araucan and *caupolican* provide a link between compartment subdivisions and patterning of sensory organs and veins in the *Drosophila* wing

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The homeo box prepattern genes *araucan* (*ara*) and *caupolican* (*caup*) are coexpressed near the anterior-posterior (AP) compartment border of the developing *Drosophila* wing in two symmetrical patches located one at each side of the dorsoventral (DV) compartment border. *ara-caup* expression at these patches is necessary for the specification of the prospective vein L3 and associated sensory organs through the transcriptional activation, in smaller overlapping domains, of *rhomboid/veinlet* and the proneural genes *achaete* and *scute*. We show that *ara-caup* expression at those patches is mediated by the Hedgehog signal through its induction of high levels of Cubitus interruptus (Ci) protein in anterior cells near to the AP compartment border. The high levels of Ci activate *decapentaplegic* (*dpp*) expression, and, together, Ci and Dpp positively control *ara-caup*. The posterior border of the patches is apparently defined by repression by *engrailed*. Wingless accumulation at the DV border sets, also by repression, the gap between the two patches. Thus, *ara* and *caup* integrate the inputs of genes effecting the primary subdivisions of the wing disc into compartments to define two smaller territories. These in turn help create the even smaller domains of *rhomboid/veinlet* and *achaete-scute* expression.

[Key Words: *araucan*; *caupolican*; *decapentaplegic*; *cubitus interruptus*; *Drosophila*; pattern formation]

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The adult epidermis of *Drosophila melanogaster* displays pattern elements, namely, sensory organs and veins, located in very precise positions. These positions are largely defined in the imaginal discs, the larval structures that give rise to most of the adult epidermis, by the spatially restricted domains of expression of the proneural genes *achaete* (*ac*) and *scute* (*sc*) and the provinc gene *rhomboid* (= *veinlet*) (*rho/ve*), respectively. Thus, in the pair of wing imaginal discs, which will give rise to the mesothorax and wings, *ac* and *sc* are coexpressed in relatively small groups of cells, the proneural clusters, that delimit the regions where the precursor cells of sensory organs will appear [Cubas et al. 1991; Skeath and Carroll 1991]. Similarly, *rho/ve* is expressed in thin stripes of cells that prefigure the future veins [Sturtevant et al. 1993].

The highly resolved patterns of expression of these genes require 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 (for review, see Ghysen and Dambly-Chaudière 1988, 1989). Prepattern factors should be present in domains broader than the *ac-sc* proneural clusters or the *rho/ve*

"preveins." *ac-sc* and *rho/ve* would be activated at sites with appropriate combinations of prepattern factors by means of the interaction of these transcriptional controllers with specific *cis*-regulatory regions (enhancers) of *ac-sc* and *rho/ve*.

Although little is known of the constituents of the prepattern, recently two of its components, encoded by *araucan* (*ara*) and *caupolican* (*caup*), two members of the *iroquois* gene complex (IRO-C), have been characterized [Gómez-Skarmeta et al. 1996; Leyns et al. 1996]. They are highly related, putative transcription factors that belong to a new family of homeoproteins. In wing imaginal discs, Ara and Caup accumulate in broad regions that cover several *ac-sc* proneural clusters and *rho/ve* domains. The absence of both Ara and Caup at specific regions eliminates the expression of *ac-sc* and *rho/ve* at overlapping sites. Moreover, Ara protein binds in vitro to an *ac-sc* enhancer, and the Ara binding site is necessary for the function of this enhancer. These data indicate that *ara* and *caup* are direct upstream activators of *ac-sc* and perhaps of *rho/ve*. In contrast, nothing is known of the regulation of *ara* and *caup* by upstream genes and of how their domains of expression are delimited.

In this work, we have studied the regulation of *ara* and

caup by genes that subdivide the imaginal wing disc into compartments defined by cell lineage restrictions (García-Bellido et al. 1973; Morata and Lawrence 1975; García-Bellido et al. 1976). The juxtaposition of anterior (A) and posterior (P), and dorsal (D) and ventral (V) compartments creates organization centers that are essential for growth and patterning of the imaginal discs (Meinhardt 1983; Díaz-Benjumea and Cohen 1993; Basler and Struhl 1994; Tabata and Kornberg 1994; Tabata et al. 1995; Zecca et al. 1995). Several genes are known to be important to define these compartments and to mediate cell interactions in compartment boundaries. In the case of the A and P compartments, *engrailed* (*en*), which encodes a homeo domain protein and is expressed in all P cells, is necessary to confer to these cells a posterior identity (Morata and Lawrence 1975; Kornberg et al. 1985; Tabata et al. 1995; Zecca et al. 1995). *en* directs the expression in P cells of *hedgehog* (*hh*), a gene that encodes a secreted protein that antagonizes the function of *patched* (*ptc*) in the A cells near the compartment border (Basler and Struhl 1994; Capdevila et al. 1994; Tabata and Kornberg 1994; Johnson et al. 1995; Sanicola et al. 1995; Tabata et al. 1995). *ptc* encodes a membrane-bound protein present in all A cells that mediates the repression of several target genes like *decapentaplegic* (*dpp*), *cubitus interruptus* (*ci*), and *ptc* itself (Capdevila et al. 1994; Tabata and Kornberg 1994; Johnson et al. 1995; Sanicola et al. 1995; Domínguez et al. 1996; Sánchez-Herrero et al. 1996). As a consequence of the Hh block of Ptc function, *ci* (which encodes a zinc finger protein) and *ptc* products accumulate at high levels in a stripe of anterior cells apposing the AP border (Phillips et al. 1990; Capdevila et al. 1994; Johnson et al. 1995; Motzny and Holmgren 1995; Slusarski et al. 1995; Domínguez et al. 1996; Sánchez-Herrero et al. 1996). The high levels of Ci at the AP border apparently activate the transcription of *dpp*, a gene encoding a member of the TGF- β family of signaling proteins, in an overlapping stripe (Alexandre et al. 1996; Domínguez et al. 1996; Sánchez-Herrero et al. 1996). *Dpp*, probably acting as a morphogen, promotes growth and organizes the patterning of both A and P compartments (Basler and Struhl 1994; Capdevila and Guerrero 1994; Kojima et al. 1994; Tabata and Kornberg 1994; Tabata et al. 1995; Zecca et al. 1995; Lecuit et al. 1996; Nellen et al. 1996). The cAMP-dependent protein kinase A (*pka*) is also important for the control of Ci, *ptc*, and *dpp* because loss of *pka* function in the A compartment induces ectopic accumulation of Ci protein and ectopic expression of *ptc* and *dpp* (Jiang and Struhl 1995; Johnson et al. 1995; Lepage et al. 1995; Li et al. 1995; Pan and Rubin 1995; Sánchez-Herrero et al. 1996).

