ax^{M1}* has been shown to reduce *ac/sc* expression in proneural clusters (Martínez-Arias et al., 2002). Hence, in *Ax^{M1}*/+ individuals, several notum macrochaetae are missing. *UAS-chni^S* almost completely eliminated the remaining bristles in *Ax^{M1}*/+ with the exception of the ANP and APA that were always present (Table 1). The *pnr* prepattern gene is necessary for the formation of the dorsocentral and scutellar macrochaetae (García-García et al., 1999; Heitzler et al., 1996). In the hypomorphic *pnr^{V1}/pnr-Gal4* background, expression of *ac/sc* is diminished and these bristles are partially removed. In this genetic background, *UAS-chni^W* almost completely eliminated all these bristles (Table 1). Interestingly, *UAS-chni^W* was able to partially suppress the extra macrochaetae that were generated by the expression of *UAS-sc* driven by *C765-Gal4* (Fig. 4F,G): the number of macrochaetae per notum decreased in the dorsocentral area from 12±1.2 to 7.5±1.2 and, in the scutellum, from 18±1.3 to 10.2±1.3. Moreover, macrochaetae that appeared away from the normal macrochaeate-bearing regions, such as the anterior notum, were almost completely eliminated (from 5.4±1.3 to 1±1.2). This suggested that these extra macrochaetae had a stronger requirement for *chn* than the extant bristles. Taken together, these and the data obtained from the *chn^{ECJ1}* clones indicate that *chn* is not essential for macrochaetae formation, but that it facilitates the process.

chn promotes *sc* expression

The strong genetic interaction between the LOF conditions for *chn* and *ac/sc*, together with the presumed activation of *chn* by *ac/sc* (Fig. 2H-J), led us to examine whether *chn* might in turn stimulate *ac/sc* expression. We first examined whether the overexpression of *chn* affected Sc accumulation in third instar wing disks. In these disks, *ac* and *sc* are coexpressed in a stereotyped pattern of well-resolved proneural clusters from which SOPs emerge (Fig. 5A) (Cubas et al., 1991; Skeath and Carroll, 1991). With the *MS248-Gal4* driver, *UAS-chn* promoted strong and generalized expression of *sc* in most of the domain of expression of the driver, namely, the medial and part of the lateral prospective notum (Fig. 5C). Many SOPs arose from this enlarged region of Sc accumulation, as detected by the Sens marker (Nolo et al., 2000), consistent with the additional macrochaetae that developed on the notum of these flies (Fig. 4D). With the *MS1096-Gal4* driver, which is expressed most strongly in the dorsal part of the wing anlage (Capdevila and Guerrero, 1994), there was also ectopic expression of *sc* and emergence of extra SOPs in the wing territory (Fig. 5B). Interestingly, expression of *UAS-chn* disrupted the characteristic double row expression of *sc* and *sens* at the wing margin, suggesting interference with its formation (Fig. 5B). This is also consistent with the presence of small, crumpled adult wings that carry many bristles and other types of sensilla (data not shown). Finally, overexpression of *UAS-chn* with the ubiquitous wing disk driver *C765-Gal4* (Gómez-Skarmeta et al., 1996) activated *sc* but failed to stimulate *atonal* (Jarman et al., 1993b), a proneural gene which is not a member of the ASC and is normally expressed in a few cells at the presumptive tegula and ventral radius (Fig. 5D). Conversely, overexpression of *atonal* did not stimulate *chn* in the wing disk (not shown).

The expression of *ac/sc* in proneural clusters is controlled by a series of separable enhancer elements in the ASC. Each enhancer is responsible for expression in one or in a few proneural clusters (Gómez-Skarmeta et al., 1995). We thus examined whether the ectopic activation of *sc* could be mediated by the overexpression of *UAS-chn* acting upon these enhancers. As shown in Fig. 5E,F, *UAS-chn* strongly stimulated the activity of a construct in which the *lacZ* gene was under the control of the ASC L3/TSM enhancer [construct *2.3-lacZ* (Culí and Modolell, 1998); *MS1096-Gal4* driver], which directs expression at the wing vein L3 and the twin sensilla of the wing margin proneural clusters. Similar observations were made with the dorsocentral (DC) enhancer [construct *AS1.4DC=DC-lacZ* (García-García et al., 1999); *C765-Gal4* ubiquitous driver], which promotes expression in the central part of the notum. It also activated expression directed by the ANP enhancer (Gómez-Skarmeta et al., 1995) (data not

shown). Since the *DC-lacZ* construct bears the heterologous *hsp70* promoter, these data indicate that the *sc* endogenous promoter is dispensable for the stimulation by *UAS-chn*. The Ac and Sc proneural proteins were also not essential for the increased activity of the enhancers, as *DC-lacZ* expression was strongly increased by Chn in an *In(1)sc*$^{10.1}$ background (Fig. 5I,J). By contrast, the *sc* SOP-dedicated enhancer (*SRV-lacZ* construct), which is responsible for the strong accumulation of Sc in SOPs (Culí and Modolell, 1998), was only clearly activated by *UAS-chn* (*C734-Gal4* driver) in the presence of *ac/sc*, and this stimulation occurred in individual cells (Fig. 5N,O). This observation suggests that the upregulation of this enhancer results from the formation of ectopic SOPs by the *UAS-chn*-induced overexpression of *sc*, rather than from a direct effect of Chn on the enhancer. Still, the possibility remains that Chn and Sc cooperate in the activation of this enhancer.

UAS-chn upregulated the activity of these enhancers, but it did not lead to a generalized expression of *lacZ* in all the domains of *UAS-chn* expression. These data indicate that despite the elevated activation, the enhancers are still dependent on the prepattern factors that define their spatial domains of activity. This fact was verified by the observation that the overstimulation of *DC-lacZ* was strongly dependent on its prepattern activator, the transcription factor Pnr (Fig. 5K-M). Moreover, *2.3-lacZ*, which is active only in the wing pouch, was not stimulated by the overexpression of *UAS-chn* in the prospective notum (*MS248-Gal4*), which indicates that the *sc* promoter present in this construct was not responsive to *UAS-chn* (data not shown).

