u-shaped encodes a zinc finger protein that regulates the proneural genes *achaete* and *scute* during the formation of bristles in *Drosophila*

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The pattern of the large sensory bristles on the notum of *Drosophila* arises as a consequence of the expression of the *achaete* and *scute* genes. The gene *u-shaped* encodes a novel zinc finger that acts as a transregulator of *achaete* and *scute* in the dorsal region of the notum. Viable hypomorphic *u-shaped* mutants display additional dorsocentral and scutellar bristles that result from overexpression of *achaete* and *scute*. In contrast, overexpression of *u-shaped* causes a loss of *achaete-scute* expression and consequently a loss of dorsal bristles. The effects on the dorsocentral bristles appear to be mediated through the enhancer sequences that regulate *achaete* and *scute* at this site. The effects of *u-shaped* mutants are similar to those of a class of dominant alleles of the gene *pannier* with which they display allele-specific interactions, suggesting that the products of both genes cooperate in the regulation of *achaete* and *scute*. A study of the sites at which the dorsocentral bristles arise in mosaic *u-shaped* nota, suggests that the levels of the *u-shaped* protein are crucial for the precise positioning of the precursors of these bristles.

[Key Words: *Drosophila*; *u-shaped*; zinc finger protein; bristle pattern; *achaete*; *scute*]

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The stereotyped positions of the large sensory bristles, or macrochaetes, of the *Drosophila* imago, provide a good model system for the study of pattern formation, and the analysis of genetic variants has allowed the identification of specific genes involved in the generation of this pattern (Lindsley and Zimm 1992). Each bristle organ is unique and develops within the imaginal disc from a single precursor cell, the sensory organ mother cell. Sensory mother cells (SMCs) arise as the result of a series of sequential steps. First, the competence to become a SMC is conferred to groups of cells at defined positions that prefigure the site of each future bristle. These cells are characterized by the localized expression of the proneural genes *achaete* (*ac*) and *scute* (*sc*), two members of the *ac-sc* complex (AS-C; Ghysen and Dambly-Chaudière 1988; Campuzano and Modolell 1992). *ac* and *sc* encode transcriptional factors of the basic helix-loop-helix family first isolated in *Drosophila* whose activity confers

neural potential to the cells (Villares and Cabrera 1987; Gonzalez et al. 1989). Loss of *ac* and *sc* causes a loss of all bristles on the thorax, whereas ectopic expression of these genes results in the formation of additional or ectopic bristles (García-Bellido 1979; Balcells et al. 1988; García-Alonso and García-Bellido 1988). Neural potential is then progressively refined to a single cell, the future SMC within the proneural cluster by means of cell-cell interactions mediated by the neurogenic genes. This process is called lateral inhibition and involves an inhibitory signal from the nascent precursor that prevents the remaining cells of the cluster from becoming SMCs (for review, see Simpson 1990; Campos-Ortega and Jan 1991).

Proneural clusters arise in a precise spatial and temporal pattern that defines the positions at which each SMC and its corresponding macrochaete will arise (Romani et al. 1989; Cubas et al. 1991; Skeath and Carroll 1991). This dynamic expression pattern is controlled through the action of *cis*-regulatory sequences that modulate the expression of both *ac* and *sc* and that are scattered throughout the length of the AS-C (Ruiz-Gomez and Modolell 1987; Ruiz-Gomez and Ghysen

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1993; Gomez-Skarmeta et al. 1995). It is thought that in the imaginal epithelium, a prepattern of asymmetrically distributed factors regulate expression of *ac* and *sc* via these enhancer sequences.

Few factors regulating transcription of *ac* and *sc* have been identified, including the products of the genes *hairy*, *extramacrochaetae*, and those of the *iroquois* complex, *araucan* and *caupolican*. *Hairy* and *Extramacrochaetae* are negative regulators and encode products that belong to the family of proteins bearing helix-loop-helix motifs (Rushlow et al. 1989; Ellis et al. 1990; Garrell and Modolell 1990). *Hairy* binds to *ac* upstream regulatory sequences and acts as a transcriptional repressor (Skeath and Carroll 1991; Ohsako et al. 1994; Van Doren et al. 1994), but *Extramacrochaetae* is thought to down-regulate *ac* and *sc* function by protein association (Van Doren et al. 1991; Gomez-Skarmeta et al. 1995). The recently described *araucan* and *caupolican* proteins are transcriptional activators that belong to a novel family of homeoproteins and that regulate both sensory organ development and wing vein formation (Gomez-Skarmeta et al. 1996).

The gene *pannier* (*pnr*) is also required for the spatial regulation of *ac* and *sc* in imaginal discs and encodes a protein with two functional domains—a zinc finger domain with homology to the vertebrate transcription factor GATA-1 and a domain comprising two putative amphipathic helices (Romain et al. 1993). A number of alleles of *pnr* have been described causing either a loss of *ac-sc* expression at some sites and the corresponding loss of specific bristles, or ectopic expression of *ac-sc* and the formation of additional bristles (Heitzler et al. 1996b). A class of dominant alleles (collectively called *pnr^D*), associated with lesions in the zinc finger domain, display an overexpression of *ac* and *sc* and the development of extra thoracic bristles. Here we describe mutants of the *u-shaped* (*ush*) gene, isolated during a screen for second-site modifiers of the *pnr^D* phenotype (P. Heitzler, unpubl.). Alleles of *ush* causing a loss of function act as dominant enhancers of *pnr^D* heterozygotes, resulting in an increase in the number of ectopic thoracic bristles. On the other hand, three copies of the wild-type *ush* gene suppress the *pnr^D/+* phenotype. This suggests that *pnr* and *ush* might act in the same developmental pathway to regulate the *ac* and *sc* genes.

