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The *achaete-scute* complex as an integrating device

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ABSTRACT A classical model to study pattern formation is provided by the epidermal sensory organs (bristles and other sensilla) that cover the body of *Drosophila*. Many of these sensory organs (SOs) arise in very constant positions. How are these positions specified? To a large extent, they are defined by the highly resolved sites of expression of the proneural genes of the *achaete-scute* complex (AS-C). These genes, which confer to cells the capacity to become SO precursors, attain their resolved patterns of expression by means of many position-specific enhancers located within the non-transcribed AS-C DNA. Each enhancer drives expression at one or very few sites. Evidence is growing that the enhancers interact with combinations of activators and repressors (prepattern) distributed in partially overlapping domains which are larger than the AS-C expressing sites. AS-C transcription is activated only at sites with appropriate combinations of factors. Thus, the AS-C integrates the positional information embodied in the relatively broad distributions of prepattern factors and creates a sharper and topographically more precise pattern.

KEY WORDS: *achaete-scute complex, pattern formation, proneural genes, nervous system, Drosophila*

Introduction

In September of 1979, one of us (J.M.) talked science with Antonio García-Bellido (AGB) for the first time. The motive was that I had been working on protein synthesis in *E. coli* for over a dozen years and wished to change into a less explored biological field. I felt that the hay-day of ribosomal function was passed. A beautiful model of how proteins were put together according to mRNA instructions had been developed. And although the ribosome was still a large black box, I thought that the next major advances would probably come from structurally-minded people, rather than from mere molecular biologists like myself. So, at some point the conversation went approximately like this:

JM: "Antonio, I would like to do molecular biology in a developmental problem in *Drosophila.*"
AGB: "Tell me, Juan, how ambitious are you?"
JM: "Very much!"
AGB: "Then, work on the *achaete-scute* system."
JM: "Fine."

And in this way, without the slightest idea of what the "*achaete-scute* system" was about, I took Antonio's advice and sealed the fate of my professional career for at least up to this day. I have not regretted it. After that conversation, Antonio gave me a reprint of his recent paper on the *achaete-scute* system (García-Bellido, 1979) and in the following days I started struggling my way through it. With my molecular biological background, however, I was incapable of making much headway. Sentences like "those (*achaete-scute*) functions are not redundant, but rather reiterative" were completely beyond me. Fortunately, some weeks later, Antonio, then director of our Center, took me into his office and on the blackboard and over two hours he enthusiastically explained the essentials of the system and I started comprehending the beauty of it and the challenge it represented. These were among the most illuminating two hours of my professional life.

The genetic view

To begin with, and for the sake of the non-Drosophilist reader, we should indicate that the *achaete-scute* system, *ac-sc* complex (AS-C) in the modern parlance, has to do, among other things, with the development of bristles. Bristles are a class of epidermal sensory organs that allow the fly to relate to the external world. Many large bristles or macrochaetae develop in very constant positions, so that each one has received a specific name. Small bristles, microchaetae, tend to appear in regular patterns covering specific areas, like the dorsal mesothorax (notum). Null *ac* and *sc* mutations, like *In(1)sc*$^{10.1}$, remove practically all bristles and other sensilla, while hypomorphic mutations remove only subsets of them. This was realized very early after the discovery of the first *ac* and *sc* mutations in the late twenties and early thirties. And what fascinated geneticists was the very complicated patterns of suppression of bristles by the different alleles and the difficulty of making much sense of the partial complementations between alleles. Early workers like Dubinin, Serebrovsky and Agol, sug-

