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Targeted expression of the signaling molecule decapentaplegic induces pattern duplications and growth alterations in *Drosophila* wings

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In the wing imaginal disc, the *decapentaplegic* (*dpp*) gene is expressed in a stripe of anterior cells near the anterior–posterior compartment boundary, and it is required solely in these cells for the entire disc to develop. In some viable segment polarity mutants, alterations in *dpp* expression have been demonstrated that correlate with changes in wing morphology. To test the hypothesis that the abnormal patterns of *dpp* expression are responsible directly for the mutant phenotypes, we have expressed *dpp* in ectopic places in wing imaginal discs, and we have found that *dpp* is able to cause overgrowth and pattern duplications in both anterior and posterior compartments of the wing disc. The alterations of the anterior compartment are strikingly similar to those observed in some viable segment polarity mutants. Thus, ectopic *dpp* alone can account for the phenotype of these mutants. We also show that ectopic expression of the segment polarity gene *hedgehog* (*hh*) gives similar morphological changes and activates *dpp* expression in the anterior compartment. This strongly suggests that the organizing activity of *hh* is mediated by *dpp*. We propose that the expression of *dpp* near the anterior–posterior compartment boundary is directed by the interaction between *patched* and *hh*, and that *dpp* itself could act as a general organizer of the patterning in the wing imaginal disc.

*Key words: decapentaplegic/hedgehog/*imaginal disc development/segment polarity genes/transforming growth factor-β

Introduction

It has been proposed that during development positional information originates from specialized organizer regions in the embryo. These regions serve as sources of inductive signals that instruct the differentiation of groups of cells (Spemann, 1938; Wolpert, 1969; Meinhardt, 1983, 1991). Thus, in the development of the vertebrate limb it has been defined the zone of polarizing activity (ZPA) in the posterior region of the limb bud. This ZPA seems to function by releasing a signal which would form a gradient across the early embryonic primordium, specifying the pattern of digits across the entire limb bud (Tickle *et al.*, 1975; reviewed by Tabin, 1991). Other sources of organizer activity are the blastopore lip of amphibians, which specifies the dorsal–ventral axis of the mesoderm (Spemann, 1938), and the vertebrate notochord, which contributes to the pattern of the spinal cord along the dorsal–ventral axis (reviewed by Smith, 1993).

In the embryonic epidermis of insects, it is thought that positional information in each metameric unit is conveyed in the form of a gradient across the segment. The organizer, in this case, would be the parasegmental border (Martínez-Arias and Lawrence, 1985; reviewed by Ingham and Martínez-Arias, 1992), being the source of a segmental gradient (Locke, 1960; Lawrence, 1966; Crick and Lawrence, 1975; Nubler-Jung, 1979; Meinhardt, 1983, 1991). In *Drosophila*, two possible morphogens have been proposed that could influence the cuticular pattern of cell-type differentiation across the embryonic segment: one is wingless (wg), a secreted factor produced by cells at one side of the parasegmental border (Van den Heuvel *et al.*, 1989; Bejsovec and Martínez-Arias, 1991; González *et al.*, 1991), and the other is hedgehog (hh; Heemskerk and DiNardo, 1994), a transmembrane protein that is produced under the control of the *engrailed* (*en*) gene by cells at the other side of the border and is also secreted (Lee *et al.*, 1992; Mohler and Vani, 1992; Tabata *et al.*, 1992; Taylor *et al.*, 1993; Tabata and Kornberg, 1994). hh and decapentaplegic (dpp) molecules are also thought to play a key role in patterning the primordia of the adult imaginal discs (Gelbart, 1989; Heberlein *et al.*, 1993; Ma *et al.*, 1993; Basler and Struhl, 1994; Capdevila *et al.*, 1994; Tabata and Kornberg, 1994).

dpp protein is a member of the transforming growth factor-β (TGF-β) family of secreted factors (Padgett *et al.*, 1987), known to act as a morphogen in *Drosophila* embryos organizing the dorsal–ventral pattern (Ferguson and Anderson, 1992; Wharton *et al.*, 1993). It is needed for the development of derivatives of most of the imaginal discs (Spencer *et al.*, 1982). In the wing, for instance, *dpp* viable mutants produce flies with reduced wings. Analysis of *dpp* mutant clones indicates that *dpp* is required solely in the cells just anterior to the anterior–posterior compartment boundary of the wing disc for the entire disc to develop (Posakony *et al.*, 1991). Consistent with this, *dpp* is transcribed precisely in these cells (Masucci *et al.*, 1990; Blackman *et al.*, 1991; Raftery *et al.*, 1991). It has also been proposed that *dpp* expression provides a signal for the establishment of proximal–distal positional information in the whole wing disc (Gelbart, 1989; Campbell *et al.*, 1993). Furthermore, we have shown previously that some segment polarity products are required to restrict the expression of *dpp* to its normal domain, and that this restriction may be essential for a normal morphogenesis (Capdevila *et al.*, 1994).

The *hh* gene is expressed in the posterior compartments of imaginal discs (Lee *et al.*, 1992; Tabata *et al.*, 1992; Tashiro *et al.*, 1993; Tabata and Kornberg, 1994). The hh

protein might act as a diffusible signal from the compartment border, regulating expression of other genes transcribed in the anterior compartment: patched (*ptc*) (Capdevila *et al.*, 1994; Tabata and Kornberg, 1994) and *dpp* (Basler and Struhl, 1994; Tabata and Kornberg, 1994). Recently, Basler and Struhl (1994) proposed that the main role of *hh* in the wing imaginal disc is to control the expression of *dpp* in a thin stripe of anterior cells along the anterior–posterior compartment boundary. This *dpp* source would govern growth and patterning of neighboring cells located anterior and posterior to the stripe. Ectopic expression of *hh* in the anterior compartment of the imaginal discs, using the 'flp-out' technique (Struhl and Basler, 1993), causes pattern duplications (Basler and Struhl, 1994). Moreover, ectopic *hh* is accompanied by *dpp* induction (Basler and Struhl, 1994; Tabata and Kornberg, 1994).

To test directly the roles of dpp and hh gene products in the imaginal discs, we have used the GAL4 system (Brand and Perrimon, 1993) to express the *dpp* and *hh* products in localized regions of the wing disc. In this paper we show that the targeted expression of *dpp* is able to induce pattern duplications and growth alterations in both compartments of the wing disc. We find that similar alterations are produced in the anterior compartment by ectopic expression of *hh*. Furthermore, ectopic *hh* induces *ptc* and *dpp* expression. These results indicate that *hh* is acting as a regulator of *dpp* expression, and that dpp signaling molecule is able to control growth and patterning in the wing imaginal disc acting as an organizer molecule.