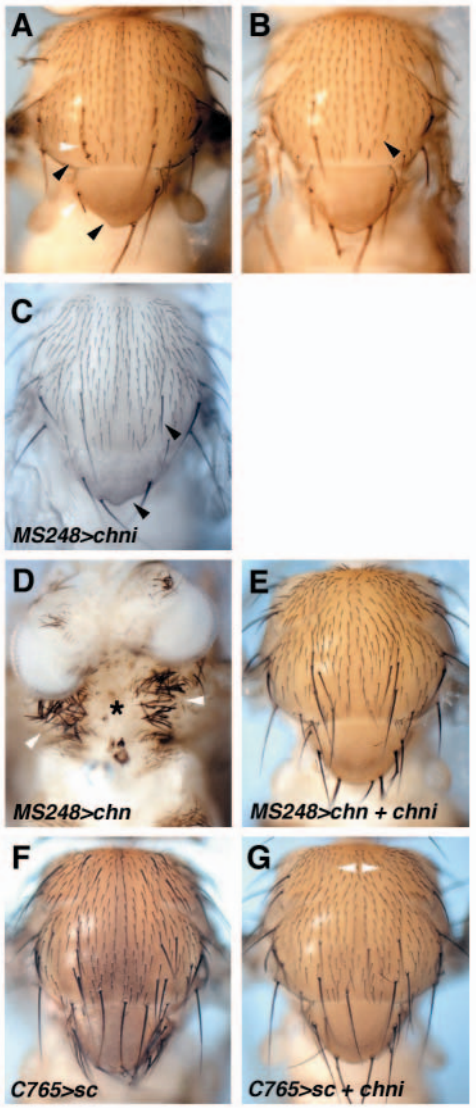

Fig. 4. Loss-of-function conditions of *chn* remove notum macrochaetae. (A,B) *f*$^{-}$, *chn*^{ECJ1} homozygous clones often lose macrochaetae (black arrowheads), although they can also develop them (white arrowheads). (C) Overexpression of *UAS-chni*^{S} directed by *MS248-Gal4* incompletely removes notum macrochaetae. The phenotype is similar to that of the *chn*^{ECJ1} homozygous clones. (D) Interference with notal fusion (asterisk) and generation of extra macrochaetae (arrowheads) due to the overexpression of *UAS-chn* with the MS248-Gal4 driver. (E) These phenotypes are largely rescued by the coexpression of *UAS-chni*^{S}. (F,G) The number of extra macrochaetae formed by overexpressing *UAS-sc* is sharply reduced by simultaneous expression of *UAS-chni*^{W} (60 to 80 heminota were examined to quantify the number of bristles, as indicated in the text). No such reduction was observed when *UAS-chni*^{W} was replaced by *UAS-GFP*. Note the essentially complete removal of extra macro- and mesochaetae from the anterior notum (arrowheads).

Loss of function of *chn* reduces *sc* and *enhancer-lacZ* construct expression

Next, we examined in mosaic wing disks whether removal of *chn* function affected expression of *sc* or *enhancer-lacZ* constructs in proneural clusters. Homozygous *chn*^{ECJ1} cells generally displayed reduced expression of *sc* (Fig. 6A) or β-galactosidase under the control of proneural enhancers (Fig. 6B,C,F,G), when compared with neighboring heterozygous *chn*^{ECJ1}/+ cells. Note, however, that the expression was not completely abolished. Similar decreased expression of *sc* was observed by misexpressing *UAS-chni*^{S} in cell clones (Fig. 6D). These effects appear to be cell-autonomous. While SOPs could still emerge from homozygous *chn*^{ECJ1} cells with reduced levels of Sc (data not shown), SOPs were often missing (Fig. 6H), in agreement with the partial suppression of macrochaetae observed within the *chn*^{ECJ1} clones. When both homozygous and heterozygous cells were near a position where an SOP emerged, a heterozygous cell appeared to be preferentially selected (Fig. 6C,E). These findings clearly indicate that *chn*$^{+}$ was required for proneural proteins to accumulate in proneural clusters at levels sufficient to ensure SOP selection. Moreover, the observation that expression of *enhancer-lacZ* constructs was reduced in *chn*$^{-}$ cells and increased in *chn* overexpressing cells indicates that the effect of *chn*$^{+}$ was not due to an enhanced perdurance of the Sc protein, but to the increased transcription of the *sc* gene.

Chn may physically interact with the DC enhancer

To analyze whether Chn is a direct regulator of the ASC enhancers, we assayed the ability of the Chn protein to bind to the DC enhancer in vitro. A fragment of the Chn protein containing the five zinc fingers was produced in and purified from *E. coli*. The enhancer DNA was divided into six partially overlapping fragments of approximately 300 bp each, and each of them was assayed in gel retardation experiments (Fig. 7A). Only the fragment that comprised the proximalmost region of the enhancer (fragment DC6) showed binding of the Chn