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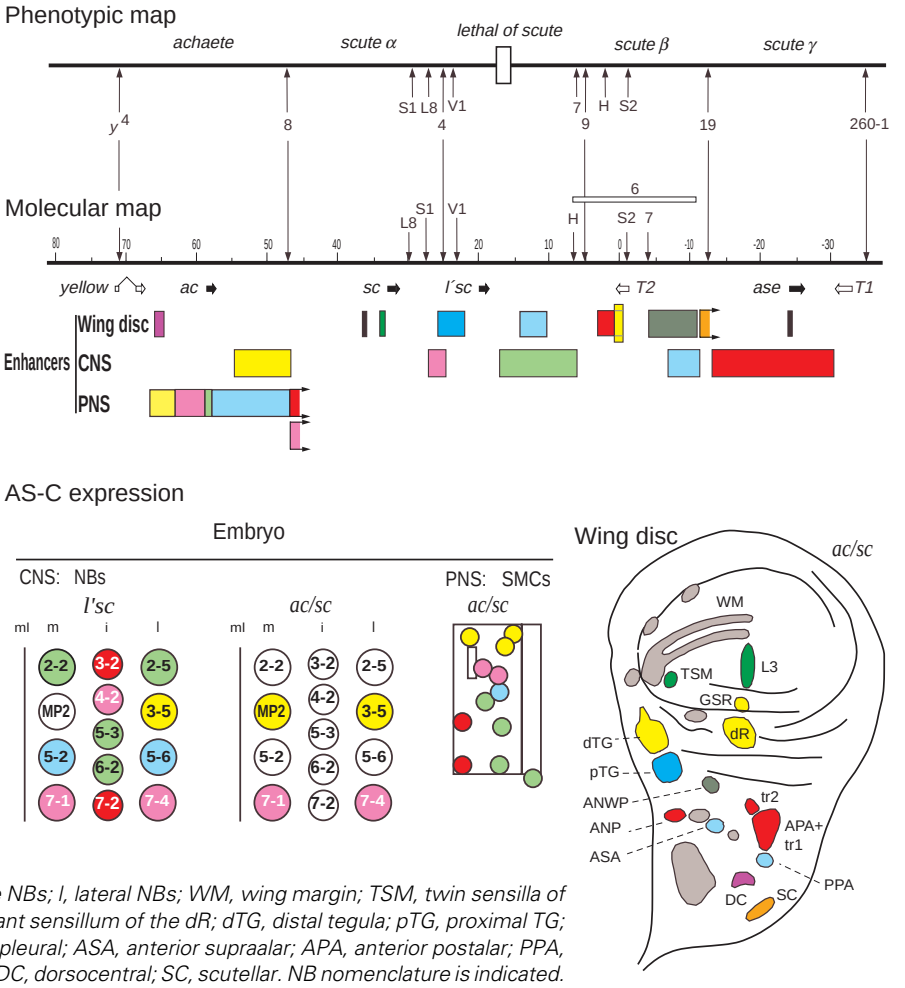

Fig. 1. Phenotypic and molecular maps of the AS-C, together with schematic representation of patterns of expression of several of its genes in embryos and wing discs. *Phenotypic map taken from (García-Bellido, 1979). Vertical arrows indicate the inferred order of the breakpoints associated with the chromosomal rearrangements used to construct the map. For simplicity, only the distinctive superindex has been indicated to name the breakpoints (so, «S1» means* sc^{S1}*). The names of the genetic regions are indicated. Rearrangements normally used to define these regions have long downwards arrows that point to the actual location of the breakpoints in the molecular map (Campuzano et al., 1985). Extent of the* sc^{6} *deletion is indicated. Coordinates on the DNA line are in kilobase pairs. Under this line, thick horizontal arrows indicate transcription units. Filled arrows correspond to proneural genes. Colored boxes indicate approximate positions on the DNA of enhancers, as determined physically (Martínez and Modolell, 1991; Gómez-Skarmeta et al., 1995; Gómez-Skarmeta et al., 1996; Culí and García, unpublished) or genetically (Skeath et al., 1992; Martín-Bermudo et al., 1993, Ruiz-Gómez and Ghysen, 1993). The colors of the enhancer boxes match the sites of expression of the corresponding gene(s) shown below for two hemisegments of germ band embryonic CNS (first wave of segregating neuroblast), one hemisegment of embryonic PNS neurogenic region, and a third instar imaginal wing disc. ac and sc are always coexpressed, so the corresponding enhancers act on both promoters. Two enhancers near the sc and ase promoters, marked as black boxes, drive expression exclusively in SMCs. Nomenclature: NBs, neuroblasts; ml, midline; m, medial NBs; i, intermediate NBs; l, lateral NBs; WM, wing margin; TSM, twin sensilla of the wing margin; L3, vein L3; dR, dorsal radius; GSR, giant sensillum of the dR; dTG, distal tegula; pTG, proximal TG; ANWP, anterior notal wing process; ANP, anterior notopleural; ASA, anterior supraalar; APA, anterior postalar; PPA, posterior postalar; tr1, tr2, sensillum trichoideum 1 or 2; DC, dorsocentral; SC, scutellar. NB nomenclature is indicated.*

gested a subdivision of the locus into subgenes, each of which would be responsible for the differentiation of specific bristles (summarized in García-Bellido, 1979).

