Development 121, 2265-2278 (1995)
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Sequential organizing activities of engrailed, hedgehog and decapentaplegic in the *Drosophila* wing

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SUMMARY

The *Drosophila* wing is formed by two cell populations, the anterior and posterior compartments, which are distinguished by the activity of the selector gene *engrailed* (*en*) in posterior cells. Here, we show that *en* governs growth and patterning in both compartments by controlling the expression of the secreted proteins hedgehog (hh) and decapentaplegic (dpp) as well as the response of cells to these signaling molecules. First, we demonstrate that *en* activity programs wing cells to express hh whereas the absence of e*n* activity programs them to respond to hh by expressing dpp. As a consequence, posterior cells secrete hh and induce a stripe of neighboring anterior cells across the compartment boundary to secrete dpp. Second, we demonstrate that dpp can exert a long-range organizing influence on surrounding wing tissue, specifying anterior or posterior pattern depending on the compartmental provenance, and hence the state of *en* activity, of the responding cells. Thus, dpp secreted by anterior cells along the compartment boundary has the capacity to organize the development of both compartments. Finally, we report evidence suggesting that dpp may exert its organizing influence by acting as a gradient morphogen in contrast to hh which appears to act principally as a short range inducer of dpp.

Key words: *engrailed*, *decapentaplegic*, *hedgehog*, *Drosophila*, wing, organizing activity, pattern formation, inducer, morphogen

INTRODUCTION

The adult appendages of *Drosophila* are each subdivided into precisely defined regions, the anterior and posterior compartments, which derive from adjacent but immiscible cell populations established early in development (Garcia-Bellido et al., 1973, 1976; Morata and Lawrence, 1975). In most regions of the body, these populations are founded around the time that the embryo becomes segmented (Steiner, 1976; Lawrence and Morata, 1977) by a process that involves the heritable activation of the homeodomain protein engrailed (en) in posterior, but not anterior, founder cells (Morata and Lawrence, 1975; Lawrence and Morata, 1976; Kornberg et al., 1985; DiNardo et al., 1985; Hama et al., 1990; Vincent and O'Farrell, 1992). *en* functions subsequently as a 'selector' gene (Garcia-Bellido, 1975), directing posterior cells to form posterior rather than anterior pattern, and to avoid mixing with anterior cells (Morata and Lawrence, 1975; Lawrence and Morata, 1976; Lawrence and Struhl, 1982).

Recently, we and others have found evidence that hedgehog (hh), a protein secreted by posterior cells (Lee et al., 1992; Mohler and Vani, 1992; Tabata et al., 1992; Tashiro et al., 1993) is responsible for organizing wing development, possibly by inducing neighboring anterior cells to express another secreted protein, decapentaplegic (dpp) (Basler and Struhl, 1994; Capdevila et al., 1994; Capdevila and Guerrero, 1994; Tabata and Kornberg, 1994). Here we show (i) that *en* activity in posterior cells directs them to express hh while at the same time blocking their ability to respond to hh, (ii) that the absence of *en* activity in anterior cells allows these cells to be induced by hh to express dpp, and (iii) that dpp can exert a long-range organizing influence on growth and patterning in both compartments. These results indicate that the selective deployment of *en* in posterior cells has two distinct roles. First, it directs the expression of the organizing signals hh and dpp in cells on opposite sides of the compartment boundary. Second, it controls the different ways anterior and posterior cells respond to these signals to generate distinct cell patterns in each compartment.

MATERIALS AND METHODS

Composition of transgenes

Tubα1>f+>dpp and *Tubα1>f+>en*

A plasmid containing the *Tubα1>y+>hh* gene (Basler and Struhl, 1994) in the *Carnegie20* transformation vector (Rubin and Spardling, 1983) was modified as follows: (i) the y^+ gene in the $>y^+>$ flp-out cassette was replaced by a 13 kb *Not*I-*Sal*I fragment containing the f^+ gene (Petersen et al, 1994), (ii) the ry^+ gene in the C20 vector was removed, (iii) the *hh* coding sequence was replaced by the coding sequence for *en* (position 1-1840, Poole et al., 1985) or *dpp* (position 1133-3096, Padgett et al., 1987), respectively. Transformants were identified by rescue of the forked mutant phenotype.

Tubα1>CD2, y+>hh

Similar to the *Tubα1>y+>hh* gene (Basler and Struhl, 1994) except

(i) the y^+ gene in the *>y+> flp*-out cassette is replaced by a *>CD2, y+>* flp-out cassette (Jiang and Struhl, 1995) and (ii) the ry^+ gene in the C20 vector was removed. The *>CD2, y+> flp*-out cassette is identical to the *>y+>* flp-out cassette present in the *Tubα1>y+>hh* gene except that it contains the coding sequence for the reporter protein rat CD2 (Dunin-Borkowski and Brown, 1995) inserted immediately downstream of the first FRT (>). Transformants were identified by rescue of the yellow mutant phenotype.

Tubα1>CD2, y+>en

Similar to *Tubα1>CD2, y+>hh*: except that the *hh* coding sequence is replaced by the *en* coding sequence.

Generation and identification of *Tubα1>en* and *Tubα1>dpp* clones in the wing

f^{36a} *hsp70-flp* females were crossed to f^{36a} males carrying a single insert of the *Tubα1>f+>en* or *Tubα1>f+>dpp* gene on the second or third chromosome, and the resulting progeny were subjected to a mild heat shock (34°C or 35°C for 30 minutes) during the first 72 hours of development. *Tubα1>f+>en* or *Tubα1>f+>dpp* adults which emerged from these crosses were collected and their wings dissected in ethanol, mounted in Euparal and screened under the compound microscope for the presence of forked wing hairs formed by f^{36a}*; Tubα1>en* or f^{36a}*; Tubα1>dpp* cells. Five independent lines of the *Tubα1>f+>en* gene, and two independent lines of the *Tubα1>f+>dpp* gene were used. All of the lines of each transgene behave similarly, so the results in each case have been pooled.