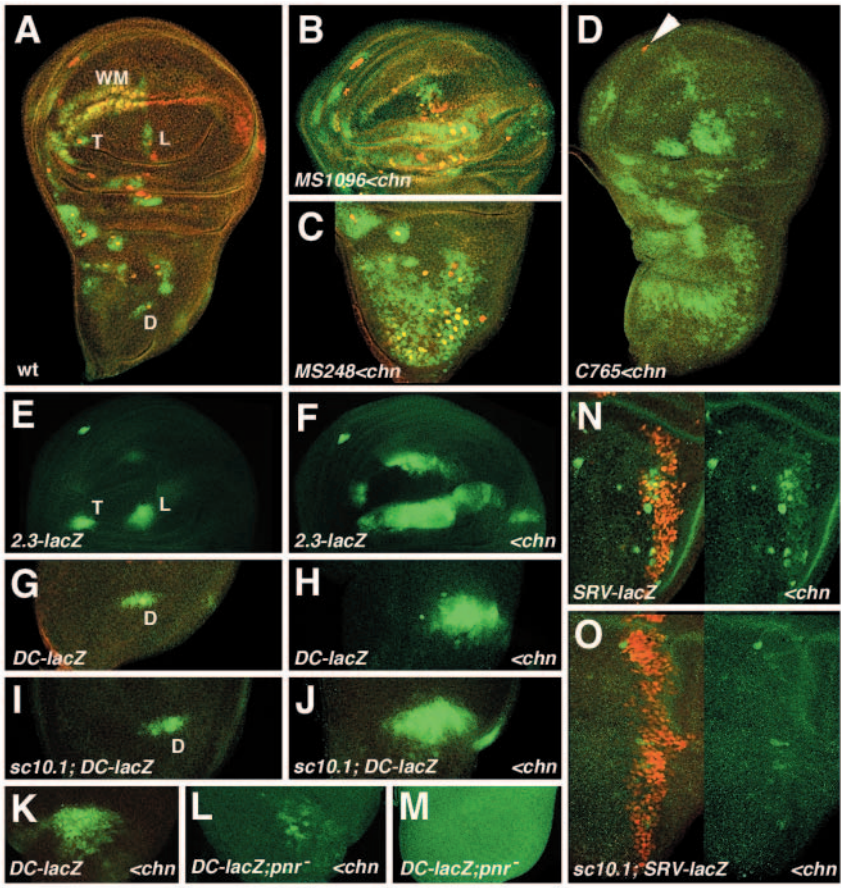

Fig. 5. Overexpression of *UAS-chn* stimulates the expression of *sc* and of constructs bearing ASC enhancers specific for individual proneural clusters. (A-C) Sc accumulation is shown in the green channel and that of Sens in the red one. (A) Wild-type late third instar disk shows the distribution of Sc in proneural clusters and that of Sens in SOPs and additional cells flanking the prospective wing margin (WM). Proneural clusters: T/TSM, twin sensilla of the WM; L, vein L3; D/DC, dorsocentral. (B) Overexpression in the wing pouch (*MS1096-Gal4* driver) induces ectopic expression of *sc* and of *sens*, but largely eliminates expression of these genes at the prospective wing margin. (C) Overexpression in the medial and central notum territory (*MS248-Gal4* driver) leads to strong, almost generalized expression of *sc* and the emergence of many ectopic SOPs. (D) Generalized *UAS-chn* expression (*C765-Gal4* driver) stimulated Sc accumulation (green) at many sites of the wing disk, but it did not enhance *atonal* expression, which remained confined to its wild-type sites (Jarman et al., 1993b), like a few cells in the prospective ventral radius (red, arrowhead). (E-O) β-galactosidase accumulation is shown in green. (E,F) Expression of the *2.3-lacZ* construct (Culí, 1998), which bears the L3 + TSM enhancer, in a wild-type disk and in a disk overexpressing *UAS-chn* with the *MS1096-Gal4* driver, respectively. (G,H) Expression of the *AS1.4DC-lacZ=DC-lacZ* construct, which bears the DC enhancer, in a wild-type disk and in a disk overexpressing *UAS-chn* with the *C765-Gal4* driver. (I,J) *DC-lacZ* expression in disks devoid of functional *ac* and *sc* genes ($In(1)sc^{10.1}$ allele). The construct is still expressed (I) and it is greatly stimulated by *UAS-chn* (J; *C765-Gal4* driver). (K,L) Expression of the *DC-lacZ* construct in the presence of *UAS-chn* driven by *pnr-Gal4* [a hypomorphic allele of *pnr* (Heitzler et al., 1996)] in the presence of a wild-type allele of *pnr* (K) or the null allele pnr^{VX6} (L). (M) This construct is not expressed in the *pnr-Gal4* /pnr^{VX6} genetic background (the green channel background has been enhanced to better appreciate the absence of expression). (N,O) Overexpression of UAS-chn with the *C734-Gal4* driver, whose pattern of expression is revealed by the accumulation of Chn protein (red), stimulates expression of the SOP-specific enhancer *SRV-lacZ* (green) in a background wild-type for the ASC (N), but fails to do so in an $In(1)sc^{10.1}$ mutant disk (O). The green channel is also shown separately.

polypeptide (Fig. 7B). Interestingly, the DC6 fragment is included within the PB0.5DC sequence (Fig. 7A) (García-García et al., 1999), the smallest subfragment of AS1.4DC, which still retains enhancer activity. For unclear reasons, the PB0.5DC enhancer only drives expression in the PDC SOP (Fig. 7C) (García-García et al., 1999). Still, misexpression of *UAS-chn* expands this expression to many cells of the posterior notum (Fig. 7D). This suggests that the binding of Chn protein to the DC6 region of the DC enhancer may prompt its response to Chn in vivo.

Discussion

We have identified *chn*, a novel gene that encodes a zinc finger factor that is involved in the development of the PNS of the *Drosophila* embryo and the adult fly. We have examined in detail the function of *chn* in the formation of the stereotyped pattern of notum macrochaetae. Complete removal of *chn* expression led to a relatively mild phenotype; namely, the failure of each notum macrochaetae to develop in 25 to 45% of the flies. Any macrochaeta was subject to loss. This loss was strongly enhanced when, concomitant to the removal of *chn*, the proneural function of *ac/sc* was reduced by either halving the doses of the ASC [a condition that normally does not cause the loss of any macrochaeta (García-Bellido, 1979)] or by introducing alleles that decreased accumulation of Ac/Sc in proneural clusters. This result suggested a positive interaction between proneural and *chn* functions in macrochaetae development, an inference that was verified by overexpression experiments. Thus, overexpression of *chn* gave rise to a large number of extra macrochaetae, an effect that was strongly dependent on the number of doses of the ASC. Reciprocally, the number of extra macrochaetae that arose when overexpressing *sc* was sharply decreased by compromising *chn* function. In all cases, the extra macrochaetae that were formed upon *chn* overexpression were not contiguous to one another and epidermal cells were present between them. This indicated that N-mediated lateral inhibition (reviewed by Artavanis-Tsakonas et al., 1995) was still operating and that *chn* was unlikely to antagonize this process.

chn and *ac/sc* establish a stimulatory loop in proneural clusters

The presence of *chn* mRNA in the proneural clusters of the wing disk is dependent on *ac/sc*. Moreover, ectopic accumulation of Sc results in ectopic expression of *chn*. These

observations place *chn* downstream of *ac/sc*, and suggest a positive, possibly direct, regulation of *chn* by *ac/sc*. Consistent with this, two clusters of four and eight E-boxes, putative binding sites for bHLH proteins of the proneural type (reviewed by Bertrand et al., 2002), were found approximately 15 kb upstream of the *chn* structural sequences and within the first intron of the gene, respectively.

