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Regulation of scute function by extramacrochaete in vitro and in vivo

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SUMMARY

The pattern of adult sensilla in *Drosophila* is established by the dosage-sensitive interaction of two antagonistic groups of genes. Sensilla development is promoted by members of the *achaete-scute* complex and the *daughterless* gene whereas it is suppressed by whereas *extramacrochaete* (*emc*) and *hairy*. All these genes encode helix-loop-helix proteins. The products of the *achaete-scute* complex and *daughterless* interact to form heterodimers able to activate transcription. In this report, we show that (1) *extramacrochaete* forms heterodimers with the *achaete, scute, lethal of scute* and *daughterless* products; (2) *extramacrochaete* inhibits DNA-binding of *Achaete, Scute* and *Lethal of Scute/Daughterless* heterodimers and *Daughterless* homodimers and (3) *extramacrochaete* inhibits transcription activation by heterodimers in a yeast assay system. In addition, we have studied the expression patterns of *scute* in wild-type and *extramacrochaete* mutant imaginal discs. Expression of *scute* RNA during imaginal development occurs in groups of cells, but high levels of protein accumulate in the nuclei of only a subset of the RNA-expressing cells. The pattern is dynamic and results in a small number of protein-containing cells that correspond to sensillum precursors. *extramacrochaete* loss-of-function alleles develop extra sensilla and correspondingly display a larger number of cells with *scute* protein. These cells appear to arise from those that in the wild type already express *scute* RNA; hence, *extramacrochaete* is a repressor of *scute* function whose action may take place post-transcriptionally.

Key words: *achaete-scute*, *extramacrochaetae*, *Drosophila*, Helix-Loop-Helix, pattern formation

INTRODUCTION

The pattern of sensilla of the adult *Drosophila* has been a recurrent theme in the study of pattern formation (Osten-Sacken, 1881; Sturtevant, 1921; Stern, 1968). A set of mechanoreceptors of the notum called macrochaeta or bristles has been particularly useful in these studies. Their conspicuous morphology and invariant position has greatly simplified the analysis of genetic variants and has allowed the identification of some gene activities involved in the construction of this pattern.

The wild-type distribution of sensilla appears to be established by the activity of two groups of genes. The *achaete-scute* gene complex (AS-C) and the *daughterless* (*da*) gene form the first group. Loss-of-function alleles of these loci prevent sensilla formation, whereas gain-of-function alleles of the AS-C elicit their ectopic appearance. This suggests that these genes are required for the specification of sensilla precursors (García-Bellido and Santamaría, 1978; García-Bellido, 1979; Dambly-Chaudière et al., 1988; García-Alonso and García-Bellido, 1986).

The second group comprises *extramacrochaetae* (*emc*) and *hairy* (*h*). In contrast to the first group, loss-of-function alleles of *emc* and *h* induce the formation of ectopic sensilla, while a gain-of-function allele of *emc* causes loss of sensilla, suggesting that their activity antagonises that of the first group (Botas et al., 1982; García-Alonso and García-Bellido, 1988; Ingham et al., 1985; Ellis et al., 1990). In addition, alleles of the two groups of genes show characteristic dosage-sensitive interactions. For example, the extra bristles caused by *emc* and *h* are partially suppressed in an AS-C heterozygote and enhanced by an AS-C duplication (Moscoso del Prado and García-Bellido, 1984). These data suggest that the wild-type pattern of sensilla results from interactions amongst the two groups of genes.

Other loci involved in sensilla patterning have been reported, the most notable being the neurogenic group. However, mutant alleles of these loci affect only the number of bristles generated at each position, rather than the position itself (see Shellenbarger and Mohler, 1978; Dietrich and Campos-Ortega, 1984; Simpson and Carteret, 1989; Hartenstein and Posakony, 1990; Mlodzik et al., 1990; Heitzler and Simpson, 1991 and reviews by Simpson, 1990a,b for further discussion)

The products of these two groups of genes contain a conserved domain, term the Helix-Loop-Helix (HLH) motif (Villares and Cabrera, 1987; Alonso and Cabrera, 1988; Caudy et al., 1988; Rushlow et al., 1989; Ellis et al., 1990; Garrell and Modolell, 1990; Jarman et al., 1993). This domain contains two amphipathic helices connected by a flexible loop (Ferré-D'Amaré et al., 1993) as originally proposed by Murre et al. (1989b). HLH proteins can form both homodimers and heterodimers, mediated by hydrophobic contacts between the two

amphipathic helices (Ferree-D'Amaré et al., 1993). However, the rules that dictate the specificity for partner selection are not yet well understood (Murre et al., 1989b; Sun and Baltimore, 1991; Cabrera and Alonso, 1991).

In addition to the HLH domain, some of these proteins contain an adjacent basic motif, which is also conserved (the entire region is referred to as the bHLH motif). It has been shown that bHLH products are sequence-specific DNA-binding proteins (Murre et al., 1989a,b) and that dimerization, mediated by the HLH domain, is a requirement for the basic region to bind DNA (Davis et al., 1990; Voronova and Baltimore, 1990).

Products of the AS-C and *da* genes are of the bHLH type; they have been shown to form DNA-binding heterodimers and to activate transcription of a reporter gene in a heterologous yeast system (Murre et al., 1989b; Cabrera and Alonso, 1991; van Doren et al., 1991). The Emc protein, however, contains only the dimerisation domain and lacks the conserved basic DNA-binding region. Consequently it has been proposed that Emc inhibits the formation of the AS-C-*DA* heterodimers by binding to their dimerisation domains (Ellis et al., 1990; Benezra et al., 1990; Garrell and Modolell, 1990). In this way, *emc* would antagonise the activity of AS-C, in a manner consistent, in principle, with the the predictions of the genetic analysis. The mode of action of the *h* product remains unclear.