Results

Ectopic expression of dpp near the dorsal–ventral compartment boundary induces mirror-image duplications in the anterior and posterior compartments of the wing

The expression of *dpp* in a stripe of cells in the anterior compartment, near the anterior–posterior compartment boundary (Masucci *et al.*, 1990; Blackman *et al.*, 1991; Posakony *et al.*, 1991; Raftery *et al.*, 1991), is required for cell proliferation and/or viability of the entire wing imaginal disc (Spencer *et al.*, 1982; Bryant, 1988; Posakony *et al.*, 1991). Recently, it has been demonstrated that *dpp* is expressed ectopically in some segment polarity mutants that display overgrowth of the anterior compartment (*patched* and *costal-2* mutants; Capdevila *et al.*, 1994). At the same time, when the segment polarity gene *hh* is ectopically expressed in the anterior compartment of the wing disc, it induces phenotypic alterations and *dpp* mis-expression (Basler and Struhl, 1994; Tabata and Kornberg, 1994). Thus, it has been suggested that dpp is responsible for the morphological alterations of the anterior compartment observed in segment polarity mutants (Capdevila *et al.*, 1994) and in experiments involving ectopic *hh* (Basler and Struhl, 1994; Tabata and Kornberg, 1994; this study).

We have used the GAL4 system (Brand and Perrimon, 1993) to activate *dpp* in different regions within the wing blade. We have generated transgenic fly lines that express the *dpp* cDNA under the control of UAS elements that respond to GAL4 protein. We have crossed these UAS*dpp* flies to several fly lines that do not express GAL4 protein in the embryo but express GAL4 in different regions of both anterior and posterior compartments of the wing discs. Figure 1A shows the wild-type pattern of expression of *dpp* (blue) in a third instar wing disc of a stock containing the *dpp–lacZ* construct, also stained to detect the wingless (wg) antigen (brown) which is expressed in the developing wing margin (arrowheads in Figure 1A and B). *wg* expression in the wing margin can be used as a marker of the dorsal–ventral compartment boundary of the wing disc. Figure 1B shows wg expression in brown and the pattern of expression of GAL4 driven by the line MS 209 (a gift from F.Jiménez) in blue. When we use this line to express *dpp*, the ectopic strong expression of *dpp* is restricted to the proximal regions of the presumptive wing blade (see endogenous and ectopic expression of *dpp* in Figure 6J). Furthermore, *dpp* is expressed in confronted dorsal and ventral cells near the prospective dorsal–ventral boundary in the anterior and posterior edges of the disc (Figure 1B). In this situation, some cells of the prospective dorsal–ventral boundary are presumably exposed to the dpp factor, as it occurs in the central region of the wing pouch where the *dpp* stripe intersects the dorsal–ventral boundary (Figure 1A, arrowheads show the prospective boundary expressing the wg antigen). Ectopic expression of *dpp* driven by the line MS 209 induces overgrowth with a mirror-image duplication of structures of the anterior and posterior compartments (see details in Figure 2). Anterior duplications involve only anterior elements (veins 1 and 2, triple and double row bristles, sensory organs, ...), while duplications in the posterior compartment include only posterior elements (veins 4 and 5, posterior crossvein and posterior row of bristles).

Using other GAL4-expressing lines, we have expressed *dpp* in different locations of the wing disc, not strictly coincident with the prospective dorsal–ventral boundary (Figure 4A), or coincident with certain cells of the dorsal wing pouch (Figure 4D). In these cases the ectopic expression of *dpp* induces overgrowth of the wing discs (not shown) and alterations of venation pattern (Figure 4C and F), but never mirror-image duplications of pattern elements which seem to be dependent on the localized expression of *dpp* in specific regions of the disc. All these phenotypes will be discussed later.

The effects of the ectopic expression of dpp in the anterior compartment are mimicked by the ectopic expression of hh in the same places

It has been shown that the ectopic expression of *hh* in the wing disc can induce *dpp* expression (Basler and Struhl, 1994; Tabata and Kornberg, 1994). The correlation between the organizing activity of hh protein and its ability to induce *dpp* expression suggests that *hh* influences wing patterning through *dpp*. To investigate this, we have studied if all the phenotypic alterations produced by the ectopic expression of *dpp* are mimicked by the expression of *hh* in the same places.

We have made transgenic fly lines that express the *hh* cDNA under the control of UAS elements (UAS*hh* flies). We intended to compare the adult phenotypes of flies which expressed either *hh* or *dpp* in the same places, crossing UAS*hh* and UAS*dpp* flies independently to the same GAL4-expressing lines. However, we found that in

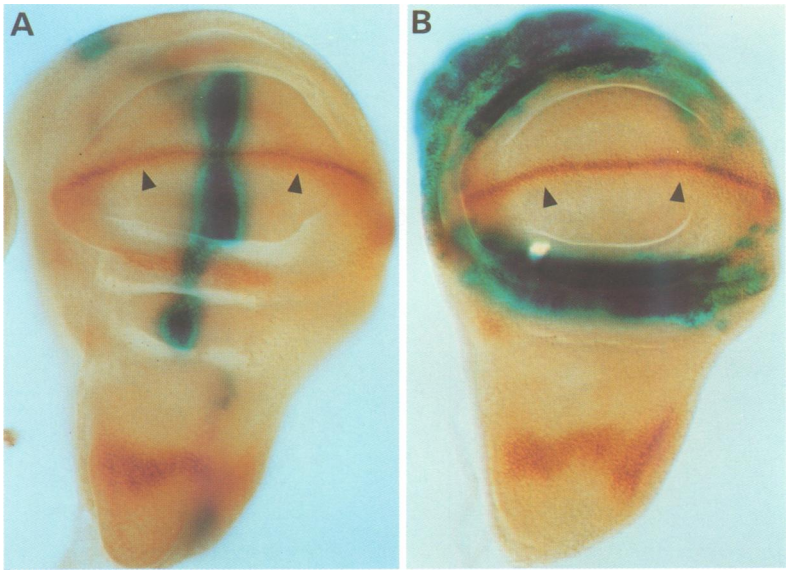

Fig. 1. Double stainings to detect wg antigen (brown) and β-galactosidase activity (blue) in wing imaginal discs from third instar larvae carrying the *dpp−lacZ* construct to show the wild-type expression of the *dpp* gene (**A**), and from third instar larvae carrying both the *UAS−lacZ* and the MS 209 constructs (**B**) to show the pattern of expression of GAL4. *dpp* is strongly expressed exactly in the same places in wing imaginal discs when MS 209 flies are crossed to UAS*dpp* flies, as shown in Figure 6J, and the corresponding adult phenotypes are shown in Figure 2. Arrowheads show *wg* expression in the presumptive wing margin.

some cases expression of *hh* resulted in lethality, while using the same GAL4 line the expression of *dpp* gave viable flies. In other cases, ectopic *dpp* expression was lethal, while ectopic *hh* gave viable flies. Thus, when we crossed UAS*hh* flies to MS 209 flies we obtained pharate adults displaying mirror-image duplications restricted to the anterior edge of the wing. The level of expression of hh has been analyzed in MS 209 UAS*hh* wing discs by staining the discs with an anti-hh polyclonal antibody (Figure 5A and B and Figure 6E). The lethality of MS 209 UAS*hh* flies could be due to a very high level of hh protein, which could be deleterious by itself, or it may be that expression of *hh* in some other tissue causes lethality. To obtain viable adults, we crossed UAS*hh* flies to flies from the GAL4 30A stock (kindly provided by N.Perrimon). This strain expresses GAL4 in a pattern similar to that described for the MS 209 line (compare Figures 3D and 1B), but apparently at lower levels which are compatible with viability. This line permits the expression of *hh* in the same places described for the UAS*dpp* experiment.