In turn *chn* stimulates the accumulation of Sc in proneural clusters, as loss of function of *chn* resulted in decreased accumulation of Sc. However, some Sc still accumulates in the complete absence of Chn, which probably explains why many SOPs and their corresponding macrochaetae developed in its absence. The upregulation of *sc* by *chn* is even more manifest by the overexpression of *UAS-chn*, which causes a strong accumulation of Sc and leads to the formation of large numbers of SOPs and extra macrochaetae. *ac* is also upregulated by overexpression of *chn* (L.M.E., unpublished). Although we cannot rule out that Chn may slow the turnover of Sc/Ac and thereby promote their accumulation, our data clearly show that Chn stimulates the transcription of *ac/sc*. Indeed, the overexpression of *chn* greatly increases in vivo the expression of the reporter gene *lacZ* driven by proneural group-specific enhancers of the ASC (Culí and Modolell, 1998; García-García et al., 1999; Gómez-Skarmeta et al., 1995) and its removal decreases the expression of these constructs. The stimulation is also observed with enhancer constructs that do not have the endogenous *sc* promoter (rather, they carry an *hsp70* minimal promoter). These data suggest that *chn* acts mainly on the ASC enhancers, but we cannot rule out at present that the endogenous promoter might additionally favor this effect. However, our results argue against a stimulatory action of Chn directly on the *sc* and/or *ac* promoters, since generalized expression of *UAS-chn* did not lead to widespread expression of the constructs carrying the *sc* promoter. Moreover, the stimulation was equally observed in the presence or absence of the endogenous *ac/sc* genes, which indicates that it is not mediated by positive feedback loops of *ac/sc* on the ASC enhancers, in agreement with previous observations (Gómez-Skarmeta et al., 1995). Considering that the ASC enhancers act in vivo on both the *sc* and the *ac* promoters (Cubas et al., 1991; Gómez-Skarmeta et al., 1995; Skeath and Carroll, 1991), it was to be expected that Chn would also stimulate *ac* expression.

Interestingly, Chn not only increased the levels of *lacZ* expression within the proneural cluster for which the enhancer was specific, but in general it also expanded the expression into a larger area surrounding the proneural cluster, so that more cells were expressing the reporter gene. Perdurance of β-galactosidase should not be responsible for this effect, because when *chn* was not overexpressed, *DC-lacZ* directed β-galactosidase accumulation only in the cells that also expressed *sc* at the DC cluster (García-García et al., 1999). Moreover, the stimulation by *chn* seemed to require the presence of at least some of the prepattern factors (reviewed by Ghysen and Dambly-Chaudière, 1988; Gómez-Skarmeta et al., 2003) that normally act on the enhancers and drive the expression of *ac* and *sc* in proneural clusters, as is the case for Pnr, the prepattern activating factor of the DC cluster (García-García et al., 1999). We propose that excess Chn makes the proneural cluster enhancers responsive to suboptimal concentrations of the prepattern activators that are normally too low to permit activity. Hence, the domains of expression of *lacZ* are

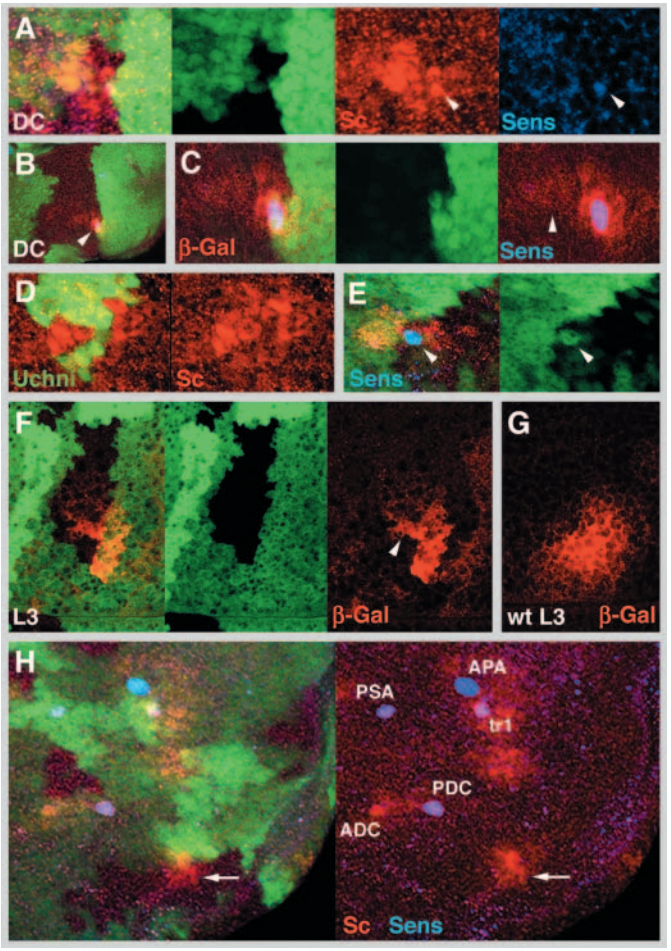

Fig. 6. Loss of *chn* function leads to decreased expression of *sc* and *enhancer-lacZ* constructs, and can impede SOP formation. All figures show parts of third instar wing disks. Except in D, clones homozygous for chn^{ECJ1} are marked by the absence of green. Anti-Sens antibody marks emerged SOPs (blue channel). (A) Clone that includes part of the DC proneural cluster. The mutant cells accumulate less Sc protein (red channel) and give the clone a split appearance. The Sens marker has just started making discernable the SOP of the posterior DC macrochaetae (arrowhead). (B) A large mutant clone that includes the anterior part of a DC proneural cluster (arrowhead), as revealed by expression of the *AS1.4DC-lacZ* construct (red). (C) Higher magnification image of the same DC cluster, showing merged, green, and red plus blue channels. Most of the cells with strong accumulation of β-galactosidase and the PDC SOP are in the heterozygous territory. The cluster appears roundish rather than elongated (Fig. 5A,F) because there is little accumulation of β-galactosidase in the homozygous territory (arrowhead). (D) Cells of a DC proneural cluster that overexpress $UAS\text{-}chni^{S}$ (green) accumulate less Sc protein than neighboring cells (red channel). (E) An SOP has been singled out from an heterozygous chn^{ECJ1} cell (arrowhead). (F) Clone that includes part of the L3 proneural cluster, as revealed by the expression of the *L3-TSM-lacZ* construct. Note the irregular shape of the cluster and the reduced expression within the homozygous territory (arrowhead). A control proneural cluster entirely within heterozygous territory has a roundish shape (G). (H) Merged and red plus blue channels views of the notum region of a late third instar wing disk harboring several clones of homozygous chn^{ECJ1} cells. Sc (red) and Sens (blue) stainings reveal SOPs. The PSC SOP, which would have to develop within a clone (arrow) and is one of the earliest SOPs to emerge, is absent. Nomenclature for other SOPs is indicated. The presence of the ADC SOP confirms the late stage of the disk.