Support for the proposed role of *emc* was first provided by results obtained with a similar set of interactive molecules involved in mouse myogenesis. Indeed, the *emc*-like protein *ID* both disrupts MYOD/E12 DNA-binding heterodimers and inhibits MyoD-dependent expression of a reporter gene in a cell transfection assay (Benezra et al., 1990; see also Sun et al., 1991 for *ID* homologues). Recently similar results have obtained with *emc* (van Doren et al., 1991 and this paper). Here we report on the activity of the *emc* product in disrupting heterodimers of three AS-C products with *DA* in vitro and in vivo in a yeast assay system. We further show that the expression of *scute* protein in the wild type and *emc* mutants is consistent with the in vitro data and note that the expression pattern of *scute* protein differs from that of *scute* RNA.

MATERIALS AND METHODS

DNA-binding assays

The *emc* cDNA (Ellis et al., 1990) was digested with *Eco*RI and transcribed with SP6 RNA polymerase. The resulting transcripts were tranlated in vitro as described previously (Cabrera and Alonso, 1991). Quantification for competition experiments was carried out by densitometric analysis of gels loaded with aliquots of [^{35}S]methionine-labelled proteins. Other clones, protein preparation by in vitro transcription/translation and the *hb* DNA-binding probe were as described (Cabrera and Alonso, 1991).

Antibody production

The peptide DDEEILDYISLWQE (kindly synthesised by Dr R. Sheppard at the LMB), corresponding to the C terminus of the translated sequence of the *sc* gene (Villares and Cabrera, 1987) was coupled to keyhole limpet hemocyanin and used to immunise rabbits. Immune serum was affinity purified against the peptide coupled to Affigel-15 (BioRad) and preadsorbed against embryos of the stock Hw^{ua}/Hw^{ua}, which carries an allele of the *sc* gene lacking the C-terminal peptide (Villares and Cabrera, unpublished). Other details and standard procedures have been described (Cabrera, 1990; Harlow and Lane, 1988).

Epitope mapping

The epitope recognised by the antipeptide antibody was mapped with six peptide sequence variants shown in Table 2. Three of these variants include the previously described T3 (*lethal of scute*) peptide (Cabrera, 1990), the T4 (*scute*) peptide described above and the T5 (*achaete*) peptide shown in Table 2. All the peptides correspond to the C-terminal sequences of the three AS-C proteins, encompassing a highly conserved domain with homology to protein tyrosine kinase substrates (Villares and Cabrera, 1987; Alonso and Cabrera, 1988). In addition three sequence variants were synthesised (Severn Biotech Ltd) substituting the tyrosine residue by phenylalanine (Table 2). These peptides were conjugated to bovine serum albumin and bound to plastic plates (Harlow and Lane, 1988). Reaction of the affinity-purified antibody with the peptides was carried out by ELISA (Harlow and Lane, 1988).

Immunoprecipitations

The various in vitro translated proteins labelled with [^{35}S]methionine were mixed in the same buffer used for DNA-binding assays, but without DTT or DNAs, and incubated 20 minutes at 24°C. 1 μl of affinity-purified rabbit anti-LSC, anti-SC and anti-AC antibodies (Cabrera, 1990 for the *LSC* antibody; *AC* antibodies were obtained likewise with peptides from the same C-terminal conserved domain and will be described elsewhere) was then added and the reactions incubated a further 20 minutes. All reactions were processed with protein-A agarose as described before (Cabrera and Alonso, 1991).

LacZ assays in yeast cells

Fragments of the *sc* and *emc* cDNAs, carrying exclusively the coding region flanked by *Bgl*II sites, were engineered by the polymerase chain reaction (Saiki et al, 1988) and cloned in, respectively, the pRS313 (Sikorski and Hieter, 1989) and pKV701 vectors (see Cabrera and Alonso, 1991 for other references and descriptions). These constructs were used to transform yeast cells harbouring a *hunchback* UAS-*lacZ* reporter plasmid and the *da* gene in the pRS314 vector (see Cabrera and Alonso, 1991 for construct description and manipulations concerning the β-galactosidase assay).

Immunohistochemistry

The wild-type stock Canton S and the mutant emc^{M7}/emc^{M7} (provided by J. Posakony) were used as a source of imaginal discs. Third instar discs were dissected in 1× BSS (Wilcox, 1986), mounted on slides coated with 500 μg/ml polylysine and fixed with 4% formaldehyde-50 mM Pipes pH 7 for 10 minutes. The discs were blocked with PBS, 1% BSA, 0.1% Triton X-100 for 30 minutes and then incubated with an 1:50 dilution of the preadsorbed SC antibody for 30 minutes, washed once with the same solution and twice with PBS, 0.1% BSA, 2% goat serum,0.1% Triton X-100. The secondary antibody, a biotinylated goat anti-rabbit (Vector Labs), was added at 1:400 and incubated 20 minutes, washed once with the same buffer and twice with PBS, 0.1% Tween 20 for 10 minutes. For enzymatic detection, a biotinylated peroxidase-streptavidin mixture was added in the above buffer following manufacturers recommendations (Vector Labs), incubated 20 minutes and washed three times with the same buffer. Staining was carried out in the presence of diaminobenzidine with or without Co and Ni ions. Discs were dehydrated through ethanol series, cleared in methyl salicylate and mounted in Araldite.

In situ hybridisation

The protocol of Tautz and Pfeifle (1989) was used. Discs were dissected as described above, but leaving epidermal tissue attached to them to provoke sinking in the buffer solutions as suggested (Philips et al., 1990). Glutaraldehyde was included on the second fixation step as suggested by Kramer and Zipurski (cited in Mlodzik et al., 1990).

After staining discs were dissected, mounted in polylysine-coated slides, dehydrated through ethanol series and mounted in GMM (Lawrence et al., 1986). Probes were generated from 0.3 μg of T4 cDNA insert by T7 DNA polymerase-mediated incorporation of a digoxigenin-dUTP/dNTP mixture (Boehringer) for 10 minutes at 37ºC using nonamer random primers.