A morphogenetic study of *ush* mutants and the molecular cloning of this gene is described. The bristle pattern of the viable recessive alleles of *ush* is similar to that of the *pnr^D* alleles. We show that the additional bristles on the dorsal part of the thorax are associated with the overexpression of *ac* and *sc* in the corresponding proneural clusters. In contrast, ectopic expression of *ush* causes a loss of *ac-sc* expression that correlates with a loss of the corresponding dorsal bristles. *ush* encodes a novel zinc finger factor that has a role in the regulation of the spatial expression of *ac* and *sc*. The effects on the dorsocentral bristles appear to be mediated through the enhancer sequences that regulate *ac* and *sc* expression at the presumptive dorsocentral site in the thoracic imaginal disc.

Results

Allele-specific interactions between pnr and ush mutants

A number of mutant alleles at the *pnr* locus have been described including a class of dominant alleles (collectively called *pnr^D*) associated with overexpression of *ac-sc* and additional dorsocentral bristles (Romain et al. 1993). This phenotype is similar to that of viable *ush* mutants and, furthermore, both *ush* hypomorphs and *pnr^D* heterozygotes lack the postvertical bristle on the head (Heitzler et al. 1996b). The phenotype of flies heterozygous for *pnr^D* mutants is strongly modified by the dosage of *ush⁺* (Fig. 1). A single copy of *ush⁺* causes a dramatic increase in the number of dorsocentral bristles, whereas three copies suppress the *pnr^D* phenotype entirely. This genetic interaction is restricted to the *pnr^D* class of alleles, no other *pnr* mutants are sensitive to the amount of *ush* product (P. Heitzler, unpubl.).

ush is required for the pattern of bristles on the dorsal notum

Mutants in the gene *ush* and their embryonic recessive lethal phenotype were first described by Nüsslein-Volhard et al. (1984). In *ush* null embryos the germ band fails to retract and lateral fusion occurs between anterior and posterior ectoderm (R.P. Ray, unpubl.). A large number of mutant alleles of *ush* have been isolated and include hypomorphic mutants that affect the pattern of bristles on the head and thorax (P. Heitzler, unpubl.). Viable transallelic combinations of *ush* mutants display additional dorsocentral and scutellar bristles on the notum and a loss of postvertical bristles on the head (Figs. 1 and 2B). They form an allelic series for the number of dorsocentral (DC), scutellar (SC), and postvertical (PV) bristles [e.g., the number of bristles per hemithorax/head was found to be (DC) 4.59 ± 0.18 , (SC) 2.44 ± 0.09 , and (PV) 0.72 ± 0.08 for *ush^{rev3}/ush⁻*; (DC) 5.44 ± 0.25 , (SC) 2.59 ± 0.10 , and (PV) 0.53 ± 0.09 for *ush^{SW42}/ush⁻*; (DC) 6.27 ± 0.14 , (SC) 2.67 ± 0.10 , and (PV) 0.15 ± 0.06 for *ush^{rev24}/ush⁻*. Wild-type flies invariably bear two DC, two SC, and one PV).

To look at the complete loss of function of *ush* on the notum, we produced clones of cells mutant for the null alleles *ush^{VX22}*, *ush^{TgR + 1}*, and *ush^{E6}* (see Materials and Methods). All three resulted in similar phenotypes and affected the development of the dorsal half of the notum only, clones in the lateral part of the notum differentiated normally (Fig. 1). Clones extending into the scutellum fail to differentiate, generating large gaps in this region. Consequently, mutant scutellar bristles are never observed. Clones touching the dorsal midline are associated with a cleft in the thorax, whereas clones extending into the dorsocentral area are associated with absence or abnormal positioning of the dorsocentral bristles. From a study of 54 clones, the anterior dorsocentral bristle was found to be missing in 22 cases (41%), the posterior dorsocentral in 14 (26%; Fig. 2E). Nevertheless four dorsocentral bristles were of the mutant genotype showing

Figure 1. Schematic representation of the bristle patterns on the head and thorax of different mutant combinations of *ush*. The wild-type pattern is shown in *a*. In *ush* mutants, the dorsocentral bristles (DC) and the scutellar bristles (SC) of the thorax and the postvertical bristle (PV) on the head are the most frequently affected. The rest of the bristle pattern is normal. For each genotype, at least 30 hemithoraxes were examined. Bristles present > 50% of the time are represented as black dots (wild-type positions) and blue circles (ectopic bristles), those present 10%–50% of the time are represented as yellow circles, and those present <10% of the time as red dots. (*a*) ANP and PNP are anterior and posterior notopleural bristles, respectively. (*b*–*d*) Three viable combinations forming an allelic series for the number of DC and SC bristles of the thorax and the PV on the head (see text). *ush^{ev18}* is a deficiency uncovering several genes, including *ush*, and will be called *ush⁻*. An allele specific interaction exists between *ush* and *pnr^D* mutants (*e*–*g*). *pnr^D / +* flies (*f*) display additional bristles (DC and SC) and loss of PV bristles (Romain et al. 1993), a phenotype that is suppressed by three doses of *ush⁺* (*e*) or enhanced when only one copy of *ush⁺* (*g*) is present in these flies. The thoracic territories affected by the lack of *ush* function as revealed by clonal analysis (see text) are presented in *h*. The pink area denotes a territory where *ush* is required for cell viability and the green area denotes a territory where *ush* is required for the normal positioning of the DC bristles, part of this territory is also required for the fusion of the dorsal midline. When both *ac* and *sc* are nonfunctional, as in *ac³ sc¹⁰⁻¹* flies, no bristles form whether or not *ush* is present (*j* and *i*).

that *ush* is not required for the construction of these bristles (Fig. 2F). In contrast, additional dorsocentral bristles were observed in only 17 cases (31%; 5 anterior and 12 posterior). Mutant bristles, when they do form, are never found at the correct locations characteristic of the wild-type flies, but are displaced to nearby positions.