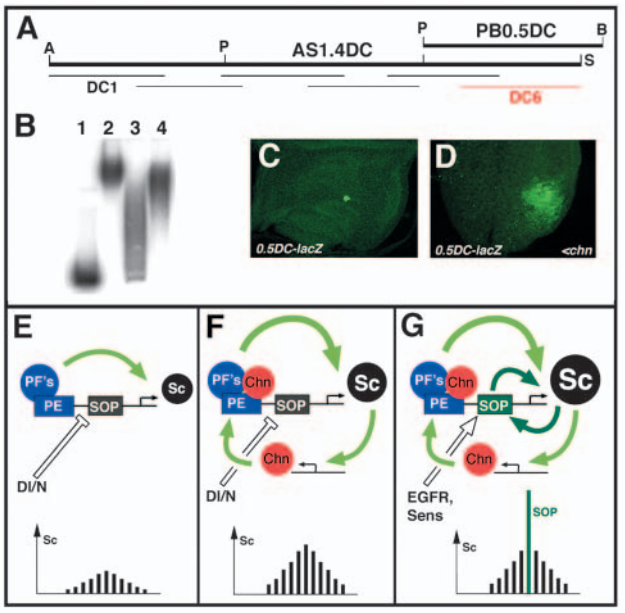

Fig. 7. Chn can bind to the DC enhancer in vitro, and model for the genetic control of macrochaeta SOP singling out. (A) Scheme of the subfragments of the AS1.4DC enhancer (thin lines under the AS1.4DC thick line) that were assayed for binding by a polypeptide containing the five zinc fingers motifs of Chn (Chn5ZF). A, *Ava*II; B, *Bgl*II; P, *Pst*I; S, *Sal*I: Only the DC6 fragment (in red) was bound by Chn5ZF in an EMSA assay, as shown in (B). A polypeptide with the five zinc fingers motifs of Chn (Chn5ZF) binds to the ^{32}P-labeled DC6 DNA probe in an EMSA assay. (1) ^{32}P-labeled DC6 DNA probe alone; (2) labeled DC6 with Chn5ZF present; (3) labeled DC6 with Chn5ZF and an 8-fold molar ratio of cold DC6 added; (4) labeled DC6 with Chn5ZF and an 8-fold molar ratio of cold control DNA (subfragment DC1) added. (C) A 0.5 kb fragment of the DC enhancer (PB0.5DC, panel A) directs *lacZ* expression only in the posterior DC SOP (García-García et al., 1999). (D) Overexpression of *UAS-chn* (*C765-Gal4*) promotes expression in many cells of the posterior notum DC region. (E) A combination of prepattern factors (PFs) acting on an ASC proneural cluster enhancer (PE) activate *sc* expression. The Dl/N signaling pathway, activated by Sc in the proneural clusters, blocks the SOP-specific enhancer (Artavanis-Tsakonas et al., 1995; Culí and Modolell, 1998; Giagtzoglou et al., 2003). An idealized representation of Sc accumulation in the proneural cluster is shown at the bottom of the panel. (F) Sc activates *chn*. This activation might be direct and mediated by E-boxes present in the *chn* gene. Chn binds to the PE and further stimulates *sc* expression, leading to higher accumulation of this protein, and also of Ac (data not shown). The *ac/sc-chn* stimulatory loop is established. Dl/N signaling still blocks the SOP-specific enhancer. (G) In a poorly understood process, a cell with high levels of Sc accumulation, and helped by the EGFR signaling pathway that is also activated by Sc (Culí et al., 2001), establishes a Sc self-stimulatory loop that is mediated by the SOP-specific enhancer (Culí and Modolell, 1998; Giagtzoglou et al., 2003). This cell accumulates much Sc, Ac and Sens and becomes the SOP. The Dl/N pathway no longer blocks the SOP enhancer in this cell, but it does so in the neighboring cells (Artavanis-Tsakonas et al., 1995).

expanded. The dependence of Chn stimulation on different prepattern factors suggests that Chn acts as a coactivator, increasing the effective interaction of prepattern activators with the *ac* and *sc* promoters. Moreover, the finding that a fragment of Chn that contains the five Zn-finger motifs of the protein can bind in vitro to a 316 bp fragment of the DC enhancer DNA further suggests that Chn stimulates *ac/sc* expression by directly binding to ASC proneural cluster-specific enhancers. The possible functional relevance of this binding is reinforced by the fact that the 316 bp fragment is found within a 508 bp segment that possesses residual DC enhancer activity and that Chn is capable of strongly stimulating this activity in vivo.

Specificity of Chn

Chn does not appear to act in vivo as a general stimulator of the enhancer action of proneural genes. The ASC enhancer(s) responsible for expression of *ac/sc* during microchaetae formation did not require Chn, as judged by the independence of microchaetae density from the activity of *chn*. Note that downregulation of *ac* and/or *sc* normally leads to a strong loss of microchaetae (Ruiz-Gómez and Modolell, 1987). By contrast, overexpression of *UAS-chn* did increase their density, suggesting that the microchaetae enhancer(s) can potentially respond to Chn. *chn*^{ECJ1} clones and *UAS-chni* did not alter the anterior wing margin bristles. However, overexpression of *UAS-chn* impaired the expression of *sc* at the anterior wing margin (Fig. 5A-C), although we favor the idea that this inhibition results from an interference of Chn with the general patterning of the wing, as suggested by the inhibition of *sens* expression even in the posterior wing margin (Fig. 5A). The lack of an identified ASC wing margin enhancer has prevented a more direct test of these possibilities. We also found that the ASC SOP-specific enhancer (Culí and Modolell, 1998) could not be activated in the absence of *ac/sc* and that the stimulation that we observed occurred in isolated cells, rather than in the majority of cells of the domain of *UAS-chn* expression. Probably, the stimulation resulted from extra SOPs arising from the overexpression of the endogenous *sc* gene. Finally, the proneural gene *atonal*, which is not a member of the ASC (Jarman et al., 1993b), was not affected in the wing or in the eye (L. M. E., unpublished) disks by *UAS-chn*. We conclude that in the wing disk, Chn is mostly specific for the ASC enhancers that direct *ac/sc* expression in the proneural clusters of the macrochaetae and other landmark sensilla, such as the twin sensilla of the anterior wing margin (TSM) and the L3 wing vein sensilla campaniformia.