As shown in Figure 3, ectopic *hh* in the proximal region of the presumptive wing blade (Figure 6E) leads to mirror-image duplications of the anterior edge of the wing (Figure 3A and B) which are very similar to that obtained expressing *dpp* ectopically in the same region (compare with Figure 2C, E and F). These phenotypes are also very similar to that described for viable mutations in the segment polarity gene *costal-2* (*cos2*, Figure 3C; Whittle, 1976; Grau and Simpson, 1987; Simpson and Grau, 1987). *cos2* imaginal discs display ectopic *dpp* in the presumptive anterior edge of the wing disc, coincident with the dorsal−ventral boundary (Capdevila *et al.*, 1994). Very high levels of *hh* in the posterior compartment directed by GAL4-expressing lines do not have any phenotypic consequence, as described previously in experiments involving *hh* 'flp-out' clones (Basler and Struhl, 1994). When we cross GAL4 30A flies to UAS*dpp* flies we also obtain mirror-image duplications in both the anterior and posterior edges of the wings (data not shown), but with lower expressivity when compared with that obtained with MS 209 flies (Figure 2).

When *hh* expression is driven by other GAL4 lines, displaying different patterns of GAL4 expression, a variety of phenotypes is obtained, including specific defects in veins (Figure 3E, when *hh* is expressed in central regions of the wing pouch, directed by the line 71B, shown in Figure 3F), the costa and the anterior margin (Figure 4B). In some cases there are extra sensory organs in wings and notum (data not shown). The phenotype shown in Figure 3E, displaying specific defects in veins 2 and 3, is similar to that observed in some *ptc* mutants, such as the ptc^{G20}/ptc^{IN} combination (Figure 3G).

The similarities with the segment polarity phenotypes extend to cases with more dramatic pattern alterations. For instance, the expression of *dpp* in large areas of the dorsal wing pouch (like that directed by the line MS 1096; Figure 4D) results in severe overgrowth of the wing discs and probably consequent cell death, giving rise to adults with necrotic wings and a highly distorted pattern (Figure 4F). This phenotype is very similar to that observed in mutant combinations such as ptc^{G20}/ptc^{IIW109}, which display strong de-repression of *dpp* in the anterior compartment of the wing disc (Capdevila *et al.*, 1994). The phenotype obtained expressing *hh* in the same place (Figure 4E) is very similar in the anterior compartment of the wing, partially necrotic and disorganized, but in the posterior compartment the pattern is quite normal (veins 4 and 5 and bristles of the posterior row are recognizable, although the whole wing is reduced in size). On the other hand, very restricted expression of *dpp* in regions of the wing disc not coincident with the prospective dorsal−ventral boundary (line MS 941; Figure 4C), gives only overgrowth phenotypes in the corresponding regions of