Remarkably, in almost all cases of mosaicism in the dorsocentral area, the dorsocentral bristles formed by wild-type cells were also found to be displaced from their

normal positions (Fig. 2D). Frequently, wild-type bristles are found on the mosaic border between mutant and wild-type cells. We counted 26 wild-type bristles in the dorsocentral region that were located precisely on the mosaic border. Furthermore, another 29 wild-type bristles, also displaced from their normal positions, were located a short distance away from the clone border outside the mutant territory. Therefore, there seems to be a nonautonomous effect of the mutant cells on the posi-

Figure 2. The notal bristle phenotypes seen after a loss or an excess of *ush* expression. In the dorsocentral area of wild-type animals, two DC macrochaetes form in each hemithorax (A), whereas *ush* hypomorphic loss-of-function mutants display an excess of DC macrochaetes (*ush^{rev24}/ush⁻* is shown in B). In contrast, overexpression of *ush* leads to loss of the DC bristles (*hs-ush* is shown in C). Absence of *ush* function also affects the formation of DC bristles, as revealed in clones of cells mutant for null alleles (D–F). Clones of mutant cells were generated during the larval stages (see Materials and Methods for details and complete genotypes). The mosaic border of the clones is indicated by a black line (D–F). A clone mutant for *ush^{TgR + 1}* (D) shows the formation of wild-type DC bristles that are displaced from the normal site and have formed at the borderline separating wild-type and mutant cells. In large clones encompassing the entire dorsocentral region, no DC bristles form (E). However, *ush* function is not required for the construction of bristles in this area because occasionally an additional mutant DC bristle develops (arrow in F). (G–I) Drawings of clones situated in the dorsocentral area. (○) Bristles of the wild-type genotype that have formed at ectopic positions at the borders of the mutant clones; (●) the normal positions at which these bristles should have been situated.

tion at which wild-type bristles will form. Taken together, these results suggest that the position at which the dorsocentral bristles form may be dependent on the relative levels of *ush* product.

To investigate these observations further, animals mosaic for the viable hypomorphic allele *ush^{rev24}* were generated. In these mosaics, wild-type *ush⁺* cells are juxtaposed to cells containing a reduced amount of Ush. In cases where the mosaic borderline fell within the dorsocentral territory, 30 bristles were located precisely on the clone border and nearly all of these were displaced from the normal positions for these bristles. In contrast, only five mutant dorsocentral bristles were situated within the clone itself. Unlike the null mutant clones, in *ush^{rev24}* mosaics, 25 (83%) of the bristles at the border were of the mutant genotype. We conclude that both the number and the position of dorsocentral bristles is dependent on the levels of *ush* product.

ush is expressed in the dorsal part of the thoracic imaginal disc

To determine the location of *ush* transcripts in the thoracic epithelium, we performed in situ hybridization with a digoxigenin-labeled *ush* cDNA probe (see below) in late third-instar imaginal discs. Expression of *ush* ap-

pears to be restricted to specific domains (Fig. 3D). Staining is detected in territories corresponding to the future dorsal-most region of the thorax, as well as in part of the hinge region and the posterior region of the pleura. In the hinge region, *ush* expression is found in a domain comprising the sites of appearance of the anterior notal wing process and the proximal tegula, expanding up to the border where anterior and posterior notopleural bristles develop (ANP and PNP in Fig. 1). In the dorsal part of the notum, staining covers the site of appearance of the scutellar bristles and extends to the border of the site at which the dorsocentral bristles form. Therefore, in the notum, the area of *ush* expression corresponds well with the region where *ush* is required for normal development. In contrast, no apparent phenotype has been detected in the hinge and the pleura in *ush* mutants, suggesting that activity of the gene is not essential at these sites.

The additional bristles formed in ush hypomorphic mutants result from overexpression of ac and sc

Formation of the thoracic sensory bristles is known to result from expression of the *ac* and *sc* genes and so we investigated the relationship between *ush* and *ac-sc*. As the effects of *ush* are strongest on the two dorsocentral macrochaetes, we focused our analysis on these bristles.