In the case of the D and V compartments, whose border demarcates the wing margin, the D compartment is specified by *apterous* (*ap*), a LIM-domain homeo box encoding gene that is required in all D cells (Blair et al. 1994; Díaz-Benjumea et al. 1994). These cells also express *Serrate* (*Ser*), a transmembrane protein that may act as a D to V signal downstream of *ap* (Díaz-Benjumea and Cohen 1995; Kim et al. 1995; de Celis et al. 1996b). Similarly, *Delta* (*DI*), another transmembrane protein,

seems to signal from V to D cells (Doherty et al. 1996). Both signals act through the receptor *Notch* (*N*) (Díaz-Benjumea and Cohen 1995; Kim et al. 1995; de Celis et al. 1996b; Doherty et al. 1996) and its activation induces *vestigial* (*vg*) and *wingless* (*wg*) expression in the DV compartment border (Rulifson and Blair 1995; Kim et al. 1996). *Vg* is essential for wing cell specification and proliferation (Kim et al. 1996), and *Wg*, a member of the Wnt family of secreted proteins, has been suggested to mediate the organizing activity of the DV boundary (Díaz-Benjumea and Cohen 1995).

Near the intersection of the AP and DV borders, *ara* and *caup* are expressed in two symmetric domains separated by the DV boundary and located near or at the AP compartment border (Gómez-Skarmeta et al. 1996; Leyns et al. 1996). These domains are necessary for activation of *rho/ve* at the presumptive vein L3 and of *ac-sc* at the proneural clusters that will give rise to the sensilla campaniformia associated with this vein (Gómez-Skarmeta et al. 1996). Here we report the relation of *ara-caup* expression in these domains with several of the genes involved in establishing both the AP and DV compartments and the disc organizing centers. The results indicate that *ara-caup* link the early subdivision of the wing disc into compartments with the final patterning of veins and sensory organs.

Results

Pattern of expression of ara-caup near the AP compartment border

In the wing pouch of third instar wing discs, the IRO-C genes *ara* and *caup* are expressed in two triangular patches located near the AP compartment border and which include the presumptive vein L3 territory (Gómez-Skarmeta et al. 1996; Leyns et al. 1996; and Fig. 1A). We refer to this domain of expression as the *ara-caup* L3 patches. To define their position more precisely, they were compared with the domains of expression of *en*, a marker for the P compartment (Kornberg et al. 1985), and *ci*, whose protein product accumulates in the A compartment and most intensely in a wide stripe adjacent to the AP border (Motzny and Holmgren 1995; Slusarski et al. 1995). Figures 2A and B show that the *ara-caup* L3 patches are contained within the Ci stripe and that their posterior borders abut to the *en*-expressing cells. It is known that in third instar wing discs *en* is expressed not only in P cells, but also in A cells located very near to the compartment boundary (Blair 1992). However, in discs mutant for the Fused serine-threonine protein kinase, *en* expression is restricted to the P compartment cells (Sánchez-Herrero et al. 1996). In a *fu* mutant background, the posterior border of the L3 patches continues to appose the *en*-expressing cells (Fig. 2D), but consistently with the retraction of the *en* expression domain, the L3 patches are expanded posteriorly (Fig. 2C). These results suggest that the posterior borders of the L3 patches are delimited by repression by *en*.