RESULTS

We have previously studied the DNA-binding properties of the products of three AS-C genes [defined by the transcripts T3, T4 and T5, which probably correspond to the genetically defined functions *lethal of scute* (*lsc*), *scute* (*sc*) and *achaete* (*ac*) respectively (Campuzano et al., 1985; Alonso and Cabrera, 1988)] and of the *da* gene product. We found that heterodimers of either one of these three AS-C products with *DA* bind strongly to the DNA sequence CAGGTG; however, *DA* homodimers bind weakly and combinations of the AS-C products do not bind at all (proteins are denoted by the gene name in capital characters, thus *AC*, *SC*, *LSC*, *DA* and *EMC*). We also showed that this behaviour correlated with the ability of these proteins to dimerize in the absence of DNA (Murre et al., 1989b; Cabrera and Alonso, 1991).

EMC inhibits DNA-binding of *AC, SC* and *LSC/DA* heterodimers and *DA* homodimers

We first asked whether *EMC* could interfere with the DNA-binding activity of *AC, SC* and *LSC/DA* heterodimers and *DA* homodimers. This was tested by monitoring the effect of increasing amounts of *EMC* on DNA-protein complexes by the gel retardation assay. The complexes were formed in the presence of the 22 bp *hb* probe as described (Cabrera and Alonso, 1991). The reactions were designed to have a slight excess of the *DA* to enhance the differential affinities of the three *AC, SC* and *LSC/DA* combinations (as only *AC, SC* and *LSC/DA* heterodimers and *DA* homodimers bind DNA, the amount of heterodimer binding is proportional to the concentration of *DA*, which is identical in all mixtures).

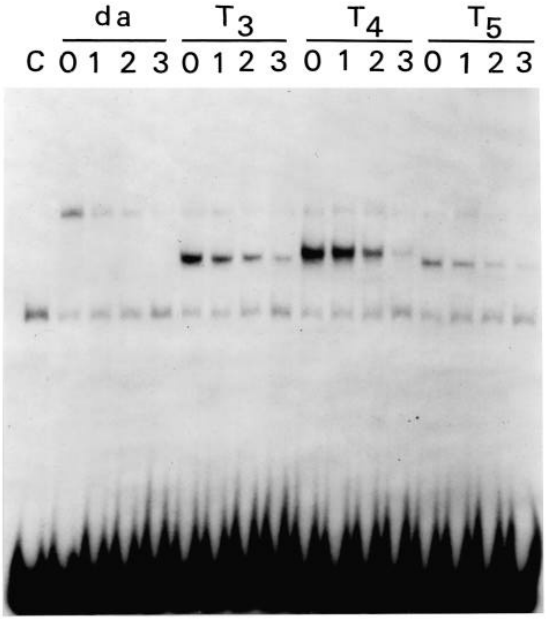

Fig. 1. Titration of *AC, SC* and *LSC/DA* DNA-binding heterodimers by *EMC*. In vitro translated *AC, SC, LSC* and *DA* were mixed with the *hb*-labelled probe and assayed in the gel retardation experiments in the absence or presence of the *EMC*. In the autoradiogram shown lane c is a control containing unprogrammed reticulocyte lysate. Other lanes are grouped as follows: da, *DA* homodimers; T3, *LSC*/*DA* heterodimers; T4, *SC*/*DA* heterodimers; T5, *AC*/*DA* heterodimers. Lanes below these groups are (0) no *EMC* added, or approximately (1) 1×, (2) 2×, (3) 4× molar excess of in vitro translated *EMC* were added. Note the progressive disappearance of the DNA-protein complexes with increasing amounts of *EMC*.

The results of these experiments show that *EMC* inhibits the DNA-binding activity of the mixtures tested in a dose-dependent fashion (see Fig. 1). In addition, these data indicate that the activity of *EMC* in this assay is greater on *SC*/*DA* than on other combinations; the data also indicate that *EMC* forms heterodimers with *DA*, as shown by the ability of the former to inhibit homodimer binding of the latter (see Fig.1).

EMC also forms heterodimers with *AC, SC* and *LSC*

The inhibitory activity of *EMC* on *AC, SC* and *LSC/DA* heterodimers could simply result from an interference with the *da* product, because combinations of *AC, SC* and *LSC* do not bind to DNA (Cabrera and Alonso, 1991). We tested these alternatives by immunoprecipitating mixtures of *AC, SC* and *LSC*/*EMC* with antibodies against the corresponding AS-C product. Co-immunoprecipitation of *EMC* was taken to indicate that heterodimers had formed.

The results of these experiments, depicted in Fig. 2, show that *EMC* co-immunoprecipitates in all the combinations tested, demonstrating its capability to form heterodimers with all the members of this interactive group. Interestingly, however, the observed stabilities of the three AS-C products with *EMC* are: *SC>AC>LSC*, in agreement with the inhibitory activity of *EMC* on DNA-binding complexes (Fig. 1). This suggests that *EMC* may break *AC, SC* and *LSC/DA* heterodimers by interfering with both components in some cases

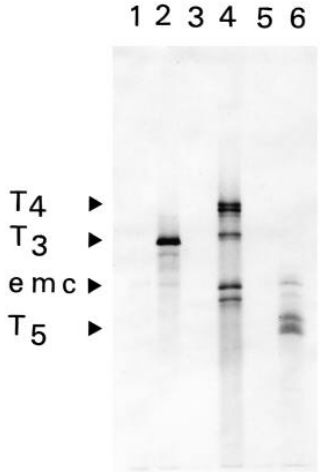

Fig. 2. *EMC* form heterodimers with the AS-C products. In vitro translated *AC, SC, LSC* and *EMC*, labelled with ^{35}S[methionine], were mixed in DNA-binding buffer without DTT or DNA and subsequently immunoprecipitated with antibodies specific for each of the AS-C proteins. Analysis of the immunoprecipitated products was carried out by SDS-PAGE. Two lanes are shown for each case, a control containing only *EMC*, to test the specificity of each antibody, and an experimental lane containing mixtures. (1) *EMC* plus *LSC* antibody; (2) *LSC*/*EMC* plus *LSC* antibody; (3) *EMC* plus *SC* antibody; (4) *SC*/*EMC* plus *SC* antibody; (5) *EMC* plus *AC* antibody; (6) *AC*/*EMC* plus *AC* antibody. The different protein bands were identified by running samples in parallel and are indicated on the left margin. (see Cabrera and Alonso, 1991 for other immunoprecipitation controls).