The more modern genetic work was initiated by Muller (1955) and brought to culmination by Antonio (García-Bellido and Santamaría, 1978; García-Bellido, 1979). The patterns of chaetae removal of a large number of *ac* and *sc* mutations (the first removed mostly microchaetae and a few macrochaetae, while the second affected essentially macrochaetae) associated with chromosomal rearrangements and of their heterozygous combinations were determined. This, together with the position of the breakpoints on the polytene chromosomes, allowed the construction of a phenotypic map of the AS-C which suggested how it might be functionally organized (Fig. 1, top). From distally to proximally, there was the *ac* region, where *ac* mutations mapped, followed by a *scα* region defined by *sc* heterochromatic rearrangements. Euchromatic rearrangements were grouped in a more proximal *scβ* region. In between *scα* and *scβ* there was a region whose absence in synthetic left-right deficiencies caused embryonic death, most likely by impairing the development of the central nervous system (García-Bellido and Santamaría, 1978, Jiménez and Campos-Ortega, 1979). It was named *lethal-of-scute* (*l'sc*). The beauty of this map was that in general, the closer the breakpoints were to *l'sc*, the stronger their phenotypes were, that is the more chaetae were suppressed. Moreover, specially in *scβ*, the strength of the phenotypes could be measured in a unique series of chaetae positions affected. That is, in general when a chaeta was removed by a mutation, the preceding ones in the series were also removed. However, this series did not have an easy topographical correlate: chaetae that were near each other on the fly's body could have widely different sensitivity to the *sc* mutations. It was proposed that the AS-C appeared "as a tandem of inverse repeat of similar functions at both sides of *l'sc*" (García-Bellido, 1979). These functions would be similar ("reiterative"), in that all promoted development of chaetae, but not redundant, in that all were necessary for a wild type chaetae pattern. It was assumed that combinations of different functions were required for the differentiation of a given chaeta or, alternatively, that all chaetae required different amounts of all the functions. In the molecular-biological framework of the seventies, these observations led to suggest that bristle development might depend on a multimeric gene product made up of several related protein monomers. However, Antonio rightly pointed out to, among others, the difficulties of explaining how *cis*-coordinated transcription and/or translation could occur over many thousands of nucleotides and why complementation did not occur among different alleles, if they affected different monomers.

Moreover, it was clear that the phenotypes of alleles like the "point mutation" *sc*^{6} did not fit the phenotypic series (see below).

The molecular view

The cloning of the AS-C DNA, accomplished by our group with the conceptual and practical help of M. Meselson, W. Bender and V. Corces, opened the way to the molecular genetics of the complex (Carramolino *et al.*, 1982; Modolell *et al.*, 1983; Campuzano *et al.*, 1985), reviewed in (Campuzano and Modolell, 1992). The mapping of the molecular lesions associated with most available *ac* and *sc* mutations showed that the AS-C spanned approximately 90 kb of DNA. An excellent correlation was found between the molecular and the phenotypic map (Fig. 1). Thus, the *ac*, *scα*, *l'sc* and *scβ* regions were readily identified. A fifth region, *scγ*, was discovered by Ghysen and Dambly-Chaudière (1987), based on the patterns of suppression of larval sensory organs by AS-C partial deficiencies, and inferred by Jiménez and Campos-Ortega (1987), attending to the enhanced loss of CNS in deletions of the X chromosome extending proximally from s*cβ*. The transcription of all this DNA, however, was surprising. Only half a dozen transcription units were detected and most of them were rather small (<2.8 kb) and lacked introns (Campuzano *et al.*, 1985; Alonso and Cabrera, 1988; González *et al.*, 1989). So, they were separated by very large stretches of non-transcribed DNA (Fig. 1). Moreover, only four of these transcription units were concerned with the development of sensory organs (SOs) and the CNS. Each one of them was neatly located in a different genetic region, but none mapped within *scβ*. Three transcription units were named after the genetic regions they were located in (*ac*, *sc*, *l'sc*) and the fourth one, *asense (ase)*, after the absence of a subset of sensory organs in larvae carrying *scγ* deletions (Ghysen and Dambly-Chaudière, 1987). Of the four transcription units, only *ac* and *sc* were found to be indispensable for the development of most adult SOs. *(l'sc* was essentially not expressed in the imaginal discs, the epithelial pouches that during metamorphosis give rise to most of the adult epidermis, and *ase* was only required for a small subset of adult SOs, like the wing margin chemosensory bristles). This was shown in *In(1)sc*$^{10.1}$ mutant flies, which do not make Ac and Sc functional proteins (Campuzano *et al.*, 1985; Villares and Cabrera, 1987) and lack almost all SOs (García-Bellido, 1979). It was also surprising that all available *ac* mutations mapped upstream from the *ac* transcription unit and most *sc* mutations mapped downstream of *sc* in a region extending over 50 kb. In general, the stronger the *sc* mutations were (the more chaetae they suppressed), the closer the associated molecular lesions were to the *sc* transcription unit.

The proneural proteins

Three key discoveries were made in the succeeding years. The first one was the realization that the four transcription units encoded related proteins (Villares and Cabrera, 1987; Alonso and Cabrera, 1988; González *et al.*, 1989) and that the largest common conserved region was similar to the basic region-helix-loop-helix (bHLH) domain of known transcriptional controllers, like the mammalian MyoD and E12/E47 (Murre *et al.*, 1989). This indicated that the AS-C proteins might function by activating or repressing other genes. In fact, as heterodimers with E12/E47 or their *Drosophila* homolog *Daughterless*, they could activate transcription in a yeast model system (Cabrera and Alonso, 1991) or in *Drosophila* cells (Van Doren *et al.*, 1992). It is now believed that the AS-C proteins are essential to commit cells to a neural fate by helping implement a neural differentiation program. Moreover, their largely similar although not identical sequences within the bHLH domain suggested that the four proteins had similar and at least partially redundant functions. Indeed, many later observations based on overexpression of any of these proteins showed that they can largely replace one another and that each of them can induce development of similar SOs (Balcells *et al.*, 1988; García-Alonso and García-Bellido, 1988; Rodríguez *et al.*, 1990; Brand *et al.*, 1993, Domínguez and Campuzano, 1993; Hinz *et al.*, 1994). So, if the different products of the AS-C had similar functions, how could one explain the specificity of the AS-C mutations?