Figure 1. Patterns of *caup* expression in wild type and mutant discs. All discs were hybridized with a *caup* DIG-labeled probe. In this and all other figures, discs are shown with anterior side to the left and ventral part to the top. (A) Wild-type disc. Arrowheads point to the L3 patches. Other expressions in the wing pouch correspond to the territories of vein L5 (arrows) and vein L1 (at the opposite side of L3 and symmetrically located with respect to L5). (B,C) *hh*^{Mrt} and *ptc*^{llw109}/*ptc*^{G20} discs, respectively. Note that the L3 patches (arrowheads) are expanded anteriorly concomitant with the overgrowth of the A compartment as a result of excess *hh* or insufficient *ptc* function, respectively. (D) *fu*¹/*Y*; *ptc*^{llw109}/*ptc*^{G20} disc. The expansion of the L3 patches and overgrowth of the A compartment are largely reverted.

ara-caup expression at the L3 territory depends on *hh* signaling

The overlap of the *ara-caup* L3 patches with the band of increased Ci accumulation, which depends on the Hh signal, suggests that this signal regulates *ara-caup*. This has been verified by examining *ara-caup* expression in *hh*^{Mrt} discs. In these discs, *hh* is ectopically expressed in the A compartment, and, consequently, *ptc* is antagonized in large areas of this compartment. This induces high levels of Ci and ectopic expression of *dpp* in most of the compartment; the latter induces its overgrowth (Tabata and Kornberg 1994; de Celis and Ruiz-Gómez 1995; Felsenfeld and Kennison 1995). Figure 1B shows that *hh* ectopic expression extends the triangular *caup* L3 patches, converting them into two bands. In between the two bands lies the presumptive wing margin. Its cells do not express *caup*.

We next examined whether *ara-caup* expression, similar to those of *ci* and *dpp*, was antagonized by *ptc*. Indeed, discs of a *ptc* hypomorphic combination, which ectopically express *dpp* and overaccumulate Ci in the A compartment (Capdevila et al. 1994; Sánchez-Herrero et

al. 1996), also displayed expanded bands of *caup* mRNA (Fig. 1C). Moreover, *fu* mutations that largely suppress the effects of *ptc* mutations (Sánchez-Herrero et al. 1996) reversed this expansion and reduced it to that observed in *fu* discs (Figs. 1D and 2C).

Recently it has been found that clones of A cells that lack *pka* show increased levels of Ci and Ptc and ectopically express *dpp* (Jiang and Struhl 1995; Johnson et al. 1995; Lepage et al. 1995; Li et al. 1995; Pan and Rubin 1995). We thus examined whether *pka*[−] cells also ectopically expressed *ara-caup*. Clones of cells homozygous for a *pka* null allele did ectopically express *ara-caup* in an autonomous way (Fig. 2E–G). Within the wing pouch, ectopic expression occurred in regions approximately corresponding to the two bands of ectopic expression of *ara-caup* observed in *hh*^{Mrt} and *ptc* discs (Fig. 1B,C). Moreover, similar to these discs, *pka*[−] cells in the prospective wing margin territory did not express *ara-caup* (Fig. 2E–G).

ara-caup expression requires both Ci and Dpp

All of the above mutant conditions (*hh*^{Mrt}, *ptc*, and *pka*) induce both increased accumulation of Ci and ectopic expression of *dpp*. Thus, to ascertain whether the ectopic expression of *ara-caup* requires either Ci, Dpp, or both we drove expression of *dpp* or *ci* using the Gal4 system (Brand and Perrimon 1993). We used Gal4 line 71B (Brand and Perrimon 1993), which drives expression most strongly in two broad domains centered at the vein L3 territory and therefore includes the areas of endogenous expression of *ara-caup* (Gómez-Skarmeta et al. 1996). *UAS-dpp* did not induce ectopic expression of *caup* or expanded the L3 patches (Fig. 3B). In contrast, *UAS-ci* expanded these patches anteriorly (Fig. 3C). Note that *UAS-ci* failed to activate *ara-caup* in the P compartment (Fig. 3C), probably because of repression by *en*.

UAS-dpp did not modify the endogenous accumulation of Ci (not shown), indicating that Dpp together with low levels of Ci was insufficient to activate *ara-caup*. However, *UAS-ci* strongly activated the endogenous *dpp* gene (Fig. 3D; Alexandre et al. 1996; Domínguez et al. 1996). Hence, the above experiments are compatible with a requirement for high levels of either Ci or both Ci and Dpp for *ara-caup* expression. To discriminate between these alternatives, clones doubly mutant for *pka* and *dpp* were induced. In contrast to anterior wing pouch *pka*[−] cells, which strongly expressed *ara-caup* provided that they were outside the wing margin domain (Fig. 2E–G), *pka*[−] *dpp*[−] cells expressed *ara-caup* to significantly lower levels, and this expression was generally lower the further the cells were from the endogenous domain of *dpp* expression at the AP border (Fig. 2H–J). Moreover, within the wing pouch, the anterior-most *pka*[−] *dpp*[−] cells did not detectably express *ara-caup*, although *pka*[−] cells in similar positions did express it (Figs. 2G and J, insets). Considering that Dpp may act over long distances from its source at the AP border, the results suggest that the expression of *ara-caup* at the L3