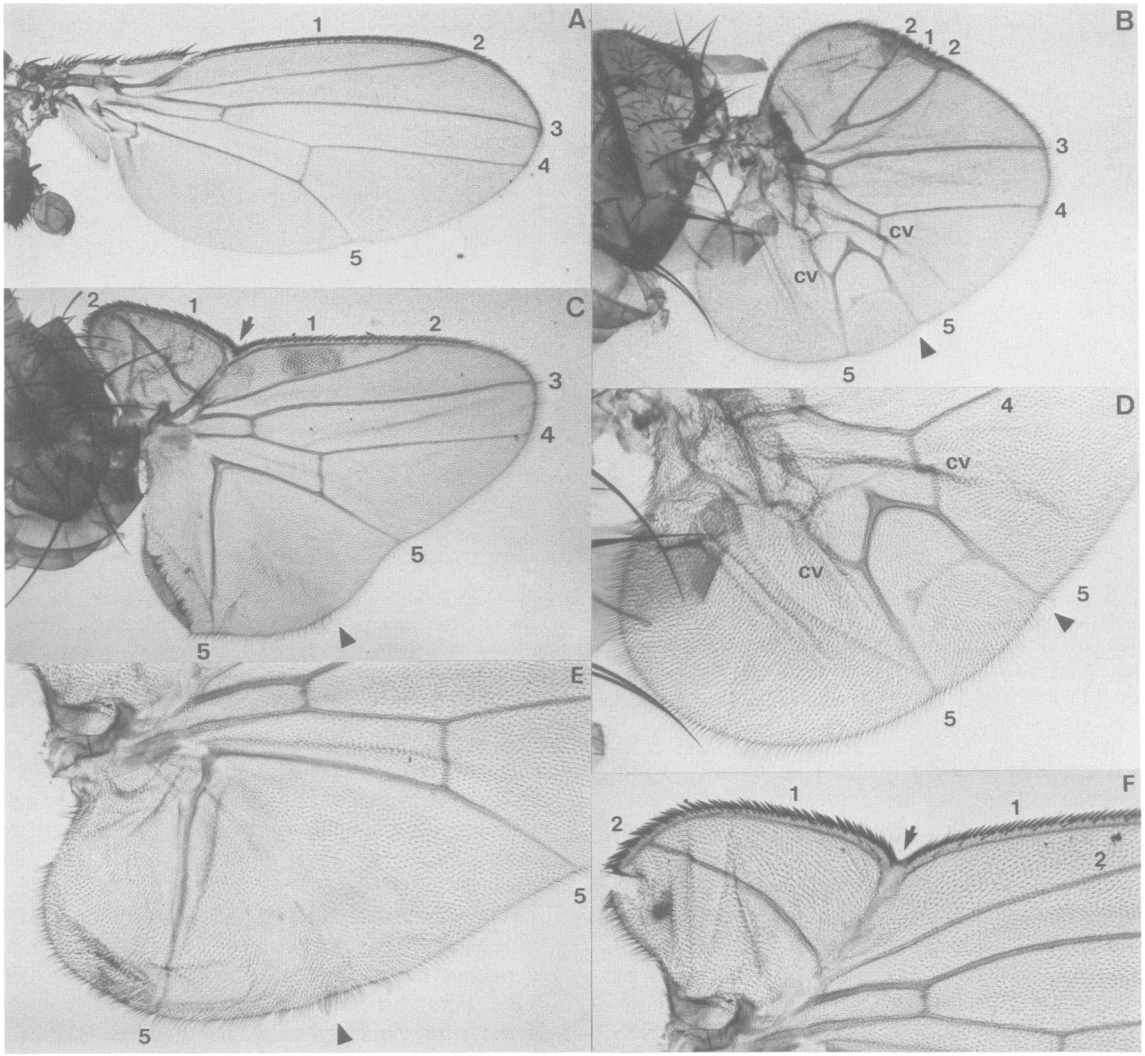

Fig. 2. Mirror-image duplications in anterior and posterior compartments of the wing are induced by targeted expression of *dpp* in specific regions. Wild-type wing (**A**) and wing phenotypes of MS 209 UAS*dpp* flies showing duplicated structures (**B**–**F**). Numbers 1–5 indicate the veins and cv indicates the posterior crossvein. A wing displaying duplications in both the anterior and posterior edges is shown in (C). Note the overgrowth in both compartments and the change in orientation of the triple row bristles in the anterior margin (arrows in C and F) and of the posterior row bristles (arrowheads in C and E). Extra veins 1, 2 and 5 are observed, as indicated in each case. (B) A mutant wing displaying an extra posterior crossvein (shown in detail in D), a change in the orientation of the posterior row bristles (arrowheads in B and D) and severe growth defects in the anterior compartment, with extra veins 1 and 2. Note that mirror-image duplications in the anterior edge are similar to that displayed by viable mutants of the segment polarity gene *cos2* (compare F with Figure 3C).

the wing and notum and alterations of the pattern of veins and sensory organs, with no mirror-image duplications. Expression of *hh* directed by the same line gives variable alterations in the same regions, the posterior compartment remaining basically unaffected.

We conclude from these experiments that the phenotypes obtained by ectopic *hh* are very similar to those obtained by ectopic expression of *dpp* in the anterior compartment. These phenotypes are also very similar to those described for segment polarity mutants. Both ectopic *hh* and *dpp* expressed in confronted dorsal and ventral cells located near the dorsal–ventral boundary of the disc are able to reorganize pattern in the anterior edge of the wing and create mirror-image duplications. We think that at least some organizing properties of the dpp factor arise by its interaction with some other factors expressed at or near the dorsal–ventral boundary, since duplications are dependent on the expression of *dpp* at this specific region of the disc. In addition, ectopic *dpp* is also able to reorganize the posterior edge of the wing.

Induction of ptc and dpp in the anterior compartment by ectopic expression of hh

It has been shown previously that ectopic expression of *hh* at the anterior compartment is accompanied by induction of *dpp* and *ptc* expression (Basler and Struhl, 1994; Tabata and Kornberg, 1994; Figure 5). Using the GAL4 system we have confirmed this and observed that ectopic *dpp* does not alter either *ptc* (data not shown) or *hh* expression (Figure 6I). We have studied in detail the control of *ptc* and *dpp* by *hh*, and found that *dpp* is not de-repressed in all the cells that express ectopic *hh*. When we use the MS 209 line, some of the cells expressing high levels of hh protein also express *dpp* (asterisk in Figure 5B), but *dpp* is mostly induced in adjacent cells located in the anterior edge of the disc (arrowheads in Figure 5B). Interestingly,