Table 1. Effect of *emc* on the transcriptional activity of *da*, *lsc*/*da* and *sc*/*da* in yeast

Construct	β-galactosidase units	
	Gal	Glu
da	217.0	<10.0
da+emc	54.0	<10.0
l'sc+da	2641.0	<10.0
l'sc+da+emc	955.0	<10.0
sc	<10.0	<10.0
sc+da	4024.0	<10.0
sc+da+emc	2285.0	<10.0
sc+da+emc (antisense)	4082.0	<10.0

(*SC*/*DA*) or by preferential association with one of them in others.

EMC inhibits transcription activation of *SC/DA* complexes

We have previously used a yeast assay system to show that *DA* homodimers and *LSC/DA* heterodimers were capable of activating transcription of a reporter gene (Cabrera and Alonso, 1991). This function was dependent on the presence of the CAGGTG motif recognised by these proteins in vitro and the magnitude of activation reflected the DNA-binding affinities displayed in the band shift experiments. We have now used the yeast assay system to determine the effect of *emc* on transcription activation by *DA*, *LSC/DA* and *SC/DA*.

As reported before for *LSC*, *SC* does not activate transcription on its own, *DA* does activate transcription over background but either *LSC/DA* or *SC/DA* result in a substantially higher activation level. Again in line with the band shift experiments the magnitude of *SC/DA* activation is higher than that of *LSC/DA* (see Fig. 1 and compare with Table 1; also Cabrera and Alonso, 1991).

The inclusion of *emc* in the system produces a reduction on the level of activation achieved by all the combinations described above. This reduction is consistent with the band shift experiments (see Fig. 1) and the level of inhibition varies between twofold and fourfold (see Table 1). It should be noted that *emc* is driven by a high copy plasmid and *da*, *lsc* and *sc* by a single copy ARS

We have tested the possibility that the low level of inhibition caused by *EMC* in vivo was due to the presence of an additional GAL promoter or plasmid in the assay. For this purpose, a control construct producing antisense *emc* was introduced in a *SC/DA* background and tested. Table 1 shows that this plasmid has no effect on the level of transcription activation achieved by *SC/DA*.

Characterisation of an anti-scute antibody: epitope mapping and specificity

The peptide used to raise antibodies against *SC* is a potential target for tyrosine protein kinase phosphorylation (Villares and Cabrera, 1987). Indeed, when a similar peptide was used to raise antibodies against *LSC* a post-transcriptional level of regulation was observed, suggesting that the antibody may recognise sequences including and surrounding the tyrosine residue (Cabrera, 1990). To test this point the anti-scute antibody was reacted with six sequence variants (Table 2); and it was shown that the major reactivity of the antibody occurs around the tyrosine residue but, even when this is substituted

Table 2. Epitope mapping

Name	Sequence	ELISA reactivity
BSA		19
T3	D D E E L L D Y I S S W Q E	262
T3-F	D D E E L L D F I S S W Q E	163
T4	D D E E I L D Y I S L W Q E	838
T4-F	D D E E I L D F I S L W Q E	378
T5	E D E D L L D Y I S L W Q D D	432
T5-F	E D E D L L D F I S L W Q D D	165

by phenylalanine, some activity remains (Table 2). This suggests that either the antibody recognises two epitopes with different affinity on the peptide sequence or that the epitope is not a simple linear sequence.

Finally, the specificity of the *sc* antibody was tested by staining discs derived from a stock carrying a homozygous allele of the *sc* gene (Hw^{Ua}) that lacks the C-terminal domain of the protein, and therefore the peptide used to raise antibodies (Villares and Cabrera, unpublished). As shown in Fig. 3, this material does not stain.

Expression of *scute* RNA and protein in wild-type and *emc* imaginal discs

The above data show that *EMC* functions by disrupting *AC*, *SC* and *LSC/DA* heterodimers, which are the active form of these products (Cabrera and Alonso, 1991). However, it is not clear how the ability of *emc* disrupting heterodimers relates to its genetic requirement in bristle patterning. Therefore, we set out to investigate this matter by studying the spatial expression of the AS-C during the early phases of bristle development.

RNA expression studies have been undertaken with the three members of the AS-C studied here, both during embryonic and imaginal development (Cabrera et al., 1987; Alonso and Cabrera, 1988; Cabrera, 1990; Romaní et al., 1987, 1989; Cubas et al., 1991). These studies showed that transcription of these genes occurs in small groups of cells and that the onset

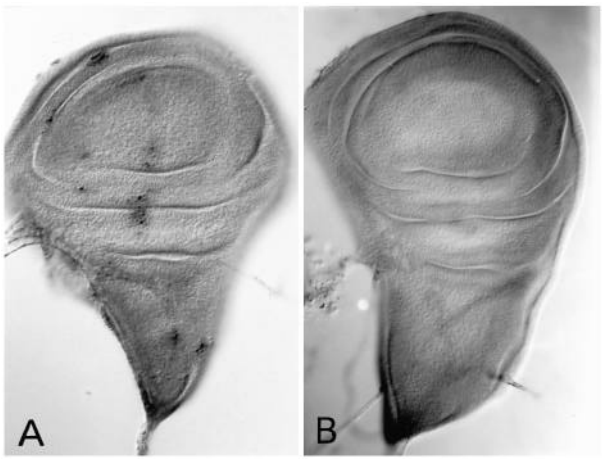

Fig. 3. Specificity of the anti-scute antibody. Wing imaginal discs of similar age stained with the anti-scute antibody. (A) Wild-type; (B) Hw^{ua} discs. Note discrete groups of cells showing specific nuclear staining in the wild type and lack of staining in the mutant that lacks the antigenic determinant to which the antibody was raised (see Materials and Methods).

of their expression precedes and parallels the segregation of sensory cell precursors.