Figure 2. (A–D) The L3 patches of *ara-caup* expression are comprised within the domain of high Ci accumulation and are delimited posteriorly by *en*. Fluorescent double stainings were performed in *iro^{rF209}* (A,B), which expresses *lacZ* in the same pattern as *ara* and *caup*, or *fu¹*; *iro^{rF209}* (C,D) discs with antibodies against β -galactosidase (red) and either Ci (A,C) or En (B,D) (green). Regions of overlap appear in yellow. Only the wing pouch region of late third instar discs, as imaged by confocal microscopy, is shown. In A, the band of high accumulation of Ci is comprised between arrowheads and the *ara-caup* L3 patches are marked with asterisks. Note in C that the Ci band of high accumulation is broader than in A and that the posterior border of the L3 patches exactly matches that of the Ci stripe (arrowheads). (E–J) Mitotic recombination *pka⁻* (E–G) or *pka⁻ dpp⁻* (H–J) clones induced in *iro^{rF209}* discs. Green channel (E,H) shows the Ci accumulation, which allowed visualization of clones (some of them are marked by arrowheads). Red channel (F,I) shows *lacZ* expression, which corresponds to sites of *ara-caup* transcription. Some sites of ectopic activation of *ara-caup* in clones are indicated by arrowheads. Wild-type expression at the prospective dorsal vein L1 is marked by asterisks. Merges of both channels (G,J) show (in yellow or red-yellow) autonomous *ara-caup* (*lacZ*) activation in clones. For clones in similar regions, this activation is lower in *pka⁻ dpp⁻* than in *pka⁻ dpp⁺* clones (cf. F,G with I,J). Insets show clones anterior to the dorsal L1 domain. The *pka⁻ dpp⁺* clone (G), but not the *pka⁻ dpp⁻* clone (J; see also H, arrow) expresses *ara-caup*. Note that in *pka⁻ dpp⁺* clones, only cells outside the prospective wing margin territory, which runs horizontally across the middle of the figure, ectopically express *ara-caup* (G). Ectopic expression of *caup* in *pka⁻* cells was verified by detection of *caup* mRNA accumulation (not shown).

domains of wild-type discs requires high levels of both Ci and Dpp.

Rescue of the *ara-caup* transcription inhibited by *ptc*

We examined the effect of reduced *hh*, *ci*, and *dpp* functions on *ara-caup* expression at the vein L3 territory. We resorted to the *71ptc* line, which carries the 71B Gal4 and *UAS-ptc* transgenes in the same chromosome (Johnson et al. 1995). *ptc* overexpression in the 71B domain is equivalent to a reduced *hh* function in that accumulation of Ci and Dpp at the AP border are strongly depressed (Johnson et al. 1995). The wing pouch of *71ptc* discs had much reduced or absent *ara-caup* L3 patches (Fig. 4A, cf. 1A). Moreover, their corresponding territory was also reduced in size, probably a consequence of Dpp insufficiency (Johnson et al. 1995). The *ara-caup* L3 patches were restored by *UAS-hh* in younger discs as wild-type looking patches (Fig. 4B), and in older ones as extended stripes (Fig. 4C), and by *UAS-ci* (Fig. 4D), but not by *UAS-dpp* (Fig. 4E). As in 71B discs, *UAS-ci* induced ectopic expression of the endogenous *dpp* gene (Fig. 4F). This confirms that *dpp* by itself is insufficient

to restore *ara-caup* expression. Consistent with this observation, vein L3, which is largely absent in *71ptc* adult wings (Johnson et al. 1995), was rescued by *UAS-ci* (Fig. 4H) but not by *UAS-dpp* (not shown).

Rescue of *ara-caup* target genes inhibited by *ptc*

Consistent with the absence or very reduced expression of *ara-caup* at the L3 territory of *71ptc* discs, the *ac-sc* genes were not expressed at the vein L3 proneural clusters (Fig. 5A,B). Fig. 5C shows that *ac-sc* could still be activated in these discs because a *UAS-ara* transgene restored the clusters. As expected from its rescuing of *ara-caup* expression (Fig. 4C), *UAS-hh* did also restore these clusters (Fig. 5D). However, in this case, and because of the overgrowth of the A compartment, the clusters were transformed into long stripes of *ac-sc* expression with individual SMCs regularly spaced along the stripes. This indicates that the cells of these extended clusters are specified as vein L3 territory, and, therefore, that the extended patches of *ara-caup* expression induced by ectopic Hh (*hh^{Mrt}* or *UAS-hh*) or by *ptc* loss of function are due to the expansion of this territory.

Figure 3. Ectopic expression of *ara-caup* requires high levels of both Ci and Dpp. All discs carry the 71B Gal4 transgene. (Similar results, not shown, were obtained with Gal4 line MS1096, which promotes expression in most of the dorsal part of the wing pouch; Capdevila and Guerrero 1994). Discs carrying a *UAS-dpp* transgene and stained with a DIG-*dpp* (A) or a DIG-*caup* (B) probe show that *UAS-dpp* causes anterior and posterior overgrowth of the wing pouch. Disc in A reveals that the narrow stripe of endogenous *dpp* expression (at center of wing pouch) is overlaid by a much wider domain of *dpp* expression. Disc in B shows that the *UAS-dpp*-induced overgrowth does not essentially modify the pattern of *ara-caup* expression in the wing pouch (cf. Fig. 1A), although it decreases the accumulation of *caup*-mRNA. (C) *UAS-ci* expands anteriorly the *ara-caup* L3 domains of expression (arrowheads). Arrow points to ectopic expression in the peripodial membrane. (D) *UAS-ci* strongly activates *dpp* in the 71B domains (arrowheads). Note the lack of lateral extragrowth in the discs of C and D compared with those in A and B.

rho/ve, another *ara-caup* downstream gene necessary for vein formation (Sturtevant et al. 1993; Gómez-Skarmeta et al. 1996), was not expressed in the region corresponding to veins L3 and L4 of 71*ptc* discs (Fig. 5G,H). *UAS-ara* partially restored *rho* expression in presumptive veins L3 and L4 (Fig. 5I). As expected from its lack of rescuing of *ara-caup* activity in 71*ptc* discs, *UAS-dpp* did not restore either the L3 proneural clusters or the *rho/ve* expression in presumptive veins L3 and L4 (not shown). Moreover, in *hh^{Mrt}* discs the expression of *rho/ve* at the presumptive vein L3 is expanded anteriorly (Fig. 5F). This ectopic expression of *rho/ve* depends on *ara-caup* function because it is abolished by the *In(3)iro^{DFM2}* mutation (Fig. 5E), which specifically eliminates *ara-caup* expression in the vein L3 territory (Gómez-Skarmeta et al. 1996).