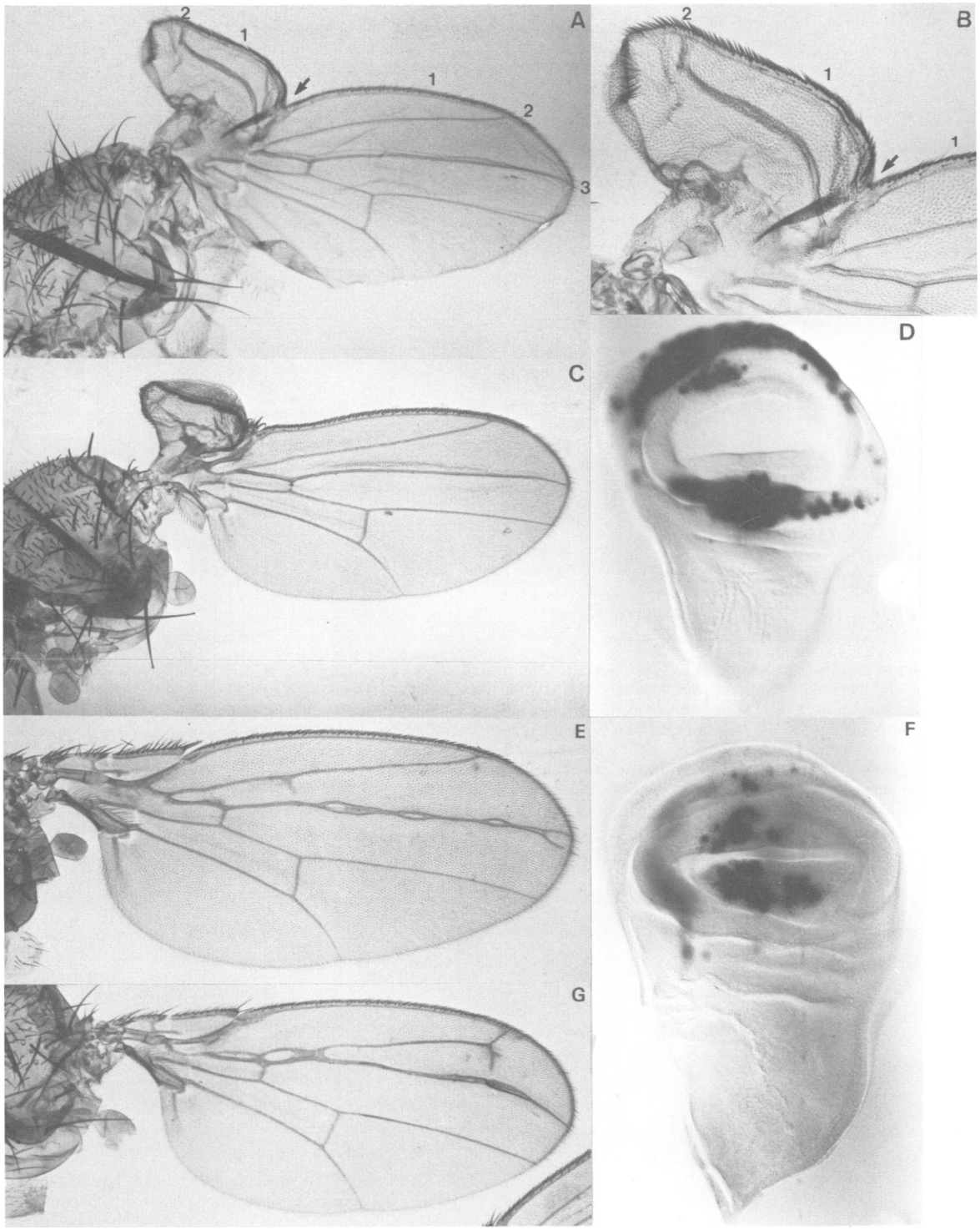

Fig. 3. Ectopic *hh* causes duplications restricted to the anterior compartment which mimic *ptc* and *cos2* phenotypes. Wing phenotype of 30A UAS*hh* flies (**A**) and detail of the anterior compartment of the same wing in (**B**). A wing of the genotype *cos2Cos1*^{2}*/CyObw* is shown in (**C**) for comparison. Note that mirror-image duplications in the anterior edges in both mutants are near indistinguishable (compare with the detail in B), and very similar to that obtained expressing *dpp* in the same places (compare with Figure 2C and F). Expression of GAL4 directed by the line 30A, as shown by XGal staining of 30A *UAS−lacZ* discs (**D**). This pattern of GAL4 expression is near identical to that directed by the line MS 209, and *hh* is expressed exactly in the same places when UAS*hh* flies are crossed to 30A flies. The expression of GAL4 directed by the line 71B is restricted to a central portion of the wing pouch, as shown by XGal staining of 71B *UAS−lacZ* discs (**F**), and correspondent wing phenotype of 71B *UAS−lacZ* flies (**E**). Vein 3 is branched and vein 2 is also affected, and the distance between veins 3 and 4 is increased. The phenotype is very similar to that displayed by some *ptc* viable mutants (a mutant wing of the ptc^{G20}/ptc^{IN} genotype is shown in **G** for comparison).

dpp is de-repressed in confronted dorsal and ventral cells near the prospective dorsal−ventral boundary, which suggests that this particular pattern of *dpp* expression suffices to create duplications. The situation is different for *ptc* expression. As shown in Figure 5C, ectopic *hh* seems to induce high levels of ptc protein expression in

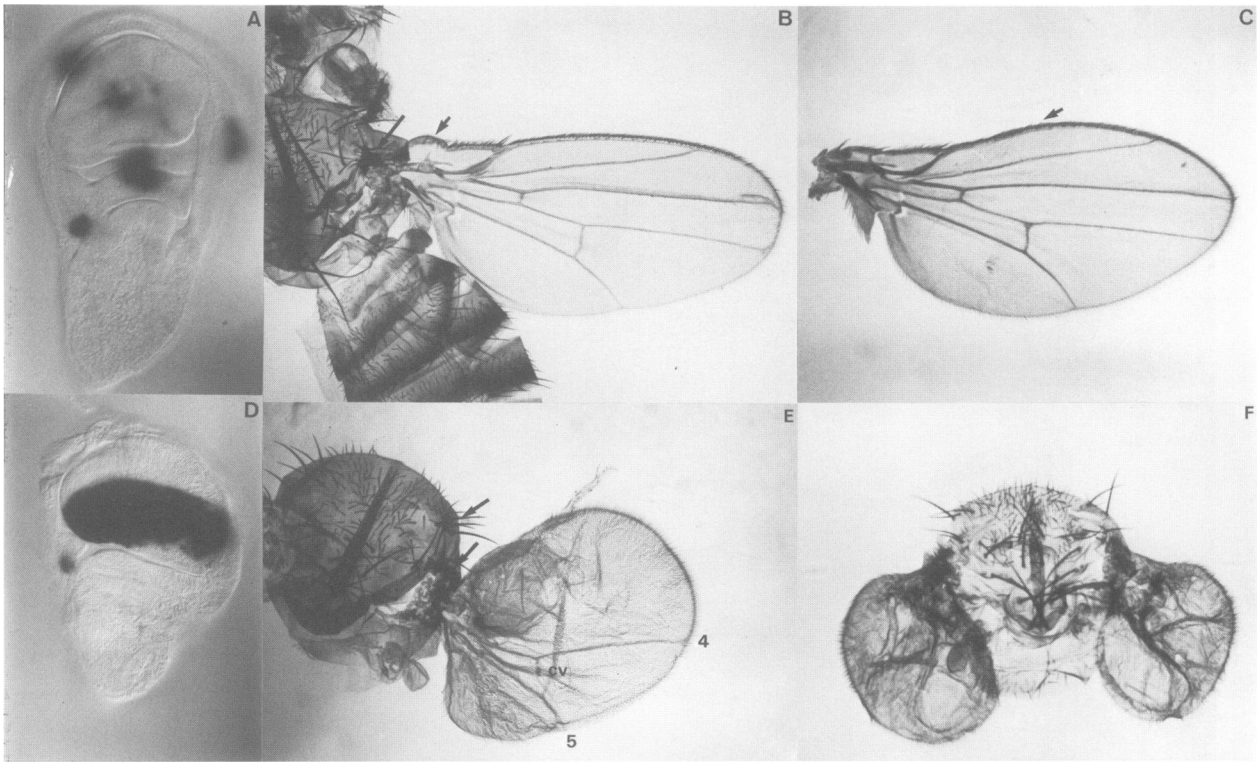

Fig. 4. Comparison of the effects of ectopic *hh* and *dpp*. Either UAS*hh* flies (**B** and **E**) or UAS*dpp* flies (**C** and **F**) were crossed to flies carrying the GAL4-expressing constructs MS 941 (**A**) and MS 1096 (**D**). The MS 941 line directs GAL4 expression in discrete spots, mostly in the anterior compartment of the wing disc. Defects in the anterior margin of the wing and in vein 3 are observed in these MS 941 UAS*hh* flies (B). A variable effect in the anterior margin and in the posterior crossvein is observed in MS 941 UAS*dpp* flies (C). Defects are observed along the anterior margin of the wing. The distance between veins 3 and 4 is usually increased with respect to the wild-type wings, and slight distortions appear near the margin of the wing. MS 1096 directs GAL4 expression in part of the dorsal wing pouch, and MS 1096 UAS*hh* flies display severe disorganization and necrosis of the anterior wing, the posterior compartment remaining unaffected (E). MS 1096 UAS*dpp* flies have severely distorted and necrotic wings, very similar to those of *ptc* mutants with strong de-repression of *dpp* in the anterior compartment.