Figure 3. Analysis of the formation of bristle precursors in *ush* mutants and after overexpression of *ush*, and the spatial expression of *ush* transcripts. Expression of *ac* and *sc* was followed by using the enhancer trap lines *ac-lacZ* (A–C) and *sca-lacZ* (E–H), which are expressed in the proneural clusters (see text). The position of the macrochaete precursors was studied with the enhancer trap line marker A101 (D,I,J,K,L). Wing discs were dissected from late third-instar mutant and wild-type larvae. Wild-type discs are shown in A, E, and I. Arrowheads in A–C and E–G point to the DC cluster detected by both enhancer trap lines, and arrows point to the DC precursor cells in D and I–L. Many more cells express *ac-lacZ* (B) and *sca-lacZ* (F) around the dorsocentral site in *ush^{rev24/ush^{rev18}}*, and staining extends into the scutellar region where more scutellar bristles develop in this genotype. A cluster of DC precursors arises in *ush^{SW42/ush^{TGR+1}}* mutant discs (arrow in J). In contrast, when *ush* is overexpressed using either a *hs-ush* (C,G,K) or *UAS-ush* driven by the GAL4 line *pnr^{MD237}* (H,L), the reduction of *ac-lacZ* (C) and the loss of expression of *sca-lacZ* (G) at the dorsocentral site indicate a severe reduction of *ac-sc* expression in the DC proneural cluster. No A101-positive cells (arrow) are seen at the dorsocentral site in *hs-ush* (K) and *pnr^{MD237}/UAS-ush* (L) discs. However, in *hs-ush* flies, the scutellar bristles develop (see Fig. 2C) and A101 *lacZ* staining is seen in the bristle precursors (K),

although they fail to appear in the *pnr^{MD237}/UAS-ush* animals (L). (D) The in situ expression pattern of *ush* in the wing disc as revealed by digoxigenin-labeled probes. Expression is strong at the dorsal side including the scutellar territory and fades rapidly at the border of the area from which the dorsocentral bristles arise. Expression is also detected in the hinge region and in the presumptive region of the posterior pleura. The arrow points to dorsocentral precursors simultaneously labeled with A101.

Several lines of evidence suggest that the effects of *ush* are mediated by *ac* and *sc*. First, a synergism between *ac-sc* and *ush* is observed—animals heterozygous for both a deletion of *ush* and a deletion of the *AS-C* lack the posterior vertical bristles on the head (PV, see Fig. 1). Second, *ac-sc* mutants are epistatic over *ush* for the bristle phenotype—in the absence of *ac-sc* function, no sensory organs develop even in *ush* mutant flies (Fig. 1). This indicates that Ush functions before Ac and Sc. The additional macrochaetes seen in *ush* mutants may be therefore be attributable to overexpression of *ac* and *sc*.

To follow expression of *ac* and *sc*, we made use of the two transgenes *ac-lacZ* and *scabrous (sca)-lacZ* (Mlodzik et al. 1990; Martinez and Modolell 1991). The *ac-lacZ* transgene, which contains the 3.8-kb *ac* promoter region fused to the *lacZ* gene, expresses *lacZ* weakly in proneural clusters attributable to activation by the endogenous Ac and Sc proteins (Martinez and Modolell 1991; Gomez-Skarmeta et al. 1995; Fig. 3A). The *sca-lacZ* line contains a *lacZ* insert in the *sca* gene, which is

known to be a direct target for Ac and Sc. It shows a pattern of expression similar to that of *ac* and *sc* in the thoracic discs and ectopic expression of either *ac* or *sc* has been shown to induce ectopic expression of *sca* (Mlodzik et al. 1990; Fig. 3E). For one allelic mutant combination, we also looked for an antibody against Ac and observed a similar expression pattern. We have also followed development of the SMCs using A101, a *lacZ* insert in the *neuralized* gene, which is expressed in all sensory organs precursors (Fig. 3I; Boulianne et al. 1991).

In hypomorphic *ush* mutants, the excess of dorsocentral bristles correlates with the segregation of an excess of SMCs (Fig. 3J). Moreover, comparison between the dorsocentral region of wild-type and *ush* mutant discs, reveals a much larger territory of expression of *ac-lacZ* and *sca-lacZ* (Fig. 3B,F) in the mutants, which extends as far as the scutellum region. Altogether, these observations indicate that overexpression of *ac* and *sc* genes is responsible for the excess of bristles formed in the *ush* mutants.

Overexpression of *ush* leads to down-regulation of *ac* and *sc* and a loss of bristles

The domain of expression of *ush* extends from the dorsal midline of the thorax to the border of the site of formation of the dorsocentral macrochaetes. Although the number and position of these bristles is affected strongly in *ush* mutants, *ush* expression is only detectable at a low level at the site of the dorsocentral precursors themselves, suggesting that the levels of *ush* product in this area may have a determining role. To test this, we have looked at the consequences on the development of these bristles of a higher level of *ush* expression.

To overexpress *ush*, we generated transgenic flies carrying the *ush* cDNA under the control of a heat shock promotor and expressed *ush* ubiquitously during the period of time when the bristle precursors are determined. Overexpression of *ush* in imaginal thoracic discs during the third-larval stage up to early pupariation (see Materials and Methods) leads to the loss of dorsocentral bristles (Fig. 2C). The precursors for these bristles could not be detected in corresponding discs stained for A101 (Fig. 3K) and levels of *ac-sc* activity were low as shown by the loss of LacZ expression of the *ac-lacZ* and *sca-lacZ* inserts (Fig. 3C,G). Using the GAL4-upstream activating sequence (UAS) system (Brand and Perrimon 1993) we also expressed *Ush* in a large domain in the dorsal thorax corresponding to that of the *pnr* gene (Raiman et al. 1993). The GAL4 line *pnr^{MD237}* (Calleja et al. 1996; Heitzler et al. 1996b) was used to drive *UAS-ush*

expression in the dorsal half of the notum, and this resulted in a loss of *sca-lacZ* expression in the dorsocentral proneural cluster, a loss of the dorsocentral precursor cells and a subsequent loss of dorsocentral bristles (Fig. 3H,L). Therefore overexpression of *ush* in the dorsocentral territory of the notum prevents formation of the dorsocentral macrochaetes.