Thus, high levels of Hh, Ci, and Dpp cannot activate *rho/ve* in the absence of *ara-caup* function.

Effects of ectopic Dpp on disc growth

Ectopic Dpp accumulation promoted by *UAS-dpp* in both 71B and 71*ptc* discs caused a large overgrowth of the anterior- and posterior-most regions of the wing pouch (Figs. 3A,B and 4E). However, the central region of the wing pouch, as visualized by *caup* expression, was not substantially modified. In contrast, *UAS-ci*, which also induced ectopic *dpp* expression (Figs. 3D and 4F), promoted extragrowth of the central region of the wing pouch in 71B discs (as seen by an increase in the folds of the epithelium; Figs. 3C,D) and it did recover the size of this region in 71*ptc* discs (Fig. 4D,F). In both cases there was no detectable overgrowth of the anterior- and posterior-most territories of the wing pouch.

ara-caup are antagonized by *wg* at the prospective wing margin

In third instar wing discs *wg* is expressed within a narrow stripe of cells that straddles the DV compartment boundary and corresponds to the prospective wing margin (Phillips and Whittle 1993; Couso et al. 1994). However, the Wg protein can be detected in a somewhat wider stripe as a result of diffusion. The D and V *ara-caup* L3 patches are separated by a gap that corresponds to the cells that detectably accumulate Wg (Fig. 6A). This suggests that Wg may repress *ara-caup* and set the width of the gap. To test this, we examined *ara-caup* expression in clones of cells mutant for both *pka* (to visualize the clone by overaccumulation of Ci) and *wg*. Clones spanning the gap between the L3 patches extended these patches toward the DV border and a narrow gap of only one or two cell diameters remained (Fig. 6C). The same narrow gap was observed in clones straddling the DV boundary in other regions of the A compartment (Fig. 6D). Conversely, in discs carrying *N* gain of function mutations that specifically derepress *wg* in most of the D and part of the V wing pouch (de Celis et al. 1996b), *ara-caup* was almost absent in this territory (Fig. 6B). Taken together, these results indicate that *wg* represses *ara-caup* expression at the prospective wing margin domain.

Discussion

Control of *ara-caup* at the compartment borders

The IRO-C genes *ara* and *caup* are expressed near the AP compartment border in two triangular patches placed symmetrically with respect to the DV axis and separated by a gap that includes the prospective wing margin. These patches overlap with the presumptive vein L3 territory and are comprised within the stripe of cells that display an increased Ci accumulation. It has been proposed that the increased levels of Ci depend on Hh sig-

Figure 4. Rescue of the *71ptc* phenotype. Discs in A–E were hybridized with a *caup*–DIG probe and that in F with a *dpp*–DIG probe. (A) *71ptc/+* disc that almost completely lacks the ventral L3 patch (arrowhead) and totally lacks the dorsal patch. Note the reduction in the size of the wing pouch, as compared with that of a wild-type disc (Fig. 1A). (B) Young third instar *71ptc/UAS–hh* disc. The L3 patches (arrowheads) are restored. The disc is anteriorly overgrown. (C) Late third instar disc of the same genotype. The L3 patches are expanded (arrowheads). (D) *71ptc/UAS–ci* disc. The L3 patches (arrowheads) and the size of the wing pouch are largely recovered. (E) *71ptc/UAS–dpp* disc. Although the anterior- and posterior-most parts of the wing pouch are expanded, the L3 patches are not recovered (cf. A). (F) *71ptc/UAS–ci* disc. *dpp* is ectopically expressed at the two broad domains of ectopic Ci accumulation (arrowheads). (G) Wild-type wing. The nomenclature of longitudinal veins is indicated. (H) Wing from a *71ptc/UAS–ci* scaper. Note the accumulation of vein material between veins L3 and L5.

naling from posterior cells (Alexandre et al. 1996; Domínguez et al. 1996; Sánchez-Herrero et al. 1996) that antagonize *ptc* function. Our results indicate that *ara-caup* expression also depends on this signal. Indeed, overexpression of *hh* or reduction of *ptc* function expand anteriorly the *ara-caup* L3 domains; conversely, overexpression of *ptc*, which antagonizes this signal, sharply reduces or abolishes these domains. Taken together, these data indicate that, in this territory, *ara* and *caup* are controlled similarly to other gene targets of Hh signaling, like *ci*, *dpp*, and *ptc* (Basler and Struhl 1994; Capdevila et al. 1994; Capdevila and Guerrero 1994; Tabata and Kornberg 1994; Johnson et al. 1995; Sanicola et al. 1995; Tabata et al. 1995; Zecca et al. 1995; Domínguez et al. 1996; Sánchez-Herrero et al. 1996).