The enhancers

An answer to this question was suggested by the second key discovery. This arose when the positions within the AS-C of the breakpoints associated with more than 70 terminal deficiencies of the X chromosome were compared with the patterns of SO suppression caused by these deficiencies (Ruiz-Gómez and Modolell, 1987). Again, similar to the mutations mapping downstream of *sc*, the removal of increasing lengths of upstream *sc* DNA suppressed increasing numbers of notum and head macrochaetae. However, the seriation of chaeta affected was different from that established by the downstream mutations. On the wing, sensilla campaniformia were also differentially removed by upstream and downstream mapping mutations (Leyns *et al.*, 1989). Both observations suggested that upstream and downstream of the *sc* structural gene there were *cis*-regulatory sequences that directed expression of this gene at specific sites. In other words, the long, non-transcribed regions of AS-C DNA probably contained enhancer-like elements which promoted *sc* expression at specific sites of the imaginal epithelia (Ruiz-Gómez and Modolell, 1987; Ghysen and Dambly-Chaudière, 1988). Enhancer-like elements could easily explain «anomalous» phenotypes like that of the *sc*^{6} mutation. *sc*^{6} is associated with a deletion of ca. 17 kb of the *sc* downstream region. While all other mutations associated with chromosomal breakpoints that map within the deleted region remove the scutellar bristles, *sc*^{6} does not do so. Assuming that an enhancer that directs *sc* expression in the scutellar territory of the wing imaginal disc is located downstream of the deletion, the breakpoints will disconnect the enhancer from the *sc* promoter, but not so the deletion of 17 kb of intervening DNA (Fig. 1). Evidently, a collection of enhancers, each with a unique spatial specificity and some of them located upstream and others downstream of the *sc* gene could explain the different seriations of SO positions affected and, therefore, the specificity of the *sc* mutations. Enhancers also explained the widely different sensitivity to the *sc* mutations found for some neighboring chaetae. This was due to the very different locations of the corresponding site-specific enhancers on the AS-C DNA: the further the enhancer was from the structural gene the larger the target for chromosomal breakpoints, and consequently the more mutations would map into it and suppress the corresponding enhancer-dependent chaetae.

The proneural clusters

Obviously, this model required that the enhancers directed *sc* expression with exquisite topographical precision within the imaginal discs. Did this in fact occur? The refinement of the techniques for *in situ* hybridization, first to serial sections (Ingham *et al.*, 1985) and later to whole-mounts (Tautz and Pfeifle, 1989), allowed examination of this point and led to the third key discovery. In the imaginal discs, the *ac* and *sc* genes were indeed expressed by small, well resolved groups of cells (Romani *et al.*, 1989; Cubas *et al.*, 1991, Skeath and Carroll, 1991). The size, position, shape, and time of appearance and disappearance of these groups of *ac* and *sc* expressing cells were very reproducible (Fig. 1, bottom). Moreover, *ac-sc* expression preceded appearance, among the cells of a cluster, of the sensory mother cell (SMC), the precursor that after two differential divisions will give rise to the specialized cells that will form a SO, namely, tormogen (socket cell), trichogen (shaft cell), a neuron that projects to the CNS, and a support (glial) cell. It was further shown that, in *ac* and *sc* mutants, the absence of an specific bristle was due to the absence of *ac-sc* expression in a cluster of cells of the imaginal disc that occupied the corresponding position and, consequently, to the non-appearance of the associated SMC. Other studies showed that the spatially restricted expression of *ac-sc* was essential to generate the wild type chaetae pattern: overexpression of *sc* led to development of extra SOs in ectopic positions (Balcells *et al.*, 1988, Rodríguez *et al.*, 1990). Hence, it was clear that *ac* and *sc* conferred to cells the capacity to become SO precursors and, consequently, they were named "proneural" genes and the groups of cells expressing them "proneural clusters" (Ghysen and Dambly-Chaudière, 1989, Romani *et al.*, 1989). The term is now applied also to *l'sc*, *ase*, *da* and to genes encoding structurally related bHLH factors from *Drosophila* (Jarman *et al.*, 1993) and other organisms whose function is to commit cells to a neural developmental pathway.