ush acts through the dorsocentral enhancer of *ac* and *sc*

The results presented in the preceding section suggest that *ush* may regulate the expression of *ac-sc* at the dorsocentral site. The dorsocentral proneural cluster of *ac-sc* expression is known to depend on enhancer sequences (the DC enhancer) located 4.0–9.8 kb upstream of the *ac* transcription start (Gomez-Skarmeta et al. 1995). To test whether the effects of *ush* are dependent on these sequences, we have examined the consequences of both loss of function and overexpression of *ush* on *lacZ* expression driven by the DC enhancer, using the *Dc-ac2* and *DC-3.2* lines described by Gomez-Skarmeta et al. (1995). Expression of both of these transgenes is increased in discs mutant for hypomorphic *ush* alleles and extends over a wider area when compared with wild-type controls (Fig. 4B,E). In contrast, ectopic expression of *ush* (*UAS-ush* driven by *pnr^{MD237}*) resulted in a loss of the dorsocentral cluster of *lacZ* expressing cells in thoracic discs from the *Dc-ac2* or *DC-3.2* lines (Fig. 4C,F). These

Figure 4. *ush* regulates *ac* and *sc* expression through a dorsocentral-specific enhancer element. The dorsocentral specific enhancer element drives *lacZ* expression in the dorsocentral region of the thoracic discs (Gomez-Skarmeta et al. 1995). *lacZ* staining is shown in thoracic discs with both the *DC-enhancer-sc-lacZ* (A–C) and the *DC-enhancer-ac-lacZ* (D–F). Compared with wild-type discs (A,D), *lacZ* expression detected in *ush^{SW42}/ush^{TgR+1}* mutant wing discs (B,E) in the dorsocentral area is stronger and covers a larger domain, whereas it is almost absent when *ush* is overexpressed (*pnr^{MD237}/UAS-ush*) (C,F).

observations are consistent with a regulatory role for *ush* in the expression of *ac* and *sc* in the dorsocentral proneural cluster.

Molecular cloning of the *ush* locus

To isolate the *ush* gene, we made use of the enhancer trap line 1513 (L. Seugnet and M. Haenlin, unpubl.), which behaves as a weak *ush* allele (*ush*¹⁵¹³). DNA surrounding the *ush*¹⁵¹³ P[*lacZ*] element was obtained by the plasmid rescue technique (see Materials and Meth-

ods) and the resulting fragments were used to probe cosmid and λ phage genomic libraries. The location of this DNA in the *ush* region (21C), was confirmed by hybridization to salivary gland polytene chromosomes (data not shown). Figure 5 shows a map of ~40 kb surrounding the *ush*¹⁵¹³ insertion (at position +0 kb on the map). Southern analysis revealed that two mutants generated by mobilization of the P[*lacZ*] insert, *ush*^{rev24} and *ush*^{rev18}, were each associated with deletions starting from the P insertion point and expanding distally and proximally to the centromere, respectively. In *ush*^{rev24},

Figure 5. Structure and protein sequence of the gene *ush*. (A) Genomic map of the *ush* region. Proximal is to the right. The localization of some mutant *ush* alleles corresponding to deletions (*ush*^{rev24}, *ush*^{rev18}), to an insertion of a TE element (*TE99*) and to an insertion of a P element (*ush*¹⁵¹³) or to an inversion [*In(2L)Tg^{R+1}*] is shown. Solid bars denote the direction of the deletions and uncertainty concerning the precise breakpoint is indicated by hatched boxes. The breakpoint associated with *In(2L)Tg^{R+1}* is localized between +24 and +28 kb on the map and is symbolized by a hatched box. The transposon insertion site of *ush*¹⁵¹³ is shown by an arrow (0 kb on the map). The insertion point of the TE element is localized in the region designed by a hatched box (between -2 and 0 kb on the map). The intron/exon structure seen by comparison between the cDNA and the genomic DNA is indicated. The genomic region included in the cosmid cos4 and located between +2 and +35 kb on the map, allows almost complete rescue of *ush* null alleles. The restriction sites shown are *EcoRI*, *BamHI*, *HindIII*, *NotI*, which correspond to E, B, H, and N, respectively. (B) The sequence of the Ush protein corresponding to 1191 amino acids was predicted from both genomic and cDNA pU4.3 clones. The acidic region located in the amino-terminal part of Ush is boxed. Putative zinc fingers of both CCHC and CCHH type motifs are shown in grey, the CCHC ones are also boxed.

analysis of *ush* cDNA transfected Cos cells with an antibody against Ush revealed a nuclear localization (data not shown). During embryogenesis, Ush is also localized in the nuclei of the cells in which it is expressed (data not shown). Unfortunately, because of the poor sensitivity of our immunodetection technique, we were unable to confirm this nuclear localization in the wing disc.

Discussion

The *ush* gene is required during both embryonic and imaginal development of *Drosophila*, and encodes a large protein bearing structural zinc finger motifs and an acidic region similar to a number of known transcription factors. No significant homology to known molecules present in the databases was found, but some of the zinc finger motifs of Ush show homologies with several transcription factors. However, as these proteins contain many other zinc finger motifs with no homology to those of Ush, it is difficult to assign a specific function to these motifs. Recently, a protein has been isolated from the mouse that displays an overall structure very similar to Ush (Tsang et al. 1997). This protein, FOG, like Ush, bears an acidic domain located at the amino-terminal part of the protein and nine zinc fingers arranged in two clusters. Interestingly, the protein contains four CCHH and five CCHC motifs, like Ush. Furthermore, a strong homology is found between the CCHC motifs of FOG and Ush that display more than 50% identical amino acids depending on which zinc finger motif is compared (Tsang et al. 1997). Altogether, these data indicate that FOG and Ush, although not homologous, are sufficiently similar to suggest they comprise a new family of zinc finger proteins.