The overlap of the stripe of high Ci accumulation with the *ara-caup* L3 domain and the fact that all mutant cells (*ptc*, *hh^{Mrt}*, and *pka*) that ectopically express *ara-caup* also accumulate high levels of Ci and Dpp suggest that these gene products regulate *ara* and *caup*. Indeed, we have found that both Ci and Dpp are necessary for *ara-caup* activation. Thus, ectopic expression of *ci*, which promotes *dpp* expression, but not that of *dpp*,

which does not promote Ci accumulation, induces *ara-caup* expression. Moreover, anterior *pka⁻* cells, which accumulate high levels of Ci and Dpp, also strongly express *ara-caup*, while *pka⁻ dpp⁻* cells, which accumulate high levels of only Ci, express *ara-caup* to a lower extent and fail to do so when they are far removed from the endogenous source of Dpp at the AP border. These data indicate that *ara* and *caup* require high levels of Ci and Dpp to be activated. These are precisely the conditions prevalent in the wild-type L3 domains. Ci, a putative transcription factor (Alexandre et al. 1996), may directly interact with the *ara-caup* cis-regulatory regions. Dpp, in contrast, should act indirectly by inducing the expression of other transcription factors. Two of them, encoded in the *optomotor blind* (*omb*) locus and the *spalt* gene complex (Sal-C), are activated by Dpp, independently of Ci, in regions of the wing pouch of different extents and centered at the *dpp* expression domain (de Celis et al. 1996a; Grimm and Pflugfelder 1996; Lecuit et al. 1996; Nellen et al. 1996). However, it is unlikely that they mediate the *dpp* requirement for *ara-caup* expression because mutant cells lacking either *omb* or the Sal-C can still differentiate vein L3 (de Celis et al. 1996a;

Figure 5. Rescue in *71ptc* discs of the expression of *ac* (A–D) and *rho/ve* (G–I) inhibited by overexpression of *ptc*. Late third instar discs were stained with an anti Ac antibody (A–D) or were hybridized with a *rho/ve*–DIG probe (E–I). (A) Wild-type disc. The dorsal L3 proneural cluster is indicated by an arrowhead. The ventral one is not visible in this disc. (B) *71ptc/+* disc. The dorsal and ventral L3 proneural clusters are absent (arrowhead). (C) *UAS-ara/+; 71ptc/+* disc. The L3 dorsal and ventral proneural clusters are restored. (D) *71ptc/UAS-hh* disc. Both proneural clusters are restored and greatly expanded and show multiple sensory mother cells (some are indicated by arrowheads). (E) *hh^{Mrt}; In(3)iro^{DFM2}* disc. There is no *rho/ve* expression in the expanded vein L3 territory (asterisks); presumptive vein L4 is indicated by arrowheads. (F) *hh^{Mrt}* disc. *rho/ve* is expressed in the expanded vein L3 territory (arrows). (G) Wild-type disc. Presumptive veins L3, L4 and L5 are marked. (H) *71ptc/+* disc. Expression corresponding to presumptive veins L3 and L4 is missing. (I) *UAS-ara/+; 71ptc/+* disc. Expression in L3 and L4 territories is restored, together with some ectopic expression promoted by *UAS-ara* (see Gómez-Skarmeta et al. 1996). Interestingly, in mitotic recombination clones, *ara-caup* are not required for the development of vein L4 (Gómez-Skarmeta et al. 1996). This suggests that *ara* replaces another gene required for *rho/ve* expression at the presumptive vein L4 and whose expression is inhibited by *ptc* overexpression.

Grimm and Pflugfelder 1996). Another candidate is the zinc finger protein encoded in the *schnurri* locus, which acts in the Dpp signaling pathway and whose removal suppresses vein differentiation (Arora et al. 1995; Grieder et al. 1995).

All conditions tested leading to high ectopic accumulation of Ci and Dpp in the A compartment failed to activate *ara* and *caup* in cells located at the prospective wing margin. We have found that this restriction depends on *wg*. Thus, in clones of anterior *pka wg* mutant cells *ara* and *caup* ectopic expression extends toward the DV compartment border, which indicates that *wg* represses *ara* and *caup* at the prospective wing margin. In fact, ectopic expression of *wg* in *N* gain of function mutant combinations eliminates *ara* and *caup* expression at the L3 territory. Interestingly, in *pka⁻ wg⁻* clones, cells

in a narrow gap of one or two cell diameters, which presumably correspond to the DV boundary, do not express *ara* and *caup*. This suggests that at least some characteristics of the wing margin may form in the absence of *wg* function, probably through the *Serrate* and *Delta* interaction with *Notch* (Díaz-Benjumea and Cohen 1995; Kim et al. 1995; Doherty et al. 1996). The specification of these cells may turn on a repressor(s) of *ara-caup* at this site. A possible candidate may be the DNA-binding protein Suppressor of Hairless, which mediates at least some of the *N* signaling (Fortini and Artavanis-Tsakonas 1994; Bailey and Posakony 1995; Lecourtois and Schweisguth 1995; Kim et al. 1996). Ectopic Ci and Dpp were also unable to activate *ara-caup* in the P compartment. This is most likely attributable to the presence of En, which appears to define by repression the posterior