The physical entity of the position-specific enhancers within the AS-C DNA (Fig. 1) has been proven by showing that relatively small fragments of this DNA direct expression of a reporter gen (bacterial *lacZ*), fused to a basal heterologous promoter *(hsp70)*, in the sites corresponding to specific proneural clusters (Martínez and Modolell, 1991; Gómez-Skarmeta *et al.*, 1995; Gómez-Skarmeta *et al.*, 1996; Culí and García, unpublished). Remarkably, within the AS-C, enhancers for most proneural clusters are unique and stimulate the expression of both *ac* and *sc* (Gómez-Skarmeta *et al.*, 1995). This is the case regardless of whether enhancers are located upstream of *ac*, between *ac* and *sc*, or downstream from *sc*. The end result is that *ac* and *sc* are coexpressed in all proneural clusters. The reason for this coexpression is unclear since most SOs develop in the presence of only *sc* (Balcells *et al.*, 1988, Rodríguez *et al.*, 1990). Still, *ac* alone, expressed at levels similar to that of the wild type, is insufficient to promote development of several macrochaetae (Gómez-Skarmeta *et al.*, 1995). Possibly, other neural precursors may have a preference for *ac*, like those for a subset of larval SOs (Ghysen and Dambly-Chaudière, 1987) or some neuroblasts (Parras *et al.*, 1996). Evolution may have solved the problem of supplying every SO with the preferred proneural protein by always providing both proteins. Clearly, our view of the AS-C has changed from a series of chaetae-specific genes envisioned by the early workers to a series of enhancers that promote the highly localized accumulation of a mixture of just two proneural proteins. The series of enhancers embody the reiterative (= similar) but not redundant (= site specific) functions proposed by Antonio. In Figure 1, we show the location of not only a few enhancers that promote expression in the wing imaginal discs, but also the genetically inferred location of some of the enhancers that direct expression in other tissues (Martín-Bermudo *et al.*, 1993; Ruiz-Gómez and Ghysen, 1993). Thus, complex patterns of proneural clusters of *l´sc* and *ac-sc* expression are observed in the embryonic neuroectoderm (Cabrera *et al.*, 1987; Romani *et al.*, 1987; Martín-Bermudo *et al.*, 1991; Skeath *et al.*, 1992; Martín-Bermudo *et al.*, 1993; Ruiz-Gómez and Ghysen, 1993) (Fig. 1). Neuroblasts rather than SMCs delaminate from these clusters and give rise to the CNS. Other clusters give rise to precursors of the larval PNS (Ruiz-Gómez and Ghysen, 1993), or to the stomatogastric nervous system (González-Gaitán and Jäckle, 1995). As shown in Figure 1, these enhancers are also scattered along most of the AS-C DNA and therefore interspersed among the wing disc enhancers (Skeath *et al.*, 1992; Martín-Bermudo *et al.*, 1993; Ruiz-Gómez and Ghysen, 1993). Near at least the *sc* and *ase* genes there are enhancers of a different type (Martínez and Modolell, 1991; Brand *et al.*, 1993; Domínguez and Campuzano, 1993): they direct expression exclusively in SMCs and provide these cells with high amounts of proneural protein. Increased accumulation of proneural proteins seems essential for proper development of SMCs into SOs (Brand *et al.*, 1993, Domínguez and Campuzano, 1993; Culí, unpublished). The *cis*-regulatory regions of the AS-C are indeed extremely complex.

The "prepattern" genes

A mature wing imaginal disc contains approximately 50.000 cells. Within it, groups of as few as 20-30 cells located in very reproducible positions express *ac* and *sc*. How is this remarkable topographic precision accomplished? The epithelium evidently contains precise positional information. This is thought to be embodied in a "prepattern" constructed by a combination of transcriptional activators and repressors distributed heterogeneously and in different landscapes (Ghysen and Dambly-Chaudière, 1988,1989) (Fig. 2). These prepattern factors would be present in domains broader than the *ac-sc* proneural clusters. *ac-sc* would be activated only at sites with combinations of prepattern factors appropriate for productive interaction with an AS-C enhancer. Since isolated enhancers direct expression at only one or very few proneural clusters, it follows that each position-specific enhancer is tuned to respond to a different combination(s) of prepattern factors. Thus, according to this model, the AS-C enhancers «read» the positional information laid down by the partially overlapping distributions of prepattern factors and activate *ac-sc* in domains that are smaller and more precisely positioned than any of the domains of the individual prepattern factors.

Although the concept of a prepattern was initially proposed by Stern in 1954 (Stern, 1954), the molecular evidence to support it has become available only recently. So far, the clearest data concern the proteins encoded in the Iroquois complex (IRO-C) (Dambly-Chaudière and Leyns, 1992; Gómez-Skarmeta *et al.*, 1996, Leyns *et al.*, 1996; McNeill *et al.*, 1997). Named Araucan (Ara), Caupolican (Caup) and Mirror (Mrr), they are highly related, putative transcription factors that belong to a new family of