The zinc finger motifs suggest a putative DNA-binding function for the Ush protein. Using immunocytochemistry we were unable to establish the presence of Ush in the nuclei of thoracic disc cells, but a nuclear localization was observed in embryos. On the basis of genetic data *ush* regulates *ac/sc* expression via specific enhancer sequences, we have since investigated the possibility that Ush may bind these sequences directly. Gel shift assays with full-length as well as truncated Ush proteins purified from *Escherichia coli* or from SF9-infected cells with baculovirus did not reveal any binding specificity for the molecule to this putative target sequence. Therefore, Ush may function together with other intermediate factors in the regulation of this potential target sequence.

Our results suggest that *ush* has three roles in the thoracic imaginal disc, one for the viability of epithelial cells in a restricted area covering the scutellum, one for the dorsal midline closure, and the other for the development of dorsocentral and scutellar bristles in a larger territory, including both the scutellum and the dorsal half of the thorax. *ush* shows a restricted pattern of expression in the thoracic disc that correlates with those areas of the notum where its function is required. No variations in intensity of expression could be detected, however, that might account for a differential require-

ment in the scutellum for cell viability. Furthermore, two other areas of *ush* expression, in the hinge and pleural regions, do not correlate with any apparent visible phenotypes in clones of null alleles covering these territories. Different functions of *ush* in the different areas of the thorax may depend on other, differentially distributed factors.

In this study, we have concentrated on the function of *ush* in the development of the macrochaetes, in particular the dorsocentral bristles.

ush regulates ac and sc expression in the dorsocentral region of the notum

Viable hypomorphic loss-of-function alleles of *ush* display additional dorsocentral bristles. In contrast, overexpression of *ush* leads to a loss of these same bristles. Our results show that the effects of Ush are mediated through expression of the *ac-sc* genes. Analysis of double mutants showed that *ac* and *sc* act after *ush* during bristle development. Observations of hypomorphic alleles revealed that the formation of ectopic dorsocentral precursors is a consequence of overexpression of *ac-sc* in the dorsocentral region. The enhanced *ac-sc* activity is reflected by the increased expression of *ac-lacZ* and *sca-lacZ*. Moreover, this effect is mediated by the dorsocentral enhancer element described by Gomez-Skarmeta et al. (1995), as, *lacZ* expression driven by these sequences is stronger in mutant *ush* discs than in the wild type. Consistent with this, overexpression of Ush leads to the loss of the dorsocentral precursor cells and a concurrent loss of *ac-sc* expression, as well as a loss of the *lacZ* expression driven by the enhancer sequences. These results are in agreement with a negative role for *ush* in the regulation of the *ac-sc* genes at the dorsocentral site.

ush and pnr participate in the same regulatory process

The *ush* protein is unlikely to regulate the *ac-sc* genes directly and may function together with other factors. A good candidate to mediate *ush* function is the product of the gene *pnr*. A number of observations support this argument. First, a specific genetic interaction exists between a class of *pnr* mutants, *pnr^D*, and *ush* mutants that act as dominant enhancers of *pnr^D*. Second, the phenotype of hypomorphic *ush* mutants are remarkably similar to that of *pnr^D/+* flies, they display additional dorsocentral and scutellar bristles but a loss of postvertical bristles. Third, both Pnr and Ush regulate *ac/sc* expression in the dorsocentral proneural cluster and both appear to do so via the dorsocentral enhancer element (Gomez-Skarmeta et al. 1995; Haenlin et al., this issue). Taken together, these data suggest that Ush and Pnr function in the same developmental pathway to regulate *ac-sc* expression at the dorsocentral site. Recent work has revealed that Pnr is a transcriptional activator, that the Ush and Pnr proteins associate with one another, and that the activating function of Pnr is lost when it is associated with Ush (Haenlin et al., this issue). Further-

more the *pnr^D* proteins have a reduced ability to associate with Ush and almost completely resistant to the modulating effects of Ush (Haenlin et al., this issue).

A role for ush in the precise positioning of the dorsocentral bristles

Clones of cells mutant for null alleles in the dorsocentral region, have a mutant phenotype showing that the mutant cells act autonomously. Unexpectedly however, the mutant patches are frequently devoid of bristles. This is in contrast to the hypomorphic alleles, which result in the differentiation of additional bristles. These observations suggest, that, although the amount of *ush* product is crucial for bristle development, bristles do not arise as a simple consequence of the presence or absence of the Ush protein. Rather, the levels of *ush* product may be involved in the precise positioning of the bristles. Two observations are relevant to this question. First, it is noteworthy, that in the wild-type disc the dorsocentral precursors arise at the very edge of the domain of detectable *ush* expression, at a site where expressing and non-expressing cells are likely to be adjacent (or where the levels of Ush may be reduced). Second, in mosaic thoraces where mutant and wild-type cells are juxtaposed, the bristles are never in the correct positions and the majority of them form precisely along the mosaic border. This observation holds whether the bristles are of the mutant or the wild-type genotype. These observations suggest strongly that bristles form at the edges of a boundary between high and low levels of Ush. We have also observed that under mild heat shock conditions, an increase of *ush* expression causes the development of additional dorsocentral bristles, at a position that would correspond to the border of the endogenous *ush* expression domain (data not shown).