We have studied the expression of the *sc* gene in the third instar imaginal wing disc, since (i) *sc* activity is required during the third larval instar for the development of most of the macrochaeta (as well as other sensilla) (García-Bellido and Santamaría, 1978); (ii) the macrochaeta form a highly stereotyped spatial pattern, thus facilitating the detection of changes in their position; (iii) cell commitment to macrochaeta differentiation also occurs during the third imaginal instar (García-Bellido and Merriam, 1971; Hartenstein and Posakony, 1989) and (iv) the layout of the wing disc fate map (Bryant, 1978) simplifies the study of gene expression in a spatial context.

In the wing imaginal disc, the expression of *sc* is dynamic. Fig. 4 shows aspects of the pattern of *sc* expression during the last 24 hours of the third larval instar, when the distribution of *sc* in the notum anlage is most apparent. *sc* RNA is transcribed in the wing disc by groups of cells with one exception: the presumptive precursor of the ventral sensillum of the third vein (L3-v), where a unique cell expresses *sc* RNA (Fig. 4B). All the groups detected correspond to presumptive sensilla precursor regions as defined by the wing disc fate map (Bryant, 1978). According to this comparison, each group of *sc* RNA-expressing cells gives rise to a minimum of two sensilla (again exception made of L3-v), but many of these groups will generate a much larger number (for example the tg, vR, dR and WM regions Fig. 4B). In other clusters, however, the number of sc-expressing cells is larger than the number of sensilla that will differentiate, for example the SA and SC (see Fig. 4C,D). The pattern that we observe is similar to that reported by others (Romaní et al., 1989; Cubas et al., 1991). However, we note the following differences from previous reports: (1) the signal around the wing pouch border, which extends into the posterior compartment of the disc, (2) the expression in the pleural (P) region, which together with L3-v, were previously unreported (Fig. 4B) and (3) the existence of multiple small clusters in the notum, clearly distinguishable but accumulating low levels of *sc* RNA (Fig. 4C).

When the expression of *sc* RNA and protein (*SC*) are compared, it is evident that the extent of protein expression in some areas is reduced in relation to the distribution of the RNA [in particular in the WM, wing pouch border and SA regions (compare Figs 4, 5)].

The expression of *SC* protein also shows a dynamic pattern. To demonstrate this, we have utilised the notum anlage, which produces a few well-mapped bristles. This allows a comparison between the number of staining cells and the number of bristles. Initially, the number of *SC*-accumulating cells is larger than that expected for