To generate *Tubα1>dpp* clones in a dpp^{d8}/dpp^{d10} background, a *Tubα1>f+>dpp* transgene on the second chromosome (#27) was recombined onto a dpp^{d8} chromosome (St. Johnston et al., 1990) and used to generate f^{36a} *hsp70-flp;* dpp^{d8} *Tubα1>f+>dpp/*dpp^{d10} progeny, which were subjected to a mild heat shock (33°C or 34°C for 30 minutes) during the first 48 hours of development. Adults of this genotype were recognized by their reduced eyes and reduced or absent wings.

Analysis of *Tubα1>en* clones in the wing

To assess the size and distribution of 'neutral' clones (Results), morphologically normal wings were scored under the compound microscope for the presence of f^{36a} *Tubα1>en* clones. The number of cells in each clone was determined by counting the forked hairs. A total of 215 neutral clones were identified in a sample of approximately 250 wings: 98 of these clones were located in the posterior compartment and the remaining 117 were in the anterior compartment (Table 1A). To assess the size and distribution of 'reorganizing' clones (Results), wings with altered venation pattern within the anterior compartment were identified under the dissecting microscope and then mounted and scored for forked hairs under the compound microscope. Clones of marked cells associated with ectopic veins both within and outside of the clone were designated as reorganizing clones (see Table 1B). To investigate the cause of venation defects in the posterior compartment, wings which were normal in shape but contained aberrant posterior venation patterns were identified under the dissecting microscope and scored under the compound microscope for the presence of clones. In 35/40 such wings, the defective venation was associated with clones which cross the line that normally demarcates the A/P-compartment boundary and autonomously fail to differentiate parts of vein 4. In the remaining 5 cases, small ectopic veins or distortions of the normal veins were associated with *Tubα1>en* clones located entirely within the posterior compartment. To assess further the frequency with which *Tubα1>en* clones cross the A/P-compartment boundary, a sample of 94 wings collected without reference to their morphology were scored for the presence of clones along the A/P compartment boundary. 16/27 clones found in the vicinity of the compartment boundary appeared to cross the A/P compartment boundary and 15/16 of these crossing clones were associated with venation defects in the posterior compartment.

Analysis of *Tubα1>dpp* clones in the wing

To assess the distribution of 'neutral' *Tubα1>dpp* clones (Results), approximately 350 wings of normal shape and size were screened for clones under the compound microscope and 238 clones identified. Of these, 89/136 anterior clones and 53/102 posterior clones contributed to only one surface of the wing. Of the remaining clones that contributed to both the dorsal and ventral surfaces, 34/47 anterior clones were located posterior to vein 2 and 47/49 posterior clones were located anterior to vein 5.

To assess the distribution of 'reorganizing' clones (Results), wings of abnormal shape or size were mounted and screened for clones. 75 reorganizing clones were identified, all of which contributed to both the dorsal and ventral surfaces of the wing. All 42 of these clones in the anterior compartment were positioned anterior to vein 2; similarly 27/33 of the posterior compartment clones were positioned posterior to vein 5.

To assess the frequency with which *Tubα1>dpp* clones reorganized pattern in regions anterior to vein 2, or posterior to vein 4, 287 wings obtained without reference to their morphology were scored for the presence of clones that contributed to both surfaces of the wing and were located either anterior to vein 2 or posterior to vein 4. 8/10 such clones located anterior to vein 2 were reorganizing, as were 2/2 clones located posterior to vein 5. In contrast, 16 such clones located between veins 2 and 5 were neutral.

Tubα1>en and *Tubα1>hh* clones in the wing disc

y hsp70-flp larvae, which carried a single copy of the *Tubα1>CD2, y+>en* gene as well as a single copy of either the *dpp-lacZBS3.0* (Blackman et al., 1991), dpp^{P10638} (R. Blackman, personal communication), or hh^{P30} (Lee et al., 1992) *lacZ* reporter gene were subjected to a mild heat shock during the first or second larval instar. Their wing discs were then recovered during the mid to late third instar, fixed and stained for lacZ, CD2 and/or en expression by standard immunofluorescence procedures (as in Basler and Struhl, 1994). The mouse monoclonal antibodies OX34 (Serotec) and 4D9 (Patel et al., 1989) were used to detect CD2 and en expression, respectively. A rabbit polyclonal antibody (Cappel) was used to detect lacZ expression. *Tubα1>hh* clones were generated similarly by using a *Tubα1>CD2, y+>hh* transgene in place of the *Tubα1>CD2, y+>en* transgene.

Clones of cells lacking *en-inv* gene activity in the wing disc

Marked clones of cells mutant for *en* and *inv* were generated by *flp*-mediated mitotic recombination (Golic, 1991; Xu and Rubin, 1993) by subjecting first instar larvae of the following genotypes to a single severe heat shock (37°C for 1 hour): (i) *y hsp70-flp/+; FRT42 πM/FRT42 Df(2R)*en^E*;* hh^{P30}*/+* (to monitor *hh-lacZ* expression), and (ii) *y hsp70-flp/+;* dpp^{P10638} *FRT42 πM/FRT42 Df(2R)*en^E (to monitor *dpp-lacZ* expression). The resulting third instar larvae were subjected to a second heat shock (37°C for 1 hour), and after a recovery period of 1 hour, their imaginal discs were fixed and stained for immunofluorescence as described above. A mouse monoclonal antibody, 9E10 (Evan et al., 1985) was used to detect expression of the πM marker protein.

RESULTS

In a previous study, we analyzed the organizing role of hh protein by ectopically expressing or eliminating *hh* function in genetically marked cells during imaginal disc development (Basler and Struhl, 1994). Here, we describe the results of similar experiments in which we have ectopically expressed or eliminated *en* or *dpp* gene function in the imaginal wing disc.