There is an additional feature of the mosaics that is of importance. The position of the wild-type bristles is modified by the neighboring mutant cells, that is, the mutant cells have a nonautonomous effect on their wild-type neighbors. Therefore, in the case of the mosaics involving the null mutant clones, the majority of bristles that form are of the wild-type genotype and they are found in altered positions demonstrating that the wild-type cells have been induced to develop bristles at ectopic sites by the presence of neighboring mutant cells. Furthermore, not only do wild-type bristles form at the clone border, they are also displaced a short distance away from the border. The distance separating the marked mutant cells from the wild-type bristles is one, two, or occasionally three epidermal cells. It is of interest that similar observations have been made in mosaics of *pnr^D* mutants (P. Heitzler and P. Simpson, unpubl.). This suggests that in the absence of Ush (or in the presence of the constitutively active Pnr^D), a diffusible factor may be generated. Therefore, at a border of *ush⁻* clones, such a hypothetical factor might diffuse into the neighboring *ush⁺* cells. Interestingly, in mosaics involving hypomorphic alleles, bristles also form at the edge of the mutant clone borders, but in this case the bristles are

mainly of the mutant genotype. Production of the postulated diffusible factor may well be stronger in the case of the null mutant clones than for the clones of the hypomorphic alleles. In this context it is of note that the gene *wingless*, which encodes a diffusible protein, is expressed in a stripe along the notum adjacent to that of *ush*, and that expression of *wingless* changes in both *pnr* and *ush* mutants (Calleja et al. 1996; Y. Cubadda et al., unpubl.). It has been shown that *wingless* expression in the notum is required for the normal development of the dorsocentral bristles (Phillips and Whittle 1993). Further studies are required to determine whether the postulated diffusible factor correspond to Wingless.

In conclusion, we suggest that Ush may function to modulate locally the levels of *ac-sc* to precisely define the position of the precursor. It is known that a group of about six cells in the proneural field express *ac-sc* to high levels and are competent to make the bristle precursor (Heitzler and Simpson 1991; Cubas and Modolell 1992). The choice of which cell will do so depends in part on lateral signaling mediated by the Notch signaling pathway (Heitzler and Simpson 1991). However, the position of the precursors from the dorsocentral proneural cluster appears to be invariant (Cubas and Modolell 1992), suggesting that specific cells are predetermined to become the SMCs. Local modulation of the Ac-Sc levels could provide a bias. A cell could gain an early advantage by expressing higher levels of *ac-sc* than its neighbors and therefore be more competitive for the neural fate (Heitzler and Simpson 1991; Heitzler et al. 1996a). Further studies are necessary to determine how Pnr and Ush cooperate in the regulation of *ac* and *sc*.

Materials and methods

Fly strains

The locus *ush* was first defined genetically by the two amorphic mutants *ush⁽²⁾¹⁹* and *ush^{IIA102}* which are lethal at embryonic stages and affect the pattern of the larval cuticle (Nüsslein-Volhard et al. 1984). A large number of mutant alleles have since been isolated and a complete description of the genetics of these will be described elsewhere (P. Heitzler, R.P. Ray, Y. Cubadda, M. Haenlin, L. Seugnet, and P. Simpson, in prep.).

The transposon *TE99(Z)*, located at 21C6 and carrying two adjacent (*w⁺*, *rst⁺*) units and an internal fold-back sequence (Ising and Block 1981, 1984) was found to be the direct cause of a viable leaky *ush* mutant phenotype (*ush^{TE99(Z)}*). The *ush^{SW42}* mutant is a spontaneous white (SW) derivative of *ush^{TE99(Z)}* that exhibits a normal cytology at 21C6, but displays a strong recessive viable adult phenotype. The transposon *TE93(R)* (Ising and Block 1981, 1984) was used as an *ush⁺* duplication (P. Heitzler, unpubl.).

ush¹⁵¹³ behaves as a hypomorphic allele of *ush* and carries a P[*lacZ*, *w⁺*] transposon inserted next to the *ush* gene. Excision of the P[*lacZ*, *w⁺*] resulted in either wild-type *ush⁺* flies, or in a series of *ush* recessive loss-of-function mutants such as *ush^{rev3}*, *ush^{rev18}*, and *ush^{rev24}*.

ush^{TgR + 1}, *ush^{VX22}*, and *ush^{E6}* correspond to amorphic mutants based on their genetic behavior. They both display the same embryonic phenotype as deletions when homozygous (P. Heitzler, unpubl.). Furthermore, embryos homozygous for *ush^{TgR + 1}*, *ush^{VX22}*, or *ush^{E6}* show no detectable Ush protein

expression using a monoclonal antibody (2us1D5) directed against the *ush* product.

The *pnr^{D1}* mutant is described in Romain et al. (1993).

The transgenic line B17 (*ac-lacZ*) harbors 3.8 kb of the *ac* promoter region fused to the *lacZ* gene (for details, see Martinez and Modolell 1991).

The transgenic lines *DC-3.2* and *DC-ac2* harbor the 5.7-kb *EcoRI* fragment that contains the dorsocentral enhancer fragment fused to 3.7*sc-lacZ* (3.7 kb of the *sc* promoter region fused to the *lacZ* gene) or to 0.8*ac-lacZ* (0.8 kb of the *ac* promoter region fused to the *lacZ* gene) (for details, see Gomez-Skarmeta et al. 1995).

Flies were raised on standard *Drosophila* medium at 25°C. More than 30 hemithoraces were examined for each of the genetic combinations presented in Figure 1.