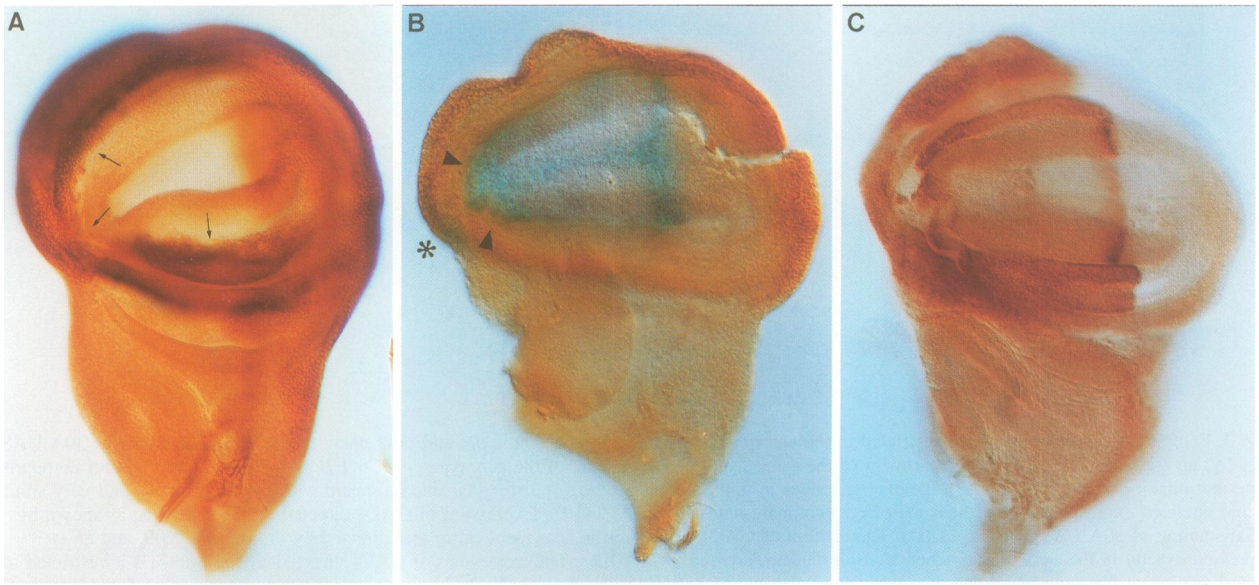

Fig. 5. Ectopic *hh* induces *dpp* expression in some *hh*-expressing cells but also in adjacent cells. Detection of hh antigen (brown) in a MS 209 UAS*hh* wing imaginal disc (**A**), and a similar disc which also carries the *dpp−lacZ* construct, doubly stained to detect both the hh antigen (brown, **B**) and β-galactosidase activity (blue, B). Arrows in (A) indicate the limits of strong expression of *hh* in the anterior compartment. Note that *dpp* de-repression is mainly found associated with, but outside of, the ectopic *hh* domain (arrowheads in B; asterisk marks a region which co-expresses *hh* and *dpp−lacZ*). A similar disc stained to detect ptc antigen (brown) is shown in (**C**), and it illustrates that *ptc* expression is enhanced by ectopic *hh* in the same cells that strongly express *hh* (compare A and C). *ptc* is strongly accumulated in the proximal regions of the anterior wing pouch where *hh* is ectopically expressed.

the anterior compartment in the same cells that express *hh*. Thus, *dpp* is de-repressed in some (but not all) of the anterior *hh*-transcribing cells (asterisk in Figure 6B), but also in a subset of adjacent cells. Similar results are obtained with the other GAL4-expressing lines used in this work. This subset of confronted dorsal and ventral cells in the anterior edge of the wing disc has been shown previously to express ectopic *dpp* in viable segment polarity mutants such as *ptc* and *cos2*, where *ptc* is more widely de-repressed in the anterior compartment than *dpp*. This suggests that these specific cells located in this anterior region are specially predisposed to de-repress *dpp*. It has also been demonstrated that in both some *ptc* mutants and *ptc* null clones, *ptc* and *dpp* are de-repressed, but not exactly in the same cells, suggesting that the cells of the anterior compartment respond differentially to the events that trigger *dpp* de-repression (Capdevila *et al.*, 1994).

Ectopic dpp modifies the expression of wg and aristaless genes

We have found that ectopic *hh* and *dpp* are able to induce expression of the homeobox-containing gene *aristaless* (*al*), which has been proposed to influence the proximal–distal specification of adult appendages (Campbell *et al.*, 1993). Using an *al* probe, ectopic *al* expression is detected in discrete regions of the anterior and posterior compartments (Figure 6H and L; compare with the wild-type in 6D). It has been proposed that the expression of *al* in imaginal discs is controlled by the combination of dpp and wg signals in discrete regions of the discs (Campbell *et al.*, 1993). Our results demonstrate that it is possible to create pattern duplications in the wing by ectopically expressing *dpp* in certain regions of the disc, and that these regions express *al*.

In addition to *al*, *ptc* and *dpp*, we have analyzed the changes in expression of other molecular markers in the wing discs (Figure 6). Ectopic wg is observed in the anterior edge of MS 209 UAS*hh* discs, close to the region where ectopic *al* arises (compare Figure 6G with H; wild-type discs are shown in Figure 6C and D). Thus, both ectopic *al* and *wg* appear in the region of the wing disc which probably will give rise to the mirror-image duplicated structures along the anterior edge. It is not clear whether ectopic *wg* appears as a consequence of ectopic *al* (Campbell *et al.*, 1993), or if *dpp* regulates *wg* directly in this region of the disc.

Ectopic *hh* and *dpp* increase the rate of incorporation of bromodeoxyuridine (BrdU) in the cells of the disc (data not shown). This is consistent with the overgrowth phenotypes observed and indicates that cell division is stimulated preferentially in regions of the anterior edge which overlap the prospective dorsal–ventral boundary. The same region in wild-type discs shows little incorporation of BrdU. This high rate of BrdU incorporation is similar to that observed analyzing mutant wing discs for *ptc* or *cos2* segment polarity genes (unpublished results), which display ectopic *dpp* in the anterior edge of the wing disc.

All these data demonstrate that ectopic *dpp* is able to induce the expression of some key genes involved in the specification of the pattern of the wing disc and in the control of cell growth.

Discussion

dpp is ectopically expressed when imaginal discs undergo regeneration (Brook *et al.*, 1993) or pattern reorganization (Basler and Struhl, 1994; Capdevila *et al.*, 1994; Tabata and Kornberg, 1994; this study). However, it was not clear in these experiments whether *dpp* expression was a cause or a consequence of these reorganization events. For instance, it could be argued that *dpp* expression in the discs could be merely required for the growth and viability of a previously patterned developing field. We have shown, however, that targeted expression of *dpp* is able to reorganize the wing by inducing pattern duplications. These results demonstrate that *dpp* plays an active role in patterning the wing discs.

It has been proposed that the adult phenotypes of some viable segment polarity mutants such as *ptc* and *cos2* are due to the de-repression of *dpp* in the anterior compartment of the wing disc (Capdevila *et al.*, 1994). Here we have substantiated this hypothesis by demonstrating that the ectopic expression of *dpp* in the anterior compartment of the wing disc mimicks the phenotypes observed in *ptc* and *cos2* viable mutants.

The segment polarity gene *ptc* (Hooper and Scott, 1989; Nakano *et al.*, 1989) is transcribed throughout the anterior compartment of the wing disc with a stronger expression near the anterior–posterior compartment boundary (Phillips *et al.*, 1990; Capdevila *et al.*, 1994). It has been shown that *ptc* acts as a repressor of *dpp* and *ptc* expression in imaginal discs (Capdevila *et al.*, 1994). On the other hand, the secreted protein *hh* is expressed in the posterior compartment of the wing disc and also affects anterior cells (Basler and Struhl, 1994; Tabata and Kornberg, 1994). Thus, the expression of *ptc* (Capdevila *et al.*, 1994; Tabata and Kornberg, 1994) and *dpp* (Basler and Struhl, 1994) near the compartment boundary is dependent on *hh*. We show here that the ectopic expression of *hh* in anterior cells leads to *dpp* and *ptc* induction. We propose that ectopic *hh* blocks the repressive activity of *ptc* on *dpp* and *ptc* expression. The same mechanism appears to work at the compartment border, where *hh* activity maintains *ptc* and *dpp* expression through inactivation of *ptc*.