Anterior cells expressing *en* develop like posterior cells and reorganize anterior compartment pattern

Clones of cells that express the *en* coding sequence under the control of the constitutive *Tubulinα1* (*Tubα1*) promoter were generated by heat shocking f^{36a} larvae carrying the transgenes *hsp70-flp* and *Tubα1>f⁺>en* during the first or second larval instar (Materials and Methods). The *Tubα1>f⁺>en* transgene contains a *>f⁺>* flp-out cassette which includes the marker gene *forked* (f^+) and is flanked by targets ('>') for the site-specific flp recombinase. Hence, flp recombinase expressed under heat-shock control can excise the cassette, thereby eliminating the f^+ gene and generating marked *Tubα1>en* cells in which the *Tubα1* promoter drives expression of the *en* coding sequence.

Three classes of *Tubα1>en* clones were obtained: those that contribute solely to the posterior compartment, those that contribute solely to the anterior compartment and a third class containing clones that cross between the two compartments. We describe these classes in turn.

Clones restricted to the posterior compartment develop normally (Fig. 1A), as expected given that the endogenous *en* gene is normally active in all cells of this compartment.

Clones restricted to the anterior compartment are of two types, which we call 'neutral' and 'reorganizing' (Table 1). Neutral clones appear morphologically normal and are generally small or found close to the compartment boundary (Table 1A). In contrast, reorganizing clones are found only in more anterior portions of the compartment and are generally larger than neutral clones in the same region of the wing (Table 1B). Moreover, they are associated with abnormal patterns both within and outside of the clone (Fig. 1C-F). *Tubα1>en* clones in the anterior compartment express only low levels of *en* protein (below), perhaps accounting for the incomplete penetrance of the 'reorganizing' phenotype.

The most striking alterations of pattern associated with reorganizing clones are those caused by single clones that contribute to both the dorsal and ventral surface of the wing (Fig. 1D), or in rare cases by the conjunction of independent dorsal and ventral clones (Fig. 1C). If the normal pattern is described as 123/45m in which the numbers stand for longitudinal veins, '/' stands for the antero-posterior compartment boundary, and 'm' stands for the wing margin posterior to vein 5 (Fig. 1A), then these clones ('*') can be described as reorganizing surrounding tissue to form double-anterior 23*32 patterns (Fig. 1C,D). Reorganizing clones restricted to only the dorsal or ventral surface do not alter the overall shape and size of the wing. However, they do alter the behavior of surrounding anterior cells on the same surface, typically causing them to form a circular vein 3 (Fig. 1E,F). Thus, reorganizing clones induce neighboring anterior cells to form structures such as vein 3 normally found close to the compartment boundary, and can exert a more long-ranging influence on anterior cells further away, causing them to form more anterior structures such as vein 2. We note that the response of surrounding wild-type cells to *Tubα1>en* clones appears to depend principally on their distance from the clone. This is apparent both in the mirror-symmetry of the 23*32 patterns associated with clones that mark the dorsal and ventral surfaces of the wing (Fig. 1C,D) and in the formation of circular vein 3's at a constant distance from the boundary of clones restricted to either of the two surfaces (Fig. 1E,F).

Table 1. Distribution and size of neutral and reorganizing *Tubα1>en* clones in the anterior compartment of the wing

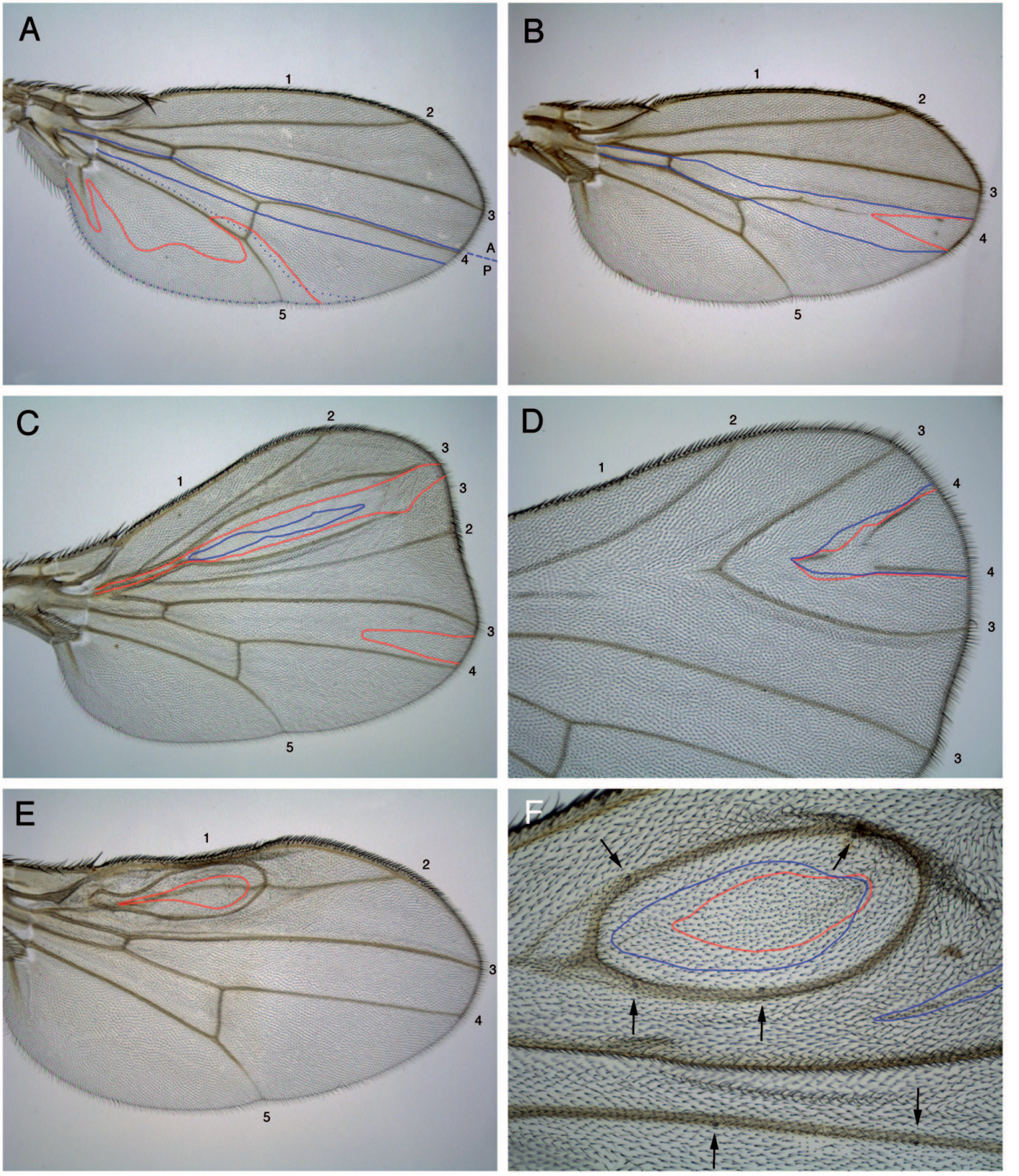
Size*	L1-L2	L2-L3	L3-L4
(A) Neutral clones			
small	8 (0)**	32 (0)	10 (0)
medium	0	11 (0)	16 (0)
large	0	4 (0)	36 (11)
total	8 (0)	47 (0)	62 (11)
(B) Reorganizing clones			
small	2 (0)	0	0
medium	17 (0)	0	0
medium/large	35 (0)	0	0
large	54 (23)	66 (46)	0
total	108 (23)	66 (46)	0