Genetic levels of control during SOP specification

Taken together, our data indicate that *chn* and *ac/sc* form a mutually stimulatory loop that enhances accumulation of Ac/Sc in the proneural clusters of the notum macrochaetae (Fig. 7F). These and other previous findings suggest the following consecutive levels of genetic control during SOP selection. The process starts by the deployment of combinations of prepattern factors that trigger the expression of *ac/sc* in proneural clusters (reviewed by Ghysen and Dambly-Chaudière, 1988; Gómez-Skarmeta et al., 2003) (Fig. 7E). Then, *ac/sc* activate *chn* and their stimulatory loop reinforces the expression of *ac/sc* (Fig. 7F). This allows increasing levels of Ac/Sc to accumulate in the cells of the proneural cluster and the formation of the proneural field, which includes the few cells of the cluster with the highest levels of Ac/Sc (Cubas and Modolell, 1992). The SOP will be selected from one of these cells by the Ac/Sc-mediated activation of *sens*, which in turn allows the autostimulatory loops of the proneural genes mediated by the SOP-specific

enhancers (Culí and Modolell, 1998; Jafar-Nejad et al., 2003; Nolo et al., 2000) (Fig. 7G). These enhancers are the targets of two antagonistic signaling systems, both triggered by the accumulation of Ac/Sc. The positive one is mediated by the EGF receptor (Culí et al., 2001) and Sens. The EGFR pathway allows the cells of the proneural cluster to signal positively to each other (lateral cooperation) and helps activate the SOP-specific enhancers, whereas Sens directly activates proneural gene expression in a positive feedback loop when the proneurals reach a certain threshhold in the SOP. Sens and EGFR are in turn antagonized by the negative loop, which is mediated by the Dl/N pathway and the E(spl) proteins and prevents more than one cell from turning on the proneural gene self-stimulation and becoming an SOP (lateral inhibition) (Artavanis-Tsakonas et al., 1995; Culí and Modolell, 1998; Giagtzoglou et al., 2003; Nolo et al., 2000; Heitzler and Simpson, 1991; Jafar-Nejad et al., 2003; Simpson, 1990; Simpson, 1997). Thus, three loops of self-stimulation of *ac/sc* exist: the first is mediated by *chn* and targets the proneural cluster enhancers; the second is mediated by the EGFR pathway and targets the SOP-specific enhancers; the third is mediated by Sens and also directly targets the SOP-specific enhancers. It is interesting to note that the first and third stimulatory loops are mediated by Zn-finger transcription factors of the C2H2 type with homologs in mammals and other species. The negative loop, mediated by Dl/N and the E(spl) proteins, maintains most cells of the proneural cluster in a non-SOP state, allowing them to differentiate as epidermal cells (Fig. 7E,F). As previously discussed, it is tempting to speculate that these consecutive layers of control facilitate the refinement of the position where SOPs arise within proneural clusters (Culí et al., 2001).

Function of *chn* as a neuronal differentiation gene

In the embryo, *chn* is expressed in regions where the neurons of the PNS will arise and later in the developing neural cells. Its removal causes loss of PNS neurons and defects in the morphology of the chordotonal organs, suggesting that *chn* is required for the proper formation of many or most elements of the PNS. So far, the reported effects of insufficiency of proneural gene function in the embryonic PNS have mostly been the removal of neurons and chordotonal organs, rather than defective morphologies (Dambly-Chaudière and Ghysen, 1987; Huang et al., 2000; Jarman et al., 1993b; Villa-Cuesta et al., 2003). Hence, we like to suggest that in the embryonic PNS *chn* acts more as a neuronal differentiation gene than a proneural gene activator. In agreement with this suggestion we observed that overexpression of *UAS-chn* did not modify the embryonic PNS, as detected with the 22c10 antibody. By contrast, overexpression of proneural genes promotes development of extra neurons and chordotonal organs (Huang et al., 2000; Jarman et al., 1993b; Villa-Cuesta et al., 2003). Moreover, loss of function of *cousin of atonal* (*cato*) and *ase*, two genes that can act as neuronal differentiation genes, also causes malformations of the lateral clusters of chordotonal organs (Goulding et al., 2000). We do not know whether the removal of *chn* may also affect the differentiation of the adult bristles, but the observation that, with low frequency, a shaft can be missing, but not the basal cell, also suggests a role of *chn* in the differentiation of these SOs. Moreover, the fact that *UAS-chni* partially suppressed the extra macrochaetae induced by *UAS-sc* (Fig. 4F,G), a transgene not subjected to *chn* modulation, may additionally indicate that *chn* favors macrochaetae formation. However, it should be kept in mind that *UAS-sc* may promote accumulation of Sc not only through its own expression, but also by the activation of *chn*, which would in turn stimulate the endogenous *ac/sc* genes. This latter stimulation should be sensitive to *UAS-chni* and its inhibition might partially suppress the formation of extra macrochaetae. At present, we cannot decide on these alternatives.

We are grateful to: S. Campuzano, S. Sotillos, J. L. Gomez-Skarmeta, L. Sanchez, R. Barrio, M. Suzanne, N. Azpiazu, E. Sanchez-Herrero, J. F. de Celis, G. Morata and colleagues of J.M.'s laboratory for advice on the work and constructive criticism of the manuscript; to C. Estella and S. Aldaz for providing reagents and stocks. Predoctoral fellowship from Comunidad Autónoma de Madrid to L.M.E. is acknowledged. H.J.B. is an investigator of the Howard Hughes Medical Institute. A grant from Dirección General de Investigación Científica y Técnica (BMC2002-411) to J.M. and an institutional grant from Fundación Ramón Areces to the Centro de Biología Molecular Severo Ochoa are acknowledged.