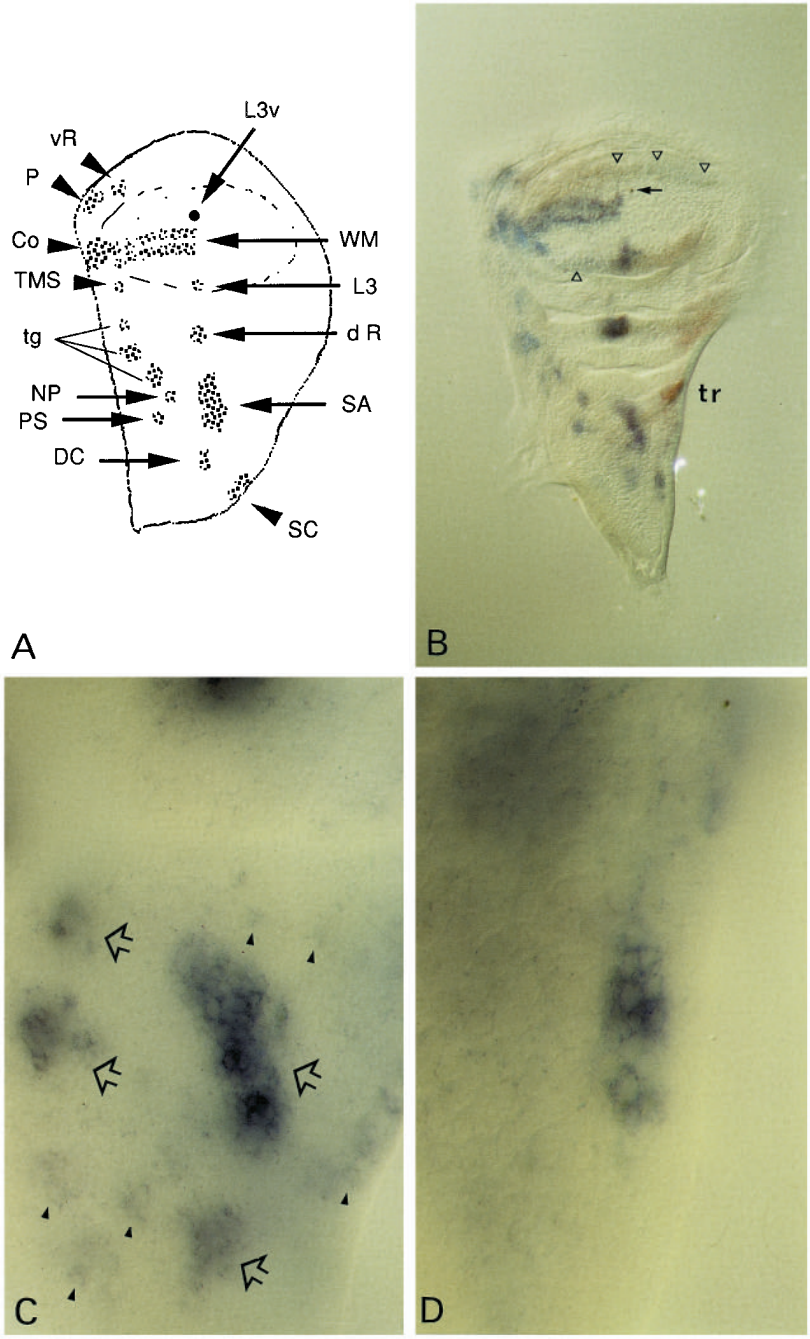

Fig. 4. Distribution of *sc* RNA in wild-type wing imaginal disc. (A) Drawing showing a wing disc approximately reproducing the *sc* RNA cell clusters in B labelled according to the fate map (Bryant, 1975) as the following sensilla positions. On the left side from top to bottom, ventral radius (vR), pleura (P), costa (CO), twin campaniform sensilla (TMS), tegula (tg), notopleurals (NP), presutural (PS) and dorsocentrals (DC). On the right side ventral and dorsal sensilla of the third vein (L3v and L3), wing margin (WM), dorsal radius (dR), supra and postalar (SA), and scutellars (SC). The wing pouch is depicted by the discontinuous elliptic trace around the wing margin. (B) In situ hybridisation with *scute* DNA probe to wild-type disc of age approximately as in Fig. 5C. Arrow points to L3v precursor and open triangles to wing pouch regions showing low level hybridisation. tr labels the trachea attachment site producing a brownish coloured background that contrasts with the deep blue of the hybridisation signal. (C) Detail of the notum region in B showing the four major *sc* RNA clusters of the notum NP, PS, SA and DC labelled with open arrows as well as several minor clusters some of which have been highlighted with filled smaller arrow heads. (D) Detail of the scutellar cluster in B.

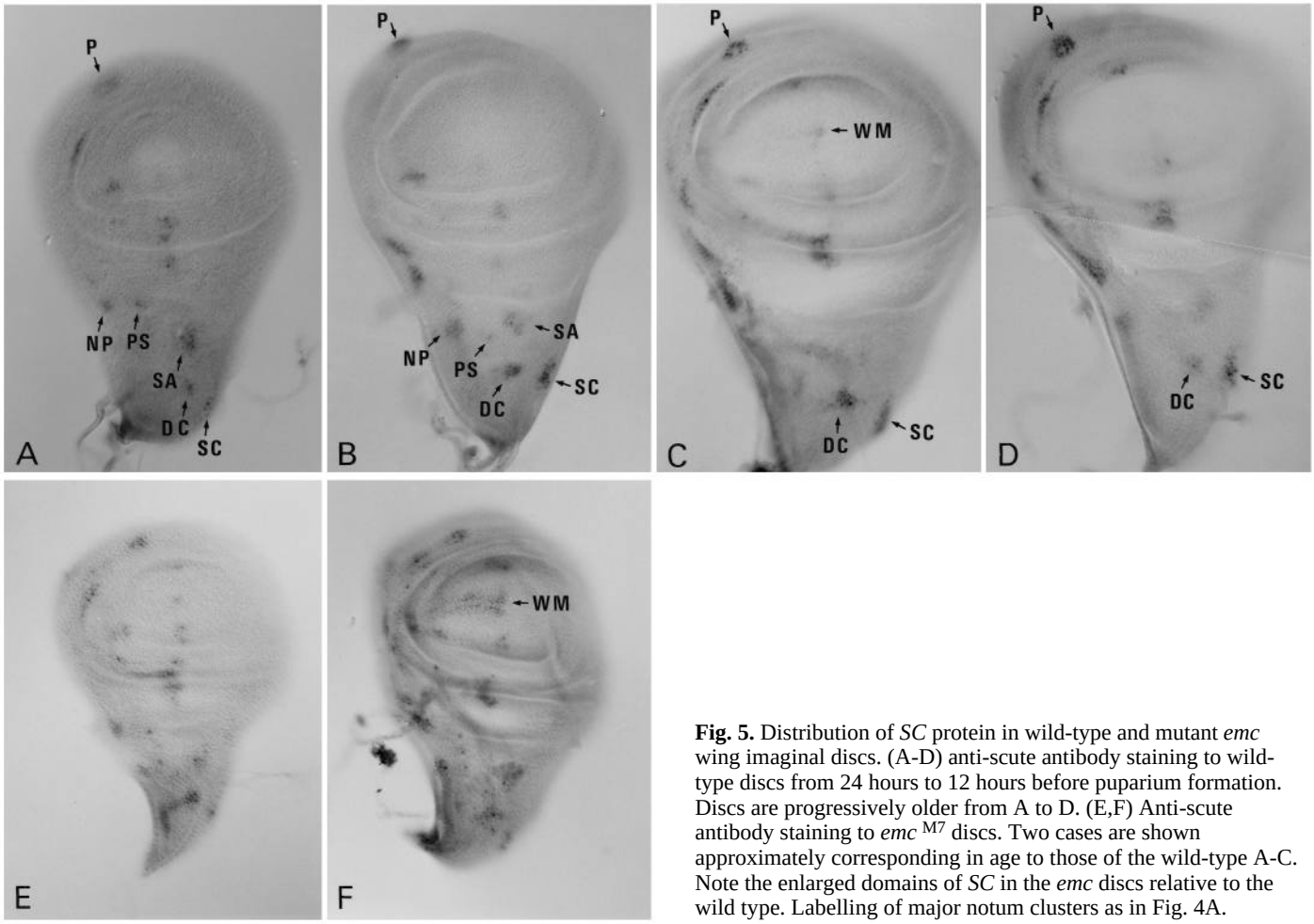

Fig. 5. Distribution of *SC* protein in wild-type and mutant *emc* wing imaginal discs. (A-D) anti-scute antibody staining to wild-type discs from 24 hours to 12 hours before puparium formation. Discs are progressively older from A to D. (E,F) Anti-scute antibody staining to *emc* ^{M7} discs. Two cases are shown approximately corresponding in age to those of the wild-type A-C. Note the enlarged domains of *SC* in the *emc* discs relative to the wild type. Labelling of major notum clusters as in Fig. 4A.

the number of bristles (Fig. 5). Subsequently these numbers diminish so that the number of cells where the protein persists longer appears to coincide with the number of bristle precursors. Eventually the staining completely fades out in late third instar discs (Fig. 5; see also Skeath and Carroll, 1991; Cubas et al., 1991).