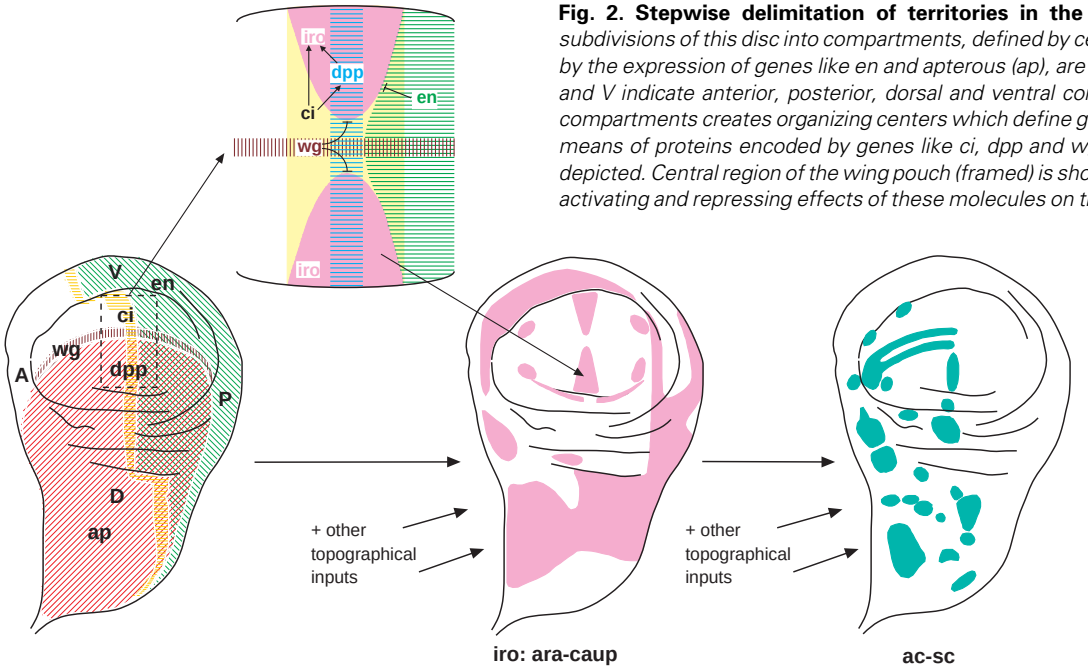

Fig. 2. Stepwise delimitation of territories in the imaginal wing disc. *The initial subdivisions of this disc into compartments, defined by cell lineage restrictions and effected by the expression of genes like en and apterous (ap), are indicated (left imaginal disc; A,P,D and V indicate anterior, posterior, dorsal and ventral compartments). The juxtaposition of compartments creates organizing centers which define growth and patterning of the disc by means of proteins encoded by genes like ci, dpp and wg, whose expression domains are depicted. Central region of the wing pouch (framed) is shown enlarged, with indication of the activating and repressing effects of these molecules on the expression of ara and caup. See text for details. Similarly, combinations of other not yet characterized factors are assumed to define the remaining domains of expression of ara and caup, two members of the «prepattern genes» (middle disc). Again, the combination of different prepattern activators and repressors, distributed in partially overlapping domains, acts on the AS-C enhancers and allows expression of ac and sc in even smaller territories (right disc). See Gómez-Skarmeta and Modolell (1996) for pertinent references.*

homeoproteins. Their role as prepattern factors has mostly been determined for Ara and Caup. Thus, in wing imaginal discs, these proteins accumulate in broad regions that cover several *ac-sc* proneural clusters (Fig. 2). The absence of both Ara and Caup at these overlapping regions remove the expression of *ac-sc* and the corresponding SOs. Moreover, as shown for Ara, this protein binds *in vitro* to the *ac-sc* enhancer that directs expression at the prospective wing vein L3 and the TSM proneural clusters, and, furthermore, the Ara binding site, which is evolutionarily conserved, is necessary for the function of this enhancer. These data indicate that Ara and Caup are direct upstream activators of *ac-sc*. Their role as prepattern factors is further supported by the observation that ectopic expression of Ara does not lead to ectopic expression of *ac-sc* in most sites of the wing disc, consistently with a requirement for additional factors to activate *ac-sc*.

Going one step higher in the genetic hierarchy, one can ask how is the relatively less resolved pattern of expression of *ara* and *caup* specified? Is there a "pre-prepattern" composed of even broader domains of expression? This seems to be the case since, at least in some domains, *ara-caup* expression is governed by a combination of inputs set up by the genes that effect the primary subdivisions of the wing disc into compartments and organize its overall patterning (Gómez-Skarmeta and Modolell, 1996). These domains consist of two symmetrical patches located one at each side of the dorso/ventral (D/V) compartment border and on the anterior territory adjacent to the anterior/posterior (A/P) compartment border (Fig. 2). They cover the territory of the prospective wing vein L3 and are necessary for the expression of *ac-sc* in the L3 proneural cluster. It has been found that *ara-caup* expression at these patches is mediated by the *Hedgehog* signal through the induction of high levels of the Gli protein Cubitus interruptus (Ci). The high levels of Ci activate *decapentaplegic (dpp)* expression and, together, Ci and Dpp, a signaling molecule of the TGF-β family, positively control *ara-caup*. The posterior border of the patches is apparently defined by repression by the homeotic protein Engrailed. The accumulation of the Wnt protein Wingless at the D/V border sets, also by repression, the gap between the two patches. Clearly, *ara* and *caup* integrate the inputs of these genes to define two smaller territories. As postulated above, these in turn should help create the even smaller domains of *achaete-scute* expression (Fig. 2). Interestingly, Ara-Caup also participate in the specification of other pattern elements like wing veins by helping define the domains of expression of the pro-vein gene *rhomboid/veinlet* (Gómez-Skarmeta *et al.*, 1996; Gómez-Skarmeta and Modolell, 1996)