*Clone size was assessed by counting the number of marked hairs (each wing cell forms a single hair): small = less than 200 cells, medium = 200-300 cells, large = more than 300 cells, medium/large = clones that contained more than 200 cells but which could not be accurately assigned to the medium or large class due to folds or other disruptions of the normal pattern.

**The number of clones in each class that contributed to both the dorsal and ventral surfaces of the wing are shown in parentheses. Neutral clones were identified following inspection of morphologically normal wings under the compound microscope; reorganizing clones were identified under the dissecting microscope and scored under the compound microscope (Materials and Methods).

The altered wing patterns formed by wild-type cells surrounding reorganizing *Tubα1>en* clones closely resemble those caused by similarly positioned *Tubα1>hh* clones (Basler and Struhl, 1994), suggesting that *Tubα1>en* cells reorganize surrounding anterior tissue because they express *hh* ectopically (see below). However, *Tubα1>en* cells within these clones differ significantly from their *Tubα1>hh* counterparts in that they appear to behave like posterior rather than anterior cells. First, they differentiate posterior structures. For example, reorganizing clones that contribute to the wing margin form 'double row' bristles characteristic of the posterior wing margin as well as longitudinal veins which meet the wing margin but do not form campaniform sensillae (Fig. 1D). In normal wings, only veins 4 and 5 behave in this way, indicating that the ectopic veins formed by *Tubα1>en* cells are of posterior type. Because these ectopic veins appear to form very close to the clone border, similar to the proximity of the normal vein 4 to the compartment boundary (Fig. 1A), we infer that they are ectopic vein 4's, which are specified in response to signals emanating from surrounding anterior tissue. Second, cells within reorganizing clones do not appear to intermix normally with surrounding anterior cells. This is apparent in clones positioned anterior to vein 2, which are generally small and circular (Table 1; Fig. 1E,F) and have an abnormally high cell density (approximately twice that of neighboring wild-type tissue, data not shown).

The last class of *Tubα1>en* clones consists of clones that typically form a broad swath spanning the normal position of the compartment boundary and sometimes fail to form part or all of longitudinal vein 4 (Fig. 1B). Because *Tubα1>en* clones restricted to the posterior compartment develop normally whereas sibling clones restricted to the anterior compartment appear to develop like posterior cells, we infer that clones belonging to this third class derive from anterior cells that have acquired 'posterior' properties including the ability to assort with normal posterior cells and hence cross into the posterior



compartment. The occasional failure of these clones to form normal posterior structures such as vein 4 may be due to the fact that they express only low levels of *en* protein.

In sum, *Tubα1>en* clones appear to exert a similar organizing influence on surrounding anterior tissue to that exerted by *Tubα1>hh* clones. However, they differ from *Tubα1>hh* clones in that cells within the clone can differentiate posterior structures and assort preferentially with posterior cells.

Ectopic *en* activity reprograms anterior cells to express *hh* and to become refractory to hh signaling

To examine the consequences of ectopic *en* expression on *hh* and *dpp* expression within the imaginal wing disc, we subjected first and second instar larvae carrying two transgenes, *Tubα1>CD2, y+>en* and *hs-flp*, to a single mild heat shock (Materials and Methods). The *Tubα1>CD2, y+>en* transgene includes the coding sequence for a reporter protein,

Fig. 1. Reorganized or abnormal wing patterns associated with clones of *Tubα1>en* cells. (A) Morphologically normal wing containing two *Tubα1>en* clones in the posterior compartment. Longitudinal veins 1-5 are indicated along the wing margin and the transition from triple row to double row bristles is apparent along the anterior wing margin just posterior to vein 2. The anteroposterior compartment boundary (A/P) is positioned just anterior to vein 4, and is defined in this wing by a *Tubα1>en* posterior clone on the ventral surface (outlined in blue) in the posterior compartment which is restricted to the ventral surface and extends along the entire length of the boundary. This wing contains a second *Tubα1>en* clone, which contributes to both the dorsal and ventral surface of the posterior compartment (outlined, respectively by red and blue dotted lines). (B-F) *Tubα1>en* clones in the anterior compartment associated with reorganized wing patterns (clones are outlined in blue ventrally and in red dorsally; cells in the clone were identified by the f^{36a} marker which is not visible at this magnification). (B) A *Tubα1>en* clone which spans the compartment boundary. Some cells in the distal portion of the clone fail to differentiate part of vein 4. (C) Symmetric 23*32 pattern organized by two *Tubα1>en* clones, one on the dorsal and the other on the ventral surface of the wing. (D) A *Tubα1>en* clone which autonomously differentiated ectopic vein 4's positioned very close to the clone border at a constant distance from ectopic vein 3's organized in the surrounding, wild-type tissue. (E) A *Tubα1>en* clone restricted to the dorsal surface of the wing which has induced surrounding tissue to form a circular vein 3; formation of vein 2 on the ventral surface underneath is normal. (F) Two *Tubα1>en* clones, one dorsal and the other ventral, positioned between veins 1 and 2 which are both encircled by ring-shaped ectopic vein 3's. The dorsal vein 3 is decorated with campaniform sensillae (marked with arrows) and each circular vein 3 maintains a constant distance from the clone (note that the more elongated ventral clone has induced a correspondingly more elongated ring than the dorsal clone).

rat CD2 (Dunin-Borkowski and Brown, 1995), immediately downstream of the first flp recombination target (Materials and Methods). Hence, excision of the *>CD2, y+>* flp-out cassette generates *Tubα1>en* cells, which can be identified in the imaginal wing disc because they do not express CD2.