This sequence does not occur concomitantly in all regions. For example, the SA region appears to resolve earlier than DC and SC (the four bristles developing from SA have practically resolved whereas the DC and SC regions are still highly represented by numerous *SC*-expressing cells, Fig. 4). We have also observed that the persistence of protein expression varies in different regions. In general, in the notum anlage, the protein turns over faster than in the tg, Co, vR, dR and third vein. However, in the SC region, expression is long lasting as well as exhibiting a late resolution to the final two *SC*-expressing cells (not shown). A similar evolution of the patterns of *SC* expression has been documented by others (see Skeath and Carroll, 1991; Cubas et al., 1991).

These data show that the number of *SC*-expressing cells eventually correlates with the number of sensilla that originate from each region and, therefore, we conclude that these cells are the precursors of sensory organs (see also Skeath and Carroll, 1991; Cubas et al., 1991).

The study of the expression of *SC* in *emc* mutant discs was carried out during the same stages as the wild type with a stock homozygous for the allele *emc*^{M7}. This is a strong, recessive and viable allele of *emc* (J. Posakony, personal communication), but clearly a hypomorph. However, since stronger allele combinations die as embryos and amorphic alleles are cell lethal (García-Alonso and García-Bellido, 1988), viable combinations provide appropriate material for the purpose of establishing a correlation between the extra-bristle phenotype of *emc* and the expression of the *sc* gene.

emc^{M7} discs stained with the *sc* antibody display a larger number of stained cells than the wild-type controls (Figs 5E,F, 6). Interestingly, the new cells that accumulate SC protein in emc mutant discs appear to correspond to those that in the wild-type accumulate the RNA but not the sc protein (see Figs 4B,C, 5A-D).

In conclusion, the appearance of ectopic sensilla in loss-of-function alleles of *emc* results from an expansion of the domain of *SC* expression. This expansion appears to involve those cells that in the wild type transcribe *sc* RNA but that do not engage in translation or stable accumulation of the protein.

DISCUSSION

We have shown that the *emc* product inhibits the DNA-binding activity of *AC, SC, LSC*/*DA* heterodimers and *DA* homodimers. Similar results and interpretations have also been reported by

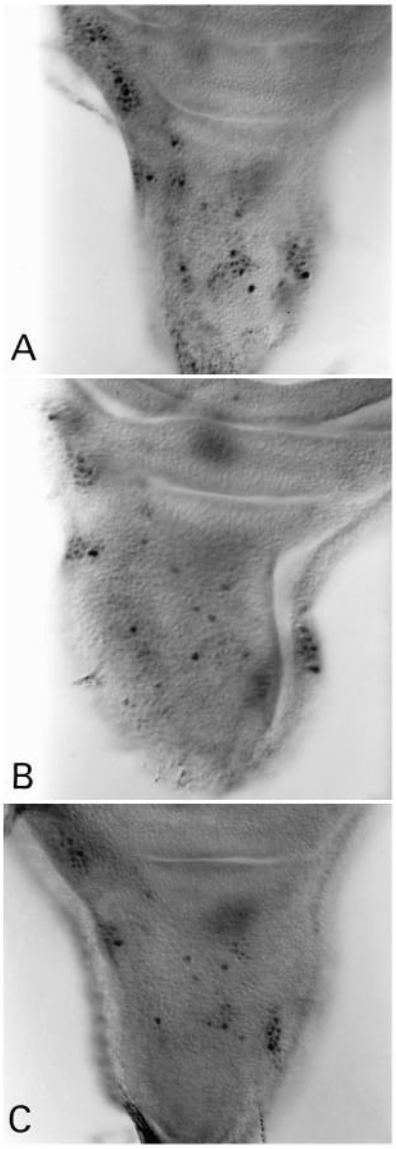

Fig. 6. Detail of *SC* protein expression in the notum anlage of mutant *emc* discs. Three progressively older discs (A-C) are shown to illustrate that similarly to the wild type, expression of *SC* in mutant *emc* discs is dynamic as well. Note how both the initial arrangement of clustered *SC*-accumulating cells as well as the number of *SC*-staining cells diminishes from A to C.

van Doren et al. (1991, 1992). This inhibition is a direct consequence of the formation of heterodimers between *EMC* and both the AS-C and *da* products. The resulting heterodimers are unable to bind DNA. Disruption of the AS-C-DA heterodimers constitutes, in effect, a mechanism of repression, because the function of these molecules as transcriptional activators requires the recognition of a specific DNA sequence (Cabrera and Alonso, 1991) and the latter requires previous dimerization (Davis et al., 1990; Voronova and Baltimore, 1990).

The inhibitory effect of *EMC* is consistent, in molecular terms, with the dosage titration analysis performed in vivo (Botas et al., 1982; Moscoso del Prado and García-Bellido, 1984). These parallels constitute evidence that *EMC* functions in vivo by sequestering AS-C and *da* products, thus rendering them inactive (van Doren et al., 1991). Similar conclusions have been reached for the function of the *Id* product of mouse (Benezra et al., 1990).

How does this suppressive function of *emc* relate to sensory organ patterning? Based on the dosage titration analysis, *emc* was envisioned as a classical repressor, controlling the spatial expression of the AS-C (Botas et al., 1982; Moscoso del Prado and García-Bellido, 1984). As the molecular nature of the genes involved became clear, it was proposed that the AS-C could be expressed in a wider region than that giving rise to sensilla in the wild type; thus a decreased *emc* activity would give raise to ectopic sensilla (Ellis et al., 1990). Nevertheless, inconsistencies with this latter view were noted, as no AS-C expression had been detected in all places where *emc* promotes sensilla development [in particular the posterior wing compartment and thoracic pleura (Romaní et al., 1989; Garrell and Modolell, 1990) but see Results and Fig. 4]. Finally, two recent studies comparing the expression of *SC* in the wild-type and *emc* mutant discs have favoured the view that the role of *emc* is to repress AS-C activation (Skeath and Carroll, 1991; Cubas et al., 1991). Notably Cubas and Modolell (1992) have concluded that *emc* refines the positioning of sensilla mother cells by reducing both the size of the proneural clusters and the number of cells within clusters that can become mother cells.