Another well known transcriptional controller that also directly regulates *ac-sc* is the bHLH factor Hairy (Rushlow *et al.*, 1989). This protein acts as a repressor and prevents ectopic expression of *ac-sc* in part of the wing and notum and helps to delimit the stripes of *ac-sc* expression in leg imaginal discs (Moscoso del Prado and García-Bellido, 1984b; Carroll and Whyte, 1989; Orenic *et al.*, 1993). Hairy binds at least to a site close to the transcriptional start of *ac* and represses this gene (Ohsako *et al.*, 1994; Van Doren *et al.*, 1994). Most likely, it also represses transcription of *sc*.

Another likely candidate to regulate *ac-sc* in the dorsocentral (DC) proneural cluster of the notum is Pannier (Pnr), a zinc finger protein with homology to the vertebrate transcription factor GATA-1 (Ramain *et al.*, 1993, Winick *et al.*, 1993). In the wing imaginal disk, Pnr accumulates in a large domain comprising the dorsalmost region of the prospective notum. The DC proneural cluster is located near the edge of this domain, and *pnr* mutant alleles can either suppress or largely expand this cluster, leading to the absence of the DC bristles or to the presence of extra bristles, respectively (Ramain *et al.*, 1993; Heitzler *et al.*, 1996). Pnr seems to bind to at least one evolutionarily conserved GATA box found in the DC enhancer and this box is necessary for optimal enhancer activity (García and Ramain, unpublished).

In the embryo, the proneural clusters of *ac*, *sc* and *l'sc*, from which neuroblasts arise at specific positions and give rise to the

CNS, are arranged in patterns reiterated in different segments (Cabrera *et al.*, 1987; Romani *et al.,* 1987; Martín-Bermudo *et al.*, 1991, Skeath *et al.*, 1992; Skeath and Carroll, 1992; Ruiz-Gómez and Ghysen, 1993). The positions of several of the responsible enhancers have been inferred from genetic data (Skeath *et al.*, 1992; Martín-Bermudo *et al.,* 1993; Ruiz-Gómez and Ghysen, 1993). Here, the AS-C genes are most likely controlled by combinations of segmentation and dorso-ventral polarity genes that create an orthogonal prepattern (Martín-Bermudo *et al.*, 1991; Skeath *et al.*, 1992; Skeath and Carroll, 1994) and the *vnd* gene (Skeath *et al.*, 1994; Jiménez *et al.*, 1995). Unfortunately, neither the AS-C enhancers nor the molecular interactions between transcriptional controllers and enhancers have been characterized in detail.

In summary, although the sample of AS-C regulators so far examined is small, it certainly reinforces the idea that combinatorial prepatterns are a reality and that the ability of the AS-C enhancers to productively interact with specific combinations of factors and integrate the positional information present in prepatterns permits the expression of its genes in small and precisely located domains. This is of paramount importance to define the positioning of SOs, since mutations that induce more generalized expressions of AS-C genes promote development of extra SOs in ectopic positions (García-Bellido and Santamaría, 1978; García-Alonso and García-Bellido, 1986; Balcells *et al.*, 1988). However, as explained below, the spatial restriction of *ac-sc* expression is just part of the story.

The *extramacrochaetae (emc)* gene

Soon after the discovery of the proneural clusters, it was clear that there were additional determinants of SO positioning. Thus, in the absence of the endogenous *ac* and *sc* genes, a transient and ubiquitous accumulation of Sc protein, provided by a *sc* transgene fused to the inducible *hsp70* promoter (*HSSC*), allowed development of a few macrochaetae, which were often located in correct positions (Rodríguez *et al.*, 1990). Moreover, within some proneural clusters, SMCs appeared eccentrically and always in the same position with respect to the proneural cluster (Cubas *et al.*, 1991). These observations indicated that SOs emerge in positions that are to some extent predetermined and, therefore, that the cells at the sites where SMCs emerge are specially responsive to the neuralizing effects of the proneural proteins. A similar conclusion has been reached by studying the selection of the proneural cell that becomes a neuroblast in the embryo CNS (Seugnet *et al.*, 1997).