As shown in Fig. 2A, *Tubα1>en* clones in the anterior compartment express barely detectable levels of ectopic en protein. Nevertheless, in many clones, this ectopic en expression is sufficient to activate *hh* transcription in most or all cells of the clone (Fig. 2C), as monitored by the expression of a *lacZ* enhancer trap insertion into the *hh* gene (hh^{P30}; Lee et al., 1992). Further, it correlates with the formation of ectopic 'doughnuts' of *dpp* expression (Fig. 2A,B), as monitored by the expression of either of two *dpp-lacZ* reporter genes (*dpp-lacZBS3.0*, Blackman et al., 1991; dpp^{P10638}; Blackman, personal communication; Materials and Methods). As shown in Fig. 2B, these doughnuts result from expression of the *dpp-lacZ* gene solely by wild-type cells that surround the *Tubα1>en* clones. Finally, we note that *Tubα1>en* clones tend to form circular patches with smooth borders (Fig. 2A-C), providing a further indication that the cells within these clones minimize their contact with surrounding cells.

To compare the behavior of *Tubα1>en* cells with that of *Tubα1>hh* cells, we have also examined *hh-lacZ* and *dpp-lacZ* expression in wing discs containing marked *Tubα1>hh* clones. These were generated as described above for *Tubα1>en* clones, except that a *Tubα1>CD2, y^+>hh* transgene was used in place of the *Tubα1>CD2, y^+>en* transgene (Materials and Methods). As shown in Fig. 3A, *Tubα1>hh* clones differ from *Tubα1>en* clones in that they do not induce ectopic expression of the *hh-lacZ* gene. Moreover, in contrast to *Tubα1>en* clones, they are associated with ectopic *dpp-lacZ* expression in all cells of the clone, as well as in surrounding tissue (Fig. 3B). Finally, these clones form irregularly shaped patches with ragged borders indicating that *Tubα1>hh* cells are able to interdigitate with surrounding cells (Fig. 3).

We interpret these results as follows. Anterior cells that express *en* ectopically turn 'on' the *hh* gene and thereby induce neighboring anterior cells to turn 'on' the *dpp* gene. At the same time, they become refractory to the action of hh protein, accounting for the doughnut holes in *dpp-lacZ* expression. In contrast, anterior cells that express *hh* ectopically do not lose their competence to respond to hh protein and do not turn 'on' the endogenous *hh* gene. Thus, in terms of their effects on *dpp-lacZ* and *hh-lacZ* expression, *Tubα1>en* cells that arise in the anterior compartment behave as if they are transplanted posterior compartment cells and create an ectopic compartment boundary. In contrast, their *Tubα1>hh* counterparts behave like normal anterior cells that are positioned close to the compartment boundary and hence exposed to hh protein secreted by neighboring posterior cells.

In wild-type animals, *en* gene expression is strictly limited to posterior compartment cells until the middle of the third larval instar; however, anterior cells neighboring the compartment boundary subsequently express *en* or the *en*-related gene *invected* (*inv*), or both (Blair, 1992). This late *en-inv* expression appears to play a minor role in controlling the differentiation of anterior cells in the immediate vicinity of the compartment boundary (Hidalgo, 1994; our unpublished observations). We note that *Tubα1>en* gene activity appears to be functionally distinct from this late, endogenous *en-inv* activity. Specifically, *Tubα1>en* cells positioned next to the compartment boundary can turn 'off' dpp^{P10638} gene expression (e.g., Fig. 2A), turn 'on' the hh^{P30} gene and cause neighboring anterior cells to express dpp^{P10638}, and hence differ in all three respects from wild-type *en-inv*-expressing cells in the same position. Consequently, we suggest that the late *'en-inv'* activity in the anterior compartment reflects the expression of only *inv* and that inv protein differs from en in that it cannot block *dpp* transcription or activate expression of *hh*.

Loss of *en* activity in posterior cells reprograms them to stop expressing *hh* and to respond to hh protein by expressing *dpp*

Further evidence that the state of *en* gene activity controls the ability of wing cells to send as well as to receive the hh signal was obtained by examining clones of posterior cells lacking the endogenous *en* and *inv* genes. In this case, clones of cells homozygous for $Df(2R)en^E$, a deletion of *en* and the adjacent *en*-related gene *invected* (*inv*) (T. Kornberg, personal communication), were induced in wing imaginal discs using flp-mediated mitotic recombination (Golic, 1991). In this experiment, cells within the clone were independently marked either by the loss of en protein expression, or by the simultaneous loss of the marker gene *hs-πM* (Xu and Rubin, 1993; Materials and Methods).