Pattern duplications in the wing are observed when *dpp* is expressed in confronted patches of dorsal and ventral cells near the prospective dorsal–ventral boundary. This suggests that the formation of the duplicated structures may require the cooperation of two signaling inputs coming from the anterior–posterior and dorsal–ventral boundaries. In this scenario, dpp would be the organizing signal coming from the anterior–posterior compartment boundary. On the other hand, some genes have been identified recently which are expressed exclusively or at high levels along the prospective dorsal–ventral boundary, and others that are specifically expressed at one or the other side of this boundary. The confrontation of dorsal and ventral cells has been proposed recently to act as a director of wing growth (Díaz-Benjumea and Cohen, 1993; Williams *et al.*, 1994). Coordinate growth and pattern of the wing disc presumably involve growth signals coming from the anterior–posterior and dorsal–ventral compartment boundaries. This view provides an explanation for the fact that we obtain mirror-image duplications when ectopic *dpp* is expressed in confronted dorsal and ventral

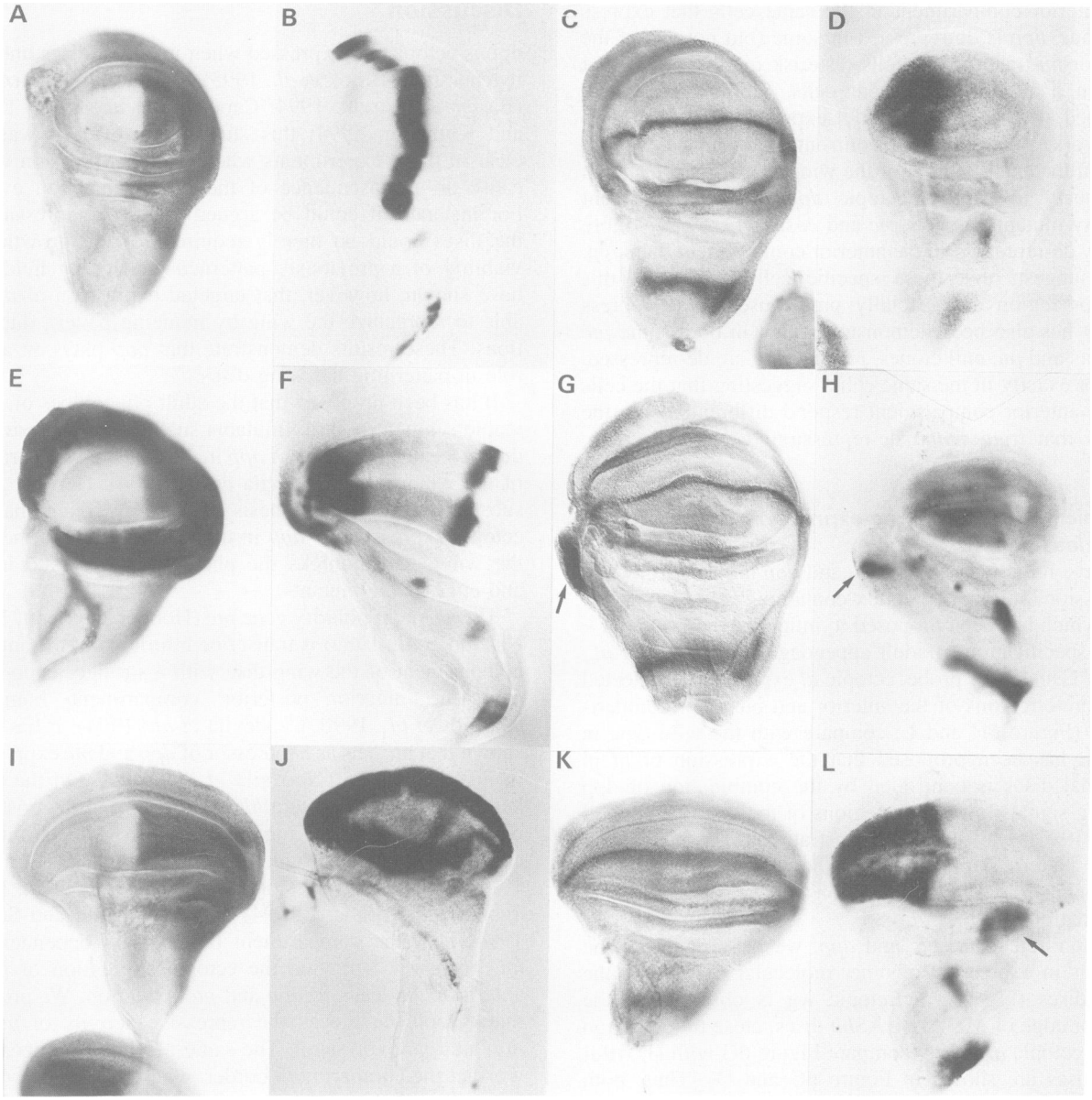

Fig. 6. Ectopic *hh* and *dpp* alter the expression of *wg* and *al* genes. Expression of different molecular markers in wild-type (**A**–**D**), MS 209 UAS*hh* (**E**–**H**) or MS 209 UAS*dpp* (**I**–**L**) third instar wing imaginal discs. Expression of hh antigen (A, E and I), detection of β-galactosidase activity in stocks bearing the *dpp–lacZ* construct (B and F), *dpp* RNA (J), wg antigen (C, G and K) and *al* RNA detection (D, H and L). Arrows indicate sites of ectopic expression of *wg* in MS 209 UAS*hh* discs (G), and sites of ectopic expression of *al* in MS 209 UAS*hh* (H) and MS 209 UAS*dpp* discs (L).

cells near the prospective dorsal–ventral boundary. Our results are in agreement with the boundary model, in which compartment borders are proposed to act as organizing centers for growth and patterning (Crick and Lawrence, 1975; Meinhardt, 1983, 1991).

Recently, a model has been proposed in which induction of the proximal–distal axis in the wing requires the juxtaposition of dpp- and wg-secreting cells. These two activities, with patterns of expression coincident with compartment boundaries, would induce the expression of *al*, which appears to be associated with the development of the proximal–distal axis (Campbell *et al.*, 1993). We show that ectopic *hh* (and *dpp*) induces *wg* and *al* expression in wing discs in the presumptive regions which will give rise to the duplicated structures. We do not know if *wg* and *al* induction occurs as a secondary effect of the pattern reorganization triggered by ectopic *hh* and *dpp*. In fact, ectopic *al* expression also activates ectopic *wg* expression (Campbell *et al.*, 1993). The regulatory interactions between these gene products are more complex than expected from a simple model in which *al* is the sole determinant of the proximal–distal axis.