Clonal analysis

Mutant clones were produced by hs-FLP/FRT-induced mitotic recombination (Golic and Lindquist 1989; Golic 1991) following procedures described in Heitzler et al. (1996a). Clones were induced in flies of the following genotypes. (1) *y FLP¹/y; ush^{VX22} P(y⁺,ry⁺)25F ck^{CH52} FRT40A/P(πM,w⁺)21C P(πM,w⁺)36F FRT40A*; (2) *y FLP¹/y; Tg^{R+1} P(y⁺,ry⁺)25F ck^{CH52} FRT40A/P(πM,w⁺)21C P(πM,w⁺)36F FRT40A*; (3) *y FLP¹/y; Df(2L)ush^{E6} P(y⁺,ry⁺)25F ck^{CH52} FRT40A/P(πM,w⁺)21C P(πM,w⁺)36F FRT40A*; and (4) *yFLP¹/y; ush^{rev24} P(y⁺,ry⁺)25F ck^{CH52} FRT40A/P(πM,w⁺)21C P(πM,w⁺)36F FRT40A*. The *FLP¹* and *FRT40A* inserts are described in Golic (1991) and Xu and Rubin (1993), respectively.

Staining for β-galactosidase activity

Lines S123, A101, and 1513 contain a *lacZ* gene inserted at the *sca* (Mlodzik et al. 1990), *neuralized* (Boulianne et al. 1991), and *ush* (L. Seugnet and M. Haenlin, unpubl.) loci, respectively. S123 was used to follow expression in the wing imaginal discs of the *ac* and *sc* genes (Mlodzik et al. 1990). A101 exhibits staining in all sensory organs precursors (Huang et al. 1991), whereas 1513 shows a staining pattern like that of the endogenous *ush* gene (data not shown). Selection of mutant or wild-type Oregon R animals at different stages, as well as dissection and treatment of imaginal discs, were performed as described in Romain et al. (1993). The *Gla*, *Bc*, and the *TM6B Tb* balancers allowed us to recognize the genotype of the larvae, before dissection and staining for β-galactosidase activity.

Molecular cloning

To recover genomic DNA flanking the *ush¹⁵¹³* insert, genomic DNA from adult *ush¹⁵¹³* flies was extracted (Bender et al. 1983) and 1 μg digested with the appropriate restriction enzymes and ligated. Clones bearing genomic DNA flanking the bacterial origin of replication and the ampicillin resistance gene of the P(lacW) were recovered by transformation of *E. coli* strain XL1-Blue according to the standard transformation protocol of Hanahan (1985). A genomic DNA fragment, flanking the P-element insertion point, was then used as a probe to screen a cosmid library (made by J. Tamkun, University of California, Santa Cruz) and a λ phage EMBL-4 Oregon genomic DNA library (constructed by V. Pirrotta, University of Geneva, Switzerland).

Isolation of *ush* cDNAs

cDNAs were isolated from a 4 to 8-hr embryonic library (Brown and Kafatos 1988) following the same procedure as the one de-

scribed in Romain et al. (1993). The cDNAs were selected through a screen with the 12-kb DNA probes (position +20 to +32 on the map shown in Fig. 5). All cDNAs belong to the same class, with one exception that displayed an artifactual deletion. The longest one, as shown by restriction enzyme mapping as well as in vitro translation using rabbit reticulocyte lysate (Promega Biotec), was analyzed further.

DNA sequencing

Sequences were obtained using the dideoxy chain termination method as described in Romain et al. (1993). A cDNA insert of 4.7 kb (*KpnI-HindIII* fragment) was cloned in pBluescript II SK+ (Stratagene) and directional deletions were generated using the exonuclease III (Stratagene kit). Sequence data were collected for both strands using the M13 universal primer as well as internal synthetic oligonucleotides. The intron/exon structure, as well as the genomic organization of the *ush* locus, were determined first by hybridization of the cDNA on genomic sequences to localize the exons, and then by sequencing the corresponding genomic fragments using internal synthetic oligonucleotides. A search for homology was performed using the University of Wisconsin GCG software packages (Devereux et al. 1984). In vitro transcription and translation of the cDNA in rabbit reticulocyte lysates and SDS-PAGE analysis revealed a product (Ush) migrating at 200 kD, which confirmed the predicted ORF. Compared with the predicted 123 kD, the difference observed might be attributable to the presence of putative sites for glycosylation in the Ush protein sequence (Hirschberg and Snider 1987).

Expression of *ush* transcripts in situ

Digoxigenin-labeled DNA was synthesized according to the Boehringer Mannheim protocol and hybridized to imaginal discs using the method of Cubas et al. (1991), modified from Tautz and Pfeifle (1989).

Overexpression of Ush

The cDNA containing the entire *ush* ORF was subcloned in the plasmid pCaSpeR-hs (a gift from C.S. Thummel, University of Utah, Salt Lake City) and in pUAST (Brand and Perrimon 1993) using appropriate restriction sites. These plasmids were used to transform embryos of a *w¹¹¹⁸* stock and homozygous lines were established for the second and third chromosomes. *UAS-ush* lines were crossed to several lines expressing GAL4, but only crosses with the *pnr^{MD237}* Gal4-expressing line (Calleja et al. 1996; Heitzler et al. 1996b) gave viable adult progeny. Early third-instar larvae were subjected throughout two days to a heat treatment regime of 30 min at 37°C followed by 2 hr at 25°C.

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u-shaped* encodes a zinc finger protein that regulates the proneural genes *achaete* and *scute* during the formation of bristles in *Drosophila

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