Figure 6. *wg* antagonizes *ara-caup* expression. (A) Wing pouch of a late third instar *iro*^{F209} disc stained with anti-β-galactosidase antibody (red) to visualize the L3 domains of *ara-caup* expression and anti-Wg antibody (green). The Wg-accumulating cells, straddling the prospective wing margin, separate these domains. (B) *Ax*¹⁶¹⁷²/*Ax*²⁸/*iro*^{F209} disc stained with X-gal to reveal the pattern of expression of *ara-caup*. Note the almost complete absence of the L3 domains. Residual expression is indicated by an arrowhead. (C,D) Wing pouches of *iro*^{F209} discs harboring clones of *pka*⁻ *wg*⁻ cells. Clones are detected by the strong accumulation of Ci protein (green). β-galactosidase (red) shows *ara-caup* expression. Regions of strong accumulation of both Ci and β-galactosidase appear in yellow. The *pka*⁻ *wg*⁻ clone in C overlaps the band of high accumulation of Ci. Only a narrow gap of cells not expressing β-galactosidase or doing it at low levels remains between both patches (arrowheads). Similarly, in a more anterior clone (D), the cells at the prospective wing margin not expressing β-galactosidase are indicated by arrowheads.

border of the L3 patches (Fig. 2). Indeed, in *fu* mutant discs, removal of En from a wedge of anterior cells apposing the AP border (Sánchez-Herrero et al. 1996) led to activation of *ara-caup* in them.

Specification of the L3 territory

Increased *ptc* function in two broad domains centered at the dorsal and ventral vein L3 territories (71*ptc* line) causes, in larvae, a reduction of *ci* and *dpp* functions and, in the adult, the reduction in size of the L2–L4 intervein regions and the partial elimination of veins L3 and L4 and associated sensory organs (Johnson et al. 1995). Consistent with these phenotypes, we find that the increased *ptc* function reduces the size of the central region of the wing pouch and eliminates the *ac-sc* L3 proneural cluster and the *rho/ve* expression at the prospective veins L3 and L4. At least part of these effects are attributable to

the strong reduction of *ara-caup* expression at the L3 patches (Fig. 4A), because restoring *ara* expression largely recovers expression of *ac-sc* and *rho/ve* (Fig. 5). However, the size of the central region of the wing pouch is not recovered. These data indicate that *ara-caup* are necessary to generate pattern elements at the L3 territory (see also Gómez-Skarmeta et al. 1996) but that they do not contribute to its growth.

Overexpression of *hh* in the A compartment causes ectopic expression of *ci*, *dpp*, and *ara-caup* in the overgrown anterior compartment (Tabata and Kornberg 1994; Felsenfeld and Kennison 1995; Johnson et al. 1995; Fig. 1B). This is associated with the expansion of the domains of expression of *rho/ve* and *ac-sc* (Fig. 5D,F). Moreover, the expanded expression of *rho/ve* depends on *ara-caup* (Fig. 5E). Thus, at least some characteristics of the vein L3 domain are imposed on the expanded territory, which in fact differentiates mainly as vein L3 (de Celis and Ruiz-Gómez 1995). These data, together with the observation that *ara-caup* are necessary for *ac-sc* expression in this territory (Gómez-Skarmeta et al. 1996), suggest that, in wild type discs, *ara-caup* are required for the proper specification of the vein L3 territory. At present we cannot distinguish whether *ara-caup* participate in providing the unique characteristics of the L3 territory or simply reveal this identity, which would be determined by other genes, among them *ci* and *dpp*.

Overexpression of *UAS-dpp* in 71*ptc* wing discs did not restore the size of the central region of the wing pouch. This could be attributable to low levels of Dpp. Alternatively, Dpp alone may not be able to direct growth at this site. Indeed, overexpressing *UAS-ci*, which provided high levels of both Ci and Dpp, restored the size of the central region of the wing pouch (and *ara-caup* expression). This, together with the observation that the expanded A compartment of discs with excess Hh function acquire at least some of the characteristics of the L3 territory, as revealed by the enlarged domains of *ara-caup*, *ac-sc*, and *rho/ve* expression, indicates that the combination of high levels of Ci and Dpp promotes the identity of this territory.

In contrast to the central region of the wing pouch, overexpression of *UAS-dpp* in both 71B and 71*ptc* lines promoted a large overgrowth of the anterior- and posterior-most regions of this pouch. The levels of Dpp might be sufficient for the overgrowth of these regions, which are far removed from the internal Dpp source, if these are the most sensitive to Dpp. Surprisingly, overexpression of *UAS-ci* in the same lines promoted growth at the central region of the wing pouch without affecting the anterior- and posterior-most regions, in spite of the fact that these are adjacent to the territory with high levels of Dpp and Ci (Figs. 3D and 4F). The reasons for this paradox are unclear.

Conclusions

Our data place *ara-caup* in an intermediate position in the genetic hierarchy that controls the patterning of the

imaginal wing disc (Fig. 7). Their expression in the L3 territory is positively regulated by the Hh signal, which induces an increased accumulation of Ci (Johnson et al. 1995; Domínguez et al. 1996; Sánchez-Herrero et al. 1996). This activates *dpp*, and both Ci and Dpp promote the expression of *ara-caup*. Dpp, a putative secreted protein, apparently acts as a morphogen. It activates the expression of individual transcription factors in domains of different extents centered at the Dpp source (de Celis et al. 1996a; Grimm and Pflugfelder 1996; Lecuit et al. 1996; Nellen et al. 1996). The combination of Dpp-dependent factors and Ci at high levels probably specifies the identity of the L3 territory (Nellen et al. 1996) and, consequently, promotes the transcription of *ara-caup*.

ara and *caup* are negatively regulated by En and Wg. En defines the posterior border of the *ara-caup* L3 domains and Wg the separation between them (Fig. 7). We surmise that another as yet unidentified repressor(s)

shapes, symmetrically to En, the anterior border of these domains. In this way, the combination of Ci, Dpp, En, Wg, and other factors defines, within the larger territory of high Ci accumulation, the two smaller domains of *ara* and *caup* expression. *ara* and *caup*, in turn, and in combination with additional activators and repressors, promote expression of *rho/ve* and *ac-sc* in still further restricted domains. Thus, the gradual subdivision of the disc epithelium by the members of this genetic hierarchy creates the positional information essential for patterning.