Clones of $Df(2R)en^E$ cells in the posterior compartment fail to express the *hh-lacZ* gene, in contrast to surrounding

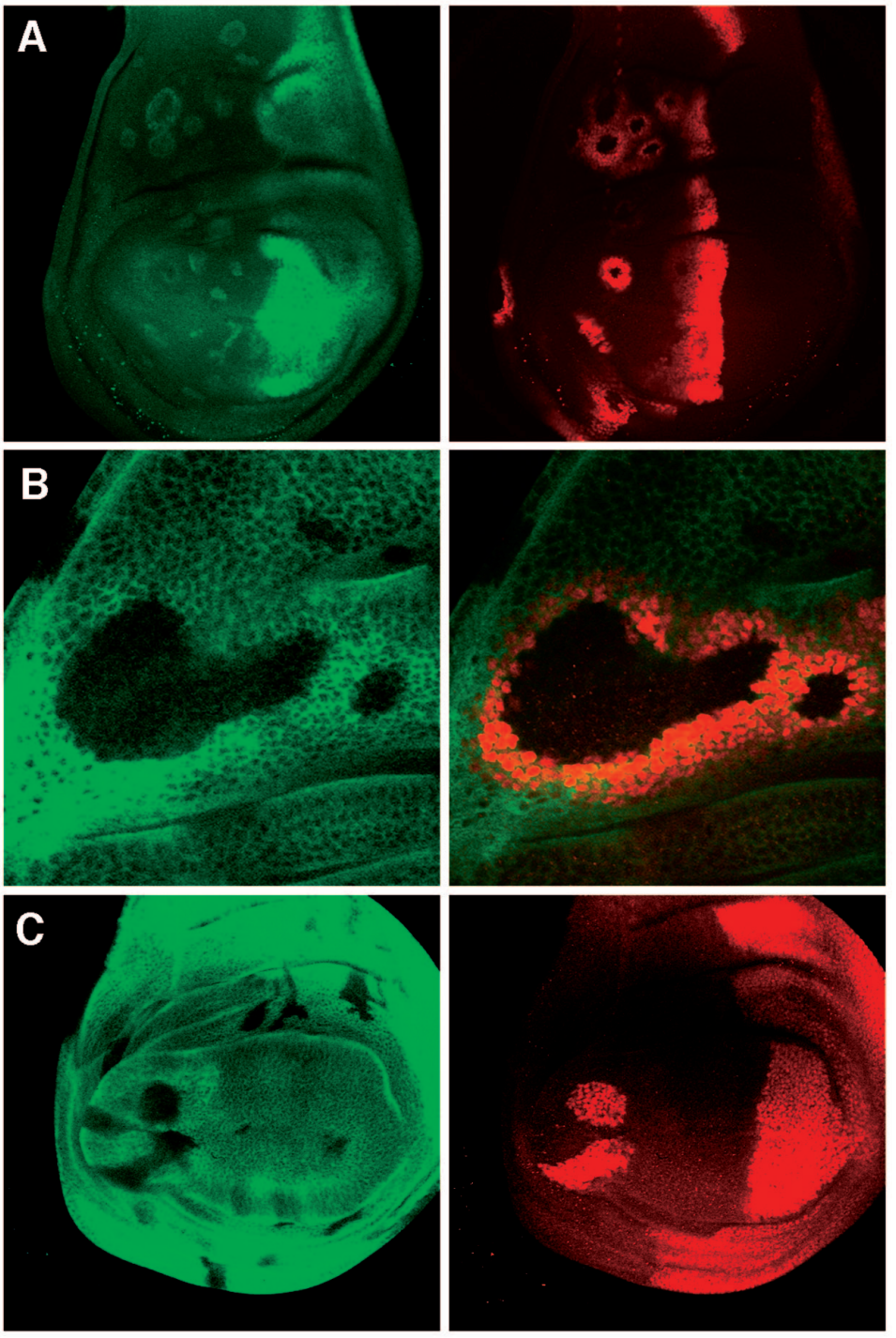


Fig. 2. Ectopic *dpp* and *hh* expression associated with anterior compartment clones of *Tubα1>en* cells. (A) *en* (green) and *dpp-lacZ* (red) expression associated with *Tubα1>en* clones in the wing disc. *en* is expressed in all cells of the posterior compartment and *dpp-lacZ* is expressed in a stripe of anterior cells running along the compartment boundary (here and in Figs 3 and 4 dorsal is up and posterior is to the right). *Tubα1>en* clones are apparent as circular patches of faint *en* expression in the anterior compartment, some of which are associated with ectopic 'doughnuts' of *dpp-lacZ* expression. (B,C) *Tubα1>en* clones in the anterior compartment marked by the absence of CD2 protein (green). Cells in these clones do not express *dpp-lacZ* themselves, but induce surrounding wild-type cells to do so (B). However, these cells can express *hh-lacZ*, shown in red (C; *hh-lacZ* is also expressed in all cells of the posterior compartment).

posterior cells (Fig. 4A,B). Moreover, all of the cells within these clones express the *dpp-lacZ* gene, in contrast to the surrounding posterior cells which do not (Fig. 4C,D). Similar results have also been reported recently by Sanicola et al. (1995). We also find that these *Df(2R)en*^{E} clones appear to stimulate excessive proliferation in surrounding wild-type cells. In particular, posterior wing compartments containing several clones appear to be abnormally large and the wild-type tissue neighboring these clones appears to contain extra folds (Fig. 4A,C). Previous analyses of *en* mutant clones in the adult have also suggested that posterior cells lacking *en* function stimulate excessive proliferation in surrounding cells (Lawrence and Morata, 1976; Hidalgo, 1994).

We interpret these results as follows. *en* gene activity normally programs wing cells to be hh signalers: that is, they secrete hh protein, but cannot respond to it by expressing dpp. Conversely, the absence of *en* activity programs them to be hh responders: they do not express hh, but respond to hh protein by expressing dpp. Consequently, both *Tubα1>en* anterior clones and *Df(2R)en*^{E} posterior clones create ectopic interfaces between signaling and responding cells, and hence lead to ectopic dpp expression.