References

Aldaz, S., Morata, G. and Azpiazu, N. (2003). The Pax-homeobox gene *eyegone* is involved in the subdivision of the thorax of *Drosophila*. *Development* **130**, 4473-4482.

Artavanis-Tsakonas, S., Matsuno, K. and Fortini, M. E. (1995). Notch signaling. *Science* **268**, 225-232.

Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J. (1999). Notch signaling: cell fate control and signal integration in development. *Science* **284**, 770-776.

Bertrand, N., Castro, D. S. and Guillemot, F. (2002). Proneural genes and the specification of neural cell types. *Nature Rev. Neurosci.* **3**, 517-530.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Brand, M., Jarman, A. P., Jan, L. Y. and Jan, Y. N. (1993). *asense* is a *Drosophila* neural precursor gene and is capable of initiating sense organ formation. *Development* **119**, 1-17.

Calleja, M., Moreno, E., Pelaz, S. and Morata, G. (1996). Visualization of gene expression in living adult *Drosophila*. *Science* **274**, 252-255.

Campuzano, S. and Modolell, J. (1992). Patterning of the *Drosophila* nervous system: the *achaete-scute* gene complex. *Trends Genet.* **8**, 202-207.

Capdevila, J. and Guerrero, I. (1994). Targeted expression of the signal molecule decapentaplegic induces pattern duplications and growth alterations in *Drosophila* wings. *EMBO J.* **13**, 4459-4468.

Cavodeassi, F., Rodríguez, I. and Modolell, J. (2002). Dpp signalling is a key effector of the wing-body wall subdivision of the *Drosophil*a mesothorax. *Development* **129**, 3815-3823.

Cooley, L., Kelley, R. and Spradling, A. (1988). Insertional mutagenesis of the *Drosophila* genome with single P elements. *Science* **239**, 1121-1128.

Cubas, P. and Modolell, J. (1992). The *extramacrochaetae* gene provides information for sensory organ patterning. *EMBO J.* **11**, 3385-3393.

Cubas, P., de Celis, J. F., Campuzano, S. and Modolell, J. (1991). Proneural clusters of *achaete-scute* expression and the generation of sensory organs in the *Drosophila* imaginal wing disc. *Genes Dev.* **5**, 996-1008.

Culí, J. (1998). Especificación de las Células Precursoras de los Órganos Sensoriales en *Drosophila*. Madrid: Universidad Autónoma de Madrid.

Culí, J. and Modolell, J. (1998). Proneural gene self-stimulation in neural precursors: an essential mechanism for sense organ development that is regulated by *Notch* signaling. *Genes Dev.* **12**, 2036-2047.

Culí, J., Martín-Blanco, E. and Modolell, J. (2001). The EGF receptor and N signalling pathways act antagonistically in *Drosophila* mesothorax bristle patterning. *Development* **128**, 299-308.

Dambly-Chaudière, C. and Ghysen, A. (1987). Independent subpatterns of sense organs require independent genes of the *achaete-scute* complex in *Drosophila* larvae. *Genes Dev.* **1**, 297-306.

Díaz-Benjumea, F. J. and García-Bellido, A. (1990). Genetic analysis of the wing vein pattern of *Drosophila*. *Roux's Arch. Dev. Biol.* **198**, 336-354.