One agent that contributes to regulate the competence of the cells to develop SOs is the *extramacrochaetae (emc)* gene. *emc* was discovered and genetically characterized in Antonio's laboratory (Botas *et al.*, 1982; Moscoso del Prado and García-Bellido, 1984a; Moscoso del Prado and García-Bellido, 1984b; García-Alonso and García-Bellido, 1988). Its properties indicated that it was a trans-regulator of the AS-C and that it antagonized its function. Thus, by varying the relative gene doses of *emc* and *ac-sc* it was shown that increasing *emc* function suppressed chaetae and corrected the phenotypes of extra chaetae corresponding to excess *ac-sc* function. And, conversely, insufficient *emc* function promoted development of extra chaetae and normalized the phenotype of absence of chaetae associated with *sc* mutations. In the framework of the first half of the eighties, it was thought that *emc* probably encoded an *ac-sc* repressor. The molecular cloning of *emc* suggested a somewhat different mechanism (Ellis *et al.*, 1990; Garrell and Modolell, 1990), reviewed in (Garrell and Campuzano, 1991). Like the proneural genes, *emc* encoded an HLH protein, but one that lacked the basic region adjacent to the HLH domain. Thus, it was proposed that Emc would antagonize proneural function by forming heterodimers with the proneural bHLH proteins. These heterodimers would be unable to interact with DNA due to the absence of a basic domain in the Emc protein. Posterior evidence has provided strong support to this molecular mechanism (Van Doren *et al.*, 1991; Cabrera *et al.*, 1994).

Evidently, if Emc sequesters proneural proteins, proneural function should occur only at sites where there is an effective excess of the second ones over the first one. Thus, the temporal and spatial distribution of the *emc* product was analyzed (Cubas and Modolell, 1992; Van Doren *et al.*, 1992). In the wing imaginal disc, *emc* mRNA was heterogeneously distributed in a complicated and evolving pattern. Most interestingly, comparing this pattern and the sites where SMCs developed, it was clear that SMCs always emerged within minima of *emc* expression. Moreover, some proneural clusters overlapped with regions of high and low *emc* expression and SMCs emerged within the area of low *emc* expression, possibly accounting for the eccentric position of the SMC within some proneural clusters. Further evidence supporting a role of *emc* in SO positioning was obtained by manipulating the patterns of expression of *ac-sc* and *emc* (Cubas and Modolell, 1992). *Hairy-wing* mutations, which expand proneural clusters, promoted development of extra SMCs, but these still emerged within minima of *emc.* However, when *emc* hypomorphic mutations, which reduced the levels of *emc* antagonist, were introduced in *Hairy-wing* larvae, many more extra SMCs appeared and now, specially in the prospective notum, they did so in erratic and variable positions. This implied that the spatial restrictions imposed by both *ac-sc* and *emc* regulate the positions where SMCs and, consequently, SOs appear. The experiments also suggested that additional factors contribute to SO positioning since in other regions of the wing disc SMCs still preferentially appeared at certain positions, or altogether failed to appear. Clearly, *emc* is one of the factors that further refines the SO positions specified by proneural clusters and shows another level of positional information input that is integrated by the AS-C: that of restricting effective proneural function to a subset of the cells expressing the proneural genes by means of interactions with the Ac and Sc proteins.

The immediate future

We have just the first hints of how the genes of the AS-C are controlled to yield the exquisite precision of their patterns of expression, but we are still far from understanding how any proneural cluster is regulated. Still, the availability of the enhancer sequences responsible for cluster-specific expression and the identification of some factors that interact with them are certainly a cause for optimism. Interspecific comparisons between AS-C enhancers have shown that only relatively short stretches of DNA are conserved (Culí and García, unpublished). This facilitates the identification of sequences potentially important for function. Their functionality can be tested by modifying the sequences *in vitro* and assaying the activity of the altered enhancers in transformant flies. The finding of known "consensus" sequences

within the functionally important DNA should help to discern the nature of the interacting factors. The sequences can also be used as "baits" to isolate binding factors. Screens can be performed in genetically sensitized backgrounds, a method pioneered by Antonio's group (Botas *et al.,* 1982), to search for interacting mutations. We are applying this "double heterozygote" method to screen sets of small deficiencies covering the *Drosophila* chromosomes to find loci that interact with an insufficiency of the *iroquois* genes. Several candidates have been found and are in the process of being characterized. These "upwards" methods, from the AS-C to the regulating genes, will surely be complemented by «downwards» methods. AS-C regulators will be most likely found among the genetic hierarchies downstream of the genes effecting the primary subdivisions and patterning of the imaginal discs and the embryo, a very fast moving area of research. Interestingly, the genetic hierarchy *hedgehog*/*cubitus interruptus*/IRO-C/AS-C seems to be conserved in vertebrates and to be important for patterning the CNS (Lee *et al*., 1997; Gómez-Skarmeta *et al*., 1998 and references therein). As recently suggested (Arnone and Davidson, 1997), the experimental analysis of *cis*-regulatory systems, like those constituted by the AS-C enhancers and the factors inputing on them, will most likely be paramount in understanding the extremely complex regulatory networks that control development in metazoans.

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