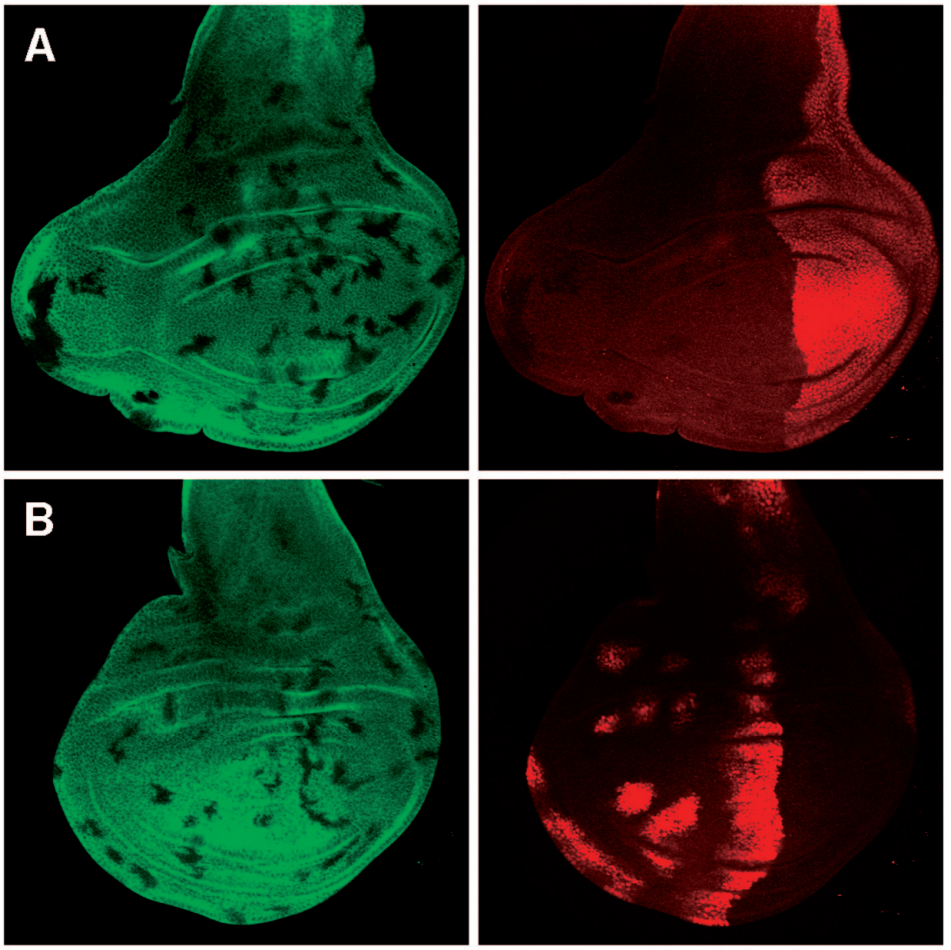


Fig. 3. *dpp-lacZ* and *hh-lacZ* expression in wing discs bearing clones of *Tubα1>hh* cells (*lacZ* expression is shown in red and the clones are marked by the absence of CD2 protein shown in green). *Tubα1>hh* clones do not. cause ectopic *hh-lacZ* expression in the anterior compartment (A), but cells within these clones express *dpp-lacZ* and induce their wild-type neighbors to do the same (B). Note also that the borders of *Tubα1>hh* clones are not smooth as in *Tubα1>en* clones in the anterior compartment (Fig. 2).

Organizing activity of dpp

The results of experiments involving gain or loss of *hh* or *en* function reveal a close association between dpp expression and the organizing activities of hh and en (Basler and Struhl, 1994; Capdevila et al., 1994; Capdevila and Guerrero, 1994; Tabata and Kornberg, 1994; here). Hence, dpp protein secreted by anterior cells may organize both anterior and posterior compartment patterns by exerting a spreading influence on cells on both sides of the boundary. If this inference is correct, ectopic dpp expression generated in either compartment should organize symmetric anterior or posterior patterns, depending on the compartmental provenance of the responding cells.

To test this prediction, we have induced clones of *Tubα1>dpp* cells marked by the loss of the f^+gene using a *Tubα1>*f^+*>dpp* transgene (Materials and Methods). As described above for the generation of *Tubα1>en* and *Tubα1>hh* clones, *Tubα1>dpp* clones were induced by a single, mild heat pulse during early larval development. We note that we have not been able to detect ectopic *dpp* protein expression associated with *Tubα1>dpp* cells with the available antibodies (Panganiban et al., 1990) indicating that the level is low relative to expression of the endogenous gene in anterior cells along the compartment boundary.

Following the induction of *Tubα1>dpp* clones, we obtained flies with two types of abnormal wings. First, we observed wings that were of normal size and shape, but which contained ectopic vein structures (e.g. Fig. 5A). In virtually all such cases, these ectopic vein structures were formed by *Tubα1>dpp* cells. Moreover, they could be formed by very small clones consisting of only a few cells. The *dpp* gene is known to have two distinct functions during wing development, a *disc* function, which is required for long-range organization of wing pattern, and a *shortvein* function, which is necessary for vein differentiation (Spencer et al., 1982; Segal and Gelbart, 1985; Posakony et al., 1990). Hence, we attribute the autonomous venation phenotype exhibited by *Tubα1>dpp* clones to the *shortvein* function of *dpp* and do not consider it further.

Second, we found wings of abnormal size and shape which displayed dramatic alterations of the normal pattern (Fig. 5B-D; see Materials and Methods for quantitative data). These abnormalities were invariably associated with clones of *Tubα1>dpp* cells that contributed to both the dorsal and ventral surface. However, unlike the autonomous ectopic vein phenotype described above, only a small fraction of the cells that contributed to these altered patterns were genotypically *Tubα1>dpp*. Hence, *Tubα1>dpp* cells can exert a long-range organizing influence on the behavior of surrounding wild-type cells. We describe the nature of these reorganized patterns below.

Anterior compartment clones associated with reorganizations of surrounding wild-type tissue were only observed in the more anterior portions of the anterior compartment (anterior to vein 2). In contrast to similarly positioned *Tubα1>en* and

Tubα1>hh clones, these *Tubα1>dpp* clones appear to organize less extensive patterns, which can be described as 12*21 or just 1*1 patterns, rather than 123*321 or 23*32 patterns (Fig. 5B,C). Thus, *Tubα1>dpp* cells in clones associated with reorganized anterior patterns appear to behave like wild-type cells positioned close to vein 2 and to organize surrounding tissue to form symmetric, more anterior patterns on either side.