Domínguez, M. and Campuzano, S. (1993). *asense*, a member of the *Drosophila achaete-scute* complex, is a proneural and neural differentiation gene. *EMBO J.* **12**, 2049-2060.
García-Bellido, A. (1979). Genetic analysis of the *achaete-scute* system of *Drosophila melanogaster*. *Genetics* **91**, 491-520.
García-García, M. J., Ramain, P., Simpson, P. and Modolell, J. (1999). Different contributions of *pannier* and *wingless* to the patterning of the dorsal mesothorax of *Drosophila*. *Development* **126**, 3523-3532.
Ghysen, A. and Dambly-Chaudière, C. (1988). From DNA to form: the *achaete-scute* complex. *Genes Dev.* **2**, 495-501.
Ghysen, A. and Dambly-Chaudière, C. (1989). Genesis of the *Drosophila* peripheral nervous system. *Trends Genet.* **5**, 251-255.
Giagtzoglou, N., Alifragis, P., Koumbanakis, K. A. and Delidakis, C. (2003). Two modes of recruitment of E(spl) repressors onto target genes. *Development* **130**, 259-270.
Gómez-Skarmeta, J. L., Rodríguez, I., Martínez, C., Culí, J., Ferrés-Marcó, M. D., Beamonte, D. and Modolell, J. (1995). Cis-regulation of *achaete* and *scute*: shared enhancer-like elements drive their coexpression in proneural clusters of the imaginal discs. *Genes Dev.* **9**, 1869-1882.
Gómez-Skarmeta, J. L., Diez del Corral, R., de la Calle-Mustienes, E., Ferrés-Marcó, D. and Modolell, J. (1996). *araucan* and *caupolican*, two members of the novel Iroquois complex, encode homeoproteins that control proneural and vein forming genes. *Cell* **85**, 95-105.
Gómez-Skarmeta, J. L., Campuzano, S. and Modolell, J. (2003). Half a century of neural prepatterning: the story of a few bristles and many genes. *Nat. Rev. Neurosci.* **4**, 587-598.
González-Crespo, S. and Levine, M. (1993). Interactions between *dorsal* and helix-loop-helix proteins initiate the differentiation of the embryonic mesoderm and neuroectoderm in *Drosophila*. *Genes Dev.* **7**, 1703-1713.
Goulding, S. E., White, N. M. and Jarman, A. P. (2000). *cato* encodes a basic helix-loop-helix transcription factor implicated in the correct differentiation of *Drosophila* sense organs. *Dev. Biol.* **221**, 120-131.
Heitzler, P. and Simpson, P. (1991). The choice of cell fate in the epidermis of *Drosophila*. *Cell* **64**, 1083-1092.
Heitzler, P., Haenlin, M., Ramain, P., Calleja, M. and Simpson, P. (1996). A genetic analysis of *pannier*, a gene necessary for viability of dorsal tissues and bristle postioning in *Drosophila*. *Genetics* **143**, 1271-1287.
Hinz, U., Giebel, B. and Campos-Ortega, J. A. (1994). The basic-helix-loop-helix of *Drosophila* lethal of scute protein is sufficient for proneural function and activates neurogenic genes. *Cell* **76**, 77-87.
Huang, F., Dambly-Chaudière, C. and Ghysen, A. (1991). The emergence of sense organs in the wing disc of *Drosophila*. *Development* **111**, 1087-1095.
Huang, M. L., Hsu, C. H. and Chien, C. T. (2000). The proneural gene *amos* promotes multiple dendritic neuron formation in the *Drosophila* peripheral nervous system. *Neuron* **25**, 57-67.
Ito, K., Awano, W., Suzuki, K., Hiromi, Y. and Yamamoto, D. (1997). The *Drosophila* mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurones and glial cells. *Development* **124**, 761-771.
Jafar-Nejad, H., Acar, M., Nolo, R., Lacin, H., Pan, H., Parkhurst, S. M. and Bellen, H. J. (2003). Senseless acts as a binary switch during sensory organ precursor selection. *Genes Dev.* **17**, 2966-2978.
Jarman, A. P., Brand, M., Jan, L. Y. and Jan, Y. N. (1993a). The regulation and function of the helix-loop-helix gene, *asense*, in *Drosophila* neural precursors. *Development* **119**, 19-29.
Jarman, A. P., Grau, Y., Jan, L. Y. and Jan, Y. N. (1993b). *atonal* is a proneural gene that directs chordotonal organ formation in the *Drosophila* peripheral nervous system. *Cell* **73**, 1307-1321.
Jarman, A. P., Sun, Y., Jan, L. Y. and Jan, Y. N. (1995). Role of the proneural gene *atonal* in formation of *Drosophila* chordotonal organs and photoreceptors. *Development* **121**, 2019-2030.
Kania, A., Salzberg, A., Bhat, M., D'Evelyn, D., He, Y., Kiss, I. and Bellen, H. J. (1995). P-element mutations affecting embryonic peripheral nervous system development in *Drosophila melanogaster*. *Genetics* **139**, 1663-1678.
Lindsley, D. L. and Zimm, G. G. (1992). *The Genome of* Drosophila melanogaster. San Diego: Academic Press.
Lunde, K., Biehs, B., Nauber, U. and Bier, E. (1998). The *knirps* and *knirps-related* genes organize development of the second wing vein in *Drosophila*. *Development* **125**, 4145-4154.
Martínez-Arias, A., Zecchini, V. and Brennan, K. (2002). CSL-independent Notch signalling: a checkpoint in cell-fate decisions during development? *Curr. Opin. Genet. Dev.* **12**, 524-533.
Modolell, J. and Campuzano, S. (1998). The *achaete-scute* complex as an integrating device. *Int. J. Dev. Biol.* **42**, 275-282.
Nagel, A. C., Maier, D. and Preiss, A. (2002). Green fluorescent protein as a convenient and versatile marker for studies on functional genomics in *Drosophila*. *Dev. Genes Evol.* **212**, 93-98.
Nolo, R., Abbott, L. A. and Bellen, H. J. (2000). Senseless, a Zn finger transcription factor, is necessary and sufficient for sensory organ development in *Drosophila*. *Cell* **102**, 349-362.
Parras, C., García-Alonso, L. A., Rodríguez, I. and Jiménez, F. (1996). Control of neural precursor specification by proneural proteins in the CNS of *Drosophila*. *EMBO J.* **15**, 6394-6399.
Romani, S., Campuzano, S., Macagno, E. and Modolell, J. (1989). Expression of *achaete* and *scute* genes in *Drosophila* imaginal discs and their function in sensory organ development. *Genes Dev.* **3**, 997-1007.
Rørth, P. (1996). A modular misexpression screen in *Drosophila* detecting tissue-specific phenotypes. *Proc. Natl. Acad. Sci. USA* **93**, 12418-12422.
Rubin, G. M. and Spradling, A. C. (1982). Genetic transformation of *Drosophila* with transposable element vectors. *Science* **218**, 348-353.
Ruiz-Gómez, M. and Ghysen, A. (1993). The expression and role of a proneural gene, *achaete*, in the development of the larval nervous system of *Drosophila*. *EMBO J.* **12**, 1121-1130.
Ruiz-Gómez, M. and Modolell, J. (1987). Deletion analysis of the *achaete-scute* locus of *D. melanogaster*. *Genes Dev.* **1**, 1238-1246.
Sánchez, L., Casares, F., Gorfinkiel, N. and Guerrero, I. (1997). The genital disc of *Drosophila melanogaster*. II. Roles of the genes *hedgehog*, *decepentaplegic* and *wingless*. *Dev. Genes Evol.* **207**, 229-241.
Sharp, P. A. (2001). RNA interference-2001. *Genes Dev.* **15**, 485-490.
Simpson, P. (1990). Lateral inhibition and the development of the sensory bristles of the adult peripheral nervous system of *Drosophila*. *Development* **109**, 509-519.
Simpson, P. (1997). Notch signalling in development: on equivalence groups and asymmetric developmental potential. *Curr. Opin. Genet. Dev.* **7**, 537-542.
Skeath, J. B. and Carroll, S. B. (1991). Regulation of *achaete-scute* gene expression and sensory organ pattern formation in the *Drosophila* wing. *Genes Dev.* **5**, 984-995.
Staehling-Hampton, K., Hoffmann, F. M., Baylies, M. K., Rushton, E. and Bate, M. (1994). *dpp* induces mesodermal gene expression in *Drosophila*. *Nature* **372**, 783-786.
Villa-Cuesta, E., de Navascués, J., Ruiz-Gómez, M., Diez del Corral, R., Domínguez, M., De Celis, J. F. and Modolell, J. (2003). *Tufted* is a gain-of-function allele that promotes ectopic expression of the proneural gene *amos* in *Drosophila*. *Genetics* **163**, 1403-1412.
Xu, T. and Rubin, G. M. (1993). Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* **117**, 1223-1237.
Zipursky, S. L., Venkatesh, T. R., Teplow, D. B. and Benzer, S. (1984). Neuronal development in the *Drosophila* retina: monoclonal antibodies as molecular probes. *Cell* **36**, 15-26.