Materials and methods

Drosophila stocks

The *UAS-dpp*, *UAS-hh* (Capdevila and Guerrero 1994), *ptc*^{llw109}, *ptc*^{G20} (Phillips et al. 1990), *fu*¹ (Díaz-Benjumea and García-Bellido 1990), *fu*¹, and *HS FLP122* stocks were provided by I. Guerrero (Centro de Biología Molecular Severo Ochoa, Madrid, Spain) and the *UAS-ci* stock by K. Basler (Zürich University, Switzerland) (Domínguez et al. 1996). The *UAS-ara*, *iro*^{F209}, and *In(3)iro*^{DFM2} stocks are described elsewhere (Gómez-Skarmeta et al. 1996; Leyns et al. 1996). *pka*^{B3} *ck* *FRT40A*/Cyo was from D. Kalderon (Columbia University, New York, NY) (Li et al. 1995). Gal4-expressing lines 71B (Brand and Perrimon 1993) and MS1096 (Capdevila and Guerrero 1994) were donated by A. Brand and F. Jiménez, respectively. 71*ptc* line was a gift from M. Scott (Johnson et al. 1995). *hh*^{Mrt} was provided by J.F. de Celis. *Ax*¹⁶¹⁷² and *Ax*²⁸ stocks were from the collection of A. García-Bellido. *pka*^{B3} *ck* *dpp*^{d12} *FRT40A*/Cyo and *pka*^{B3} *ck* *wg*^{cx4} *FRT40A*/Cyo (Pan and Rubin 1995) were provided by D. Pan.

Overexpression of *ara*, *ci*, *dpp*, and *hh*

UAS-ara, *UAS-ci*, *UAS-dpp*, and *UAS-hh* lines were crossed with Gal4-expressing lines 71B, 71*ptc*, or MS1096. Progeny were kept at 29°C to maximize expressivity of the imaginal discs and adult phenotypes.

Generation of mitotic recombination clones

HS FLP122;pka^{B3} *ck* *FRT40A*/+, *HS FLP122;pka*^{B3} *ck* *wg*^{cx4} *FRT40A*/+, or *HS FLP122;pka*^{B3} *ck* *dpp*^{d12} *FRT40A*/+ males were mated with *FRT40A;iro*^{F209} females and larvae at 48–60 hr after egg laying were heat shocked for 1 hr at 37°C. Third instar wing discs were dissected for histochemistry.

Histochemistry

Imaginal discs were dissected in PBS, fixed in 1% glutaraldehyde for 2 min at 0°C, washed twice (5 min each) in PBS, stained with X-gal (0.2%) for 2–5 hr at 37°C, dehydrated in ethanol, and mounted in Canada balsam. Antibody staining of imaginal discs for observation with visible light was performed as in Cubas et al. (1991). Fluorescent double stainings were performed similarly, except that the secondary antibodies were antimouse or antirat biotin (1:200, Amersham) and antirabbit FITC (1:40, Dako). After 2 hr at room temperature in the dark, discs were washed four times in PBT (15 min each) and incubated 1 hr with streptavidin-lissamine-rhodamine (1:200, Jackson). Finally, discs were washed in PBT as before and mounted in Mowiol (Sigma). Images were acquired with a Zeiss LSM310 confocal microscope. In situ hybridization of whole mounts of imaginal discs was performed according to Tautz and Pfeifle (1989), modified according to Cubas et al. (1991) using DIG-labeled probes prepared with the *caup* or *rho/ve* cDNAs. Mouse anti-En (Patel

Figure 7. A model for the control of *ara-caup* expression at the L3 patches of the wing disc. (A) The *hh* signal emanating from posterior cells antagonizes the *ptc* and *pka* down-regulation of Ci and creates an anterior domain of cells, adjacent to the AP border, that accumulate high levels of this protein (yellow in B). Ci activates transcription of *dpp*. Dpp is secreted outside its source (blue striped area in B) and in combination with Ci activates *ara-caup* (red striped areas in B; *ara-caup* is abbreviated *iro*). *ara-caup* are not expressed in all the Ci domain cells because of repression by *en* (green striped area in B) and *wg*, which migrates and exerts its repression outside its source (magenta striped region in B), and, possibly, in the anterior-most cells, by another as yet unidentified factor. Compartment borders are indicated by dashed lines in B.

et al. 1989) and rabbit anti-Wg (Van-der-Heuvel et al. 1989) were a gift from I. Guerrero. Rat anti-Ci was obtained from B. Holmgren (Northwestern University, Evanston, IL) (Slusarski et al. 1995). Rabbit anti-Sc was donated by S.B. Carroll (University of Wisconsin, Madison) (Skeath and Carroll 1991). Mouse anti- β -galactosidase and rabbit anti- β -galactosidase are from Promega and Cappel, respectively.

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