We have shown that cells from both the anterior and posterior compartments of the wing disc respond to the ectopic *dpp* signal in a similar way, i.e. giving rise to mirror-image duplications of pattern elements. However, these duplicated elements are compartment-specific, i.e. anterior cells give only duplicated anterior structures and posterior cells give only duplicated posterior structures. Thus, an anterior fate cannot be imposed on posterior cells by ectopic *dpp*, and this may well be because the *en* selector gene is still present. *en* could act in the posterior

compartment as a repressor of an anterior specification and, when *en* is missing, these anterior fates arise in the posterior compartment. Transformations from posterior to anterior fates have been observed in some viable *en* mutants and in null *en* mutant clones (García-Bellido and Santamaría, 1972; Lawrence and Morata, 1976). Furthermore, the viable mutants display ectopic *dpp* in the posterior compartment (Blackman *et al.*, 1991). Similarly, *en* mutant clones close to the dorsal−ventral compartment boundary are able to induce overgrowth in the posterior wing (Lawrence and Morata, 1976), probably because these clones induce ectopic *dpp* expression.

It has been found that *dpp* and *hh* homologs in vertebrates exist that are involved in patterning the limb and the neural tube (Basler *et al.*, 1993; Echelard *et al.*, 1993; Krauss *et al.*, 1993; Niswander and Martin, 1993; Riddle *et al.*, 1993; Francis *et al.*, 1994; Roelink *et al.*, 1994). Much effort is needed to understand the patterning events triggered by the TGF-β factors, and experiments involving targeted expression of *dpp* in *Drosophila* can help to identify factors involved in the TGF-β pathway. Our results in *Drosophila* imaginal discs demonstrate that targeted expression of a TGF-β molecule in specific cells is sufficient to reorganize pattern-causing growth alterations and mirror-image duplications.

Materials and methods

Fly stocks

Wild-type flies were obtained from Oregon R strain (Lindsley and Zimm, 1992). Mutant stocks are the following: w^{1118}, used for transformation experiments and obtained from the Bloomington Stock Center; ptc^{G20}, kindly provided by J.R.S.Whittle (Phillips *et al.*, 1990); ptc^{IN} (Tearle and Nüsslein-Volhard, 1987; Hooper and Scott, 1989); ptc^{IIW} (Tearle and Nüsslein-Volhard, 1987); and $cos2^{v1}Cos1^{2}$, kindly provided by P.Simpson (Grau and Simpson, 1987; Lindsley and Zimm, 1992). The *dpp−lacZ* stock (BS3.0; Blackman *et al.*, 1991) was kindly provided by W.Gelbart, GAL4-expressing lines 71B and 30A were kindly provided by N.Perrimon (Brand and Perrimon, 1993), and lines MS 209, MS 941 and MS 1096 were kindly provided by F.Jiménez.

Production of anti-hedgehog antiserum

To produce hh fusion proteins, a *Pvu*II−*Pvu*II fragment of the *hh* cDNA (889–1580 bp) was cloned into the *Sma*I site of a pGEX-2 vector (Smith and Johnson, 1988) to produce a glutathione-S-transferase fusion protein in *Escherichia coli* to immunize the mice. Purification of fusion proteins and immunization of mice were exactly as described previously for anti-ptc antiserum (Sampedro and Guerrero, 1991).

Production of UAShh and UASdpp transgenic fly lines

For the production of the UAS*hh* transgenic fly lines, a *Hin*dIII−*Eco*RI fragment of 3091 bp containing the entire *hh* ORF was isolated from a plasmid kindly provided by J.Lee and P.Beachy and, after the addition of *Eco*RI linkers, the fragment was cloned into the *Eco*RI site of the pUAST plasmid (provided by N.Perrimon). In the case of the UAS*dpp* transgenic fly lines, an *Eco*RI−*Eco*RI fragment of 2860 bp containing the entire *dpp* ORF was excised from a plasmid kindly provided by D.St Johnston and cloned into the *Eco*RI site of the pUAST vector. The recombinant plasmids containing the *hh* or *dpp* fragments in the correct orientations relative to the UAS sequences were used to transform *Drosophila* embryos from the stock w^{1118}, employing standard procedures for microinjection (Roberts, 1986). Several independent lines were obtained, all of them showing a similar level of gene expression as judged by either immunostaining of imaginal discs using our anti-hh antiserum or whole-mount *in situ* hybridization of discs using a specific *dpp* probe.

Whole-mount immunostaining of imaginal discs

Immunostainings using anti-ptc and anti-hh antisera were performed essentially as described in Capdevila *et al.* (1994). For detection of hh antigen, imaginal discs from wandering third instar larvae were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature and washed in PBS. For diaminobenzidine (DAB) staining, PBS containing 0.2% bovine serum albumin (BSA), 0.1% saponin and 5% goat serum was used for blocking and antibody incubations. Tissue was blocked for 1 h and incubated overnight at 4°C in a 1/200 dilution of anti-hh antiserum, blocked and incubated in a 1/300 dilution of biotinylated anti-rat antiserum (Amersham) for 1 h at room temperature. Discs were then washed in PBT (PBS containing 0.1% Tween 20) and incubated for 30 min in Vector AB elite solution in PBT. After several washes in PBT, the reaction was developed in 0.5 mg/ml DAB (Sigma) in PBS containing 0.06% H_2O_2. Discs were mounted under coverslips in epon-araldite (Fluka) after dehydration. Anti-wg antiserum was used at 1/300 dilution. Secondary antibody was biotinylated anti-rabbit (Amersham).

Imaginal discs were observed and photographed under a Zeiss Axiophot microscope.

X-Gal staining

Imaginal discs were first fixed in 4% paraformaldehyde in PBS for 20 min at room temperature, fixed again in 0.5% glutaraldehyde (Fluka) in PBS on ice for 2 min and washed in PBS. The reaction was developed in 5 mM $K_4[Fe^{II}(CN)_6]$, 5 mM $K_3[Fe^{III}(CN)_6]$, 1 mM $MgCl_2$ and 0.2% X-Gal in PBS containing 0.3% Triton X-100. Discs were mounted and observed as described for DAB staining.

Whole-mount RNA in situ hybridizations

Digoxigenin-labeled *al* probe was prepared as follows. A purified *Eco*RI−*Apa*I fragment of the *al* cDNA clone provided by M.Noll (Schneitz *et al.*, 1993) was digested with *Xho*I and labeled using the Genius kit (Boehringer Mannheim), following the manufacturer's instructions. Labeling of *dpp* probe and the RNA *in situ* hybridization procedure (Tautz and Pfeiffle, 1989) were as described previously (Capdevila *et al.*, 1994); D.St Johnston provided the *dpp* cDNA.

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