Given these conflicting views and inconsistencies, we undertook a detailed study of the expression of the *sc* gene during imaginal development. Our previous work on the expression of the *lsc* gene in embryos showed that only a subset of *lsc* RNA-expressing cells stably accumulate the protein possibly as a consequence of post-translational regulation (Cabrera, 1990). Similarly, we have shown here that *sc* RNA accumulates in all regions where sensilla develop in both the wild type and *emc* mutants but the protein is detected in only a fraction of the RNA-containing cells. This pattern of antibody staining evolves so that the protein only persists in a progressively smaller number of cells that eventually coincides with the expected number of sensory precursors.

In emc^{M7} discs, the number of cells accumulating *SC* is larger that in the wild type and this ectopic expression appears to occur within the groups of *sc* RNA-containing cells. This observation suggests that the regulation of *sc* by *emc* is post-transcriptional (see Ellis et al., 1990) and is in agreement with the disruption of heterodimers achieved by *emc* both in vivo and in vitro (see Figs 1, 2; Table 1; also van Doren et al., 1991). However, it should be noted that the pattern of *sc* RNA is so dynamic that a precise assessment of the extent of its expression at any given time is difficult to ascertain (see Fig. 1 in Cubas et al., 1991 and unpublished observations). In addition, *emc* mutations produce additional phenotypic effects. For example, clones of cells homozygous for lethal *emc* alleles produce extra veins and null alleles behave as cell lethals (García-Alonso and García-Bellido, 1988). These phenotypes suggest that *emc* may have other functions, some of which may be upstream of the process of sensilla determination.

The pattern of *SC* described shows narrower domain of staining than that obtained with a different antibody (Skeath and Carroll, 1991). The data derived from the epitope mapping experiments (Table 2) show that our antibody preferentially recognises sequences close to the tyrosine residue, which is a putative phosphorylation site. A possible explanation for the discrepancy is therefore that the antibody used here recognises only unmodified protein whereas that of Skeath and Carroll (1991) is insensitive to the state of modification. A second possibility is that the antibody used stains only those cells that accumulate high levels of the cognate epitope. Whatever the precise nature of the difference between the two antibodies, it

is likely that the restricted domain of *SC* expression observed here identifies a functionally distinct region.

What is the developmental role of *emc* in bristle pattern formation? Although our data are limited to a hypomorphic allele, stronger examples analysed in genetic mosaics elicit a larger number of sensilla (García-Alonso and García-Bellido, 1988). Since, in the allele, we have studied only a fraction of the cells of most RNA groups accumulate *SC*, it is likely that a more severe shortage of the amount of *EMC* will be reflected in a larger number of cells with the same characteristic nuclear staining. In agreement with this, we identify one role of *emc* as a regulator of the number of *sc* RNA-expressing cells that initially engage in the sensillum pathway.

Such a mechanism was previously unsuspected. Initially, the selection of sensilla precursors was believed to arise from competition amongst cells expressing *sc* RNA, mediated by the neurogenic genes (Simpson and Carteret, 1989; Heitzler and Simpson, 1991; reviewed in Simpson, 1990a) in the same way that the segregation of the neuroblasts is determined during embryonic neurogenesis (Cabrera, 1990). However, our interpretation of the present data is that the regulation of *SC* expression in the wild-type imaginal wing disc is a two-step process. In a first stage, the decision appears to be made as to where *sc* RNA would be translated. Within these areas of RNA expression, subsets of cells accumulating *SC* are found in tight groups that contain more cells than would be expected from the eventual number of bristles developed. A second stage would be the refining of these groups of *SC*-expressing cells. We and others (Skeath and Carroll, 1991; Cubas et al., 1991) have seen that these groups evolve towards smaller number of *SC*-expressing cells that ultimately coincide with the number of bristles generated from each region. We have shown that *emc* controls the number of *SC*-expressing cells at the first step; the neurogenic genes might regulate the second step. This interpretation accounts for the distinct phenotypes elicited by *emc* and the neurogenic genes: extra sensory organs, in the former, develop in new places but remain separated from each other, whereas in the latter, a tuft appears in the extant areas (see review by Simpson, 1990a). This model is, in principle, compatible with the proposal of Cubas and Modolell (1992), which proposes that troughs in *emc* levels in the disc derepress *AS-C* autoactivation. It follows that in an *emc*$^{-}$ hypomorph any decrease in *emc* function would first become effective in the vicinity of the proneural clusters, since it is these regions that correspond to the troughs.

An important corollary of the present data is the possible consequences that the interactions described here between *EMC* and AS-C/*DA* may have upon experimental interference or upon the interpretation of mutant phenotypes. Clearly the normal bristle pattern results from the direct physical interaction between these molecules, in accordance with the dosage titration analysis (Botas et al., 1982; Moscoso del Prado and García-Bellido, 1984). It is obvious that the most likely consequence of overexpressing any of the components will be a perturbation of the normal balance of the activator (AS-C/*DA* promoting bristle development) and the post-transcriptional repressor (*EMC* inhibiting it). Both *sc* overexpression in the *Hairy wing* alleles or by means of a heat-shock promoter leads to the production of extra bristles (Rodríguez et al., 1990; Balcells et al., 1988). These results should be interpreted with consideration of these interactions: does *sc* overexpression promote sensilla development because its activity as a transcriptional activator or because it titrates the *emc* repressor in those places where AS-C RNA is already present?

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