We also found posterior *Tubα1>dpp* clones associated with reorganized patterns of posterior wing tissue, including the formation of supernumerary double posterior wings (Fig. 5D). These clones were all positioned posterior to vein 5 and caused patterns that can be described as m5*5m or m54*45m supporting our proposal (Basler and Struhl, 1994) that the normal /45m pattern is organized by dpp protein secreted by anterior cells adjacent to the compartment boundary.

To test further the hypothesis that localized dpp expression is sufficient to organize both anterior and posterior compartment patterns in the wing, we have generated genetically marked *Tubα1>dpp* clones in dpp^{d8}/dpp^{d10} animals. This combination of *dpp* mutations reduces normal *dpp* activity in the wing imaginal disc, blocking proliferation and patterning in both compartments. Consequently, dpp^{d8}/dpp^{d10} flies form only rudimentary wings (Spencer et al., 1982; Fig. 6A). As shown in Fig. 6B-F, this 'no wing' phenotype can be partially rescued by *Tubα1>dpp* clones. In particular, we find flies that bear 'winglets' composed of symmetric

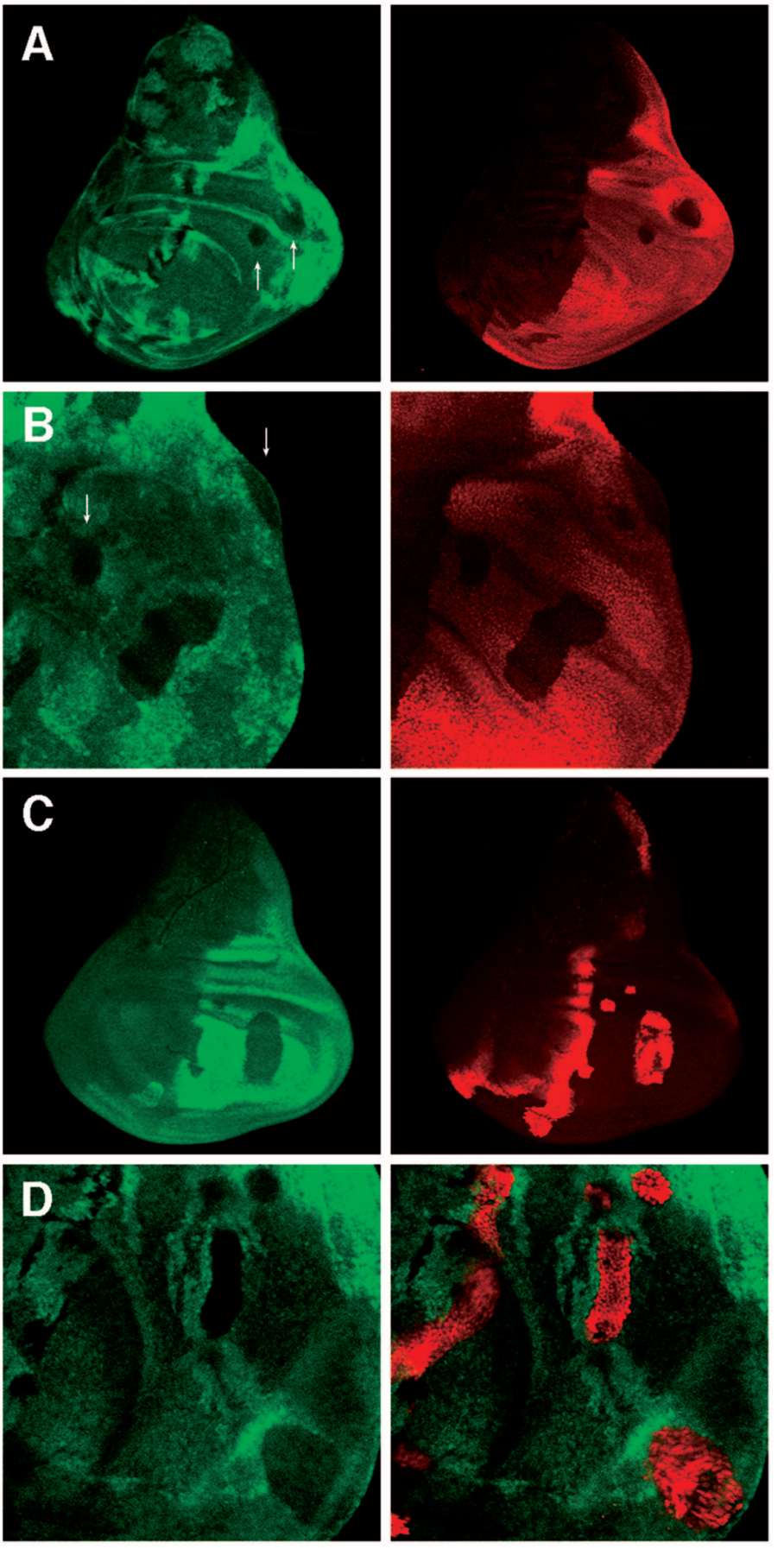


Fig. 4. Loss of *hh* expression and gain of *dpp* expression in clones of posterior compartment cells lacking *en* and *inv* gene function. *hh-lacZ* (A,B) and *dpp-lacZ* (C,D) expression (red) in wing discs containing clones of en^- inv^- cells (marked by the absence of *πM* expression (green) in A,B and D, and by the absence of endogenous *en* expression (green) in C). en^- inv^- cells in posterior compartment clones do not express *hh-lacZ* (A,B: arrows indicate representative clones). However all of the cells in these clones do express *dpp-lacZ* (C,D; in both these discs, *dpp-lacZ* is also expressed in a stripe of anterior cells running along the compartment boundary; to the left in each photograph). Note that the posterior compartments in A and C appear larger than their respective anterior compartments and that the larger clone in A is associated with extra folds, both indications that en^- inv^- cells in the posterior compartment stimulate proliferation in surrounding wild-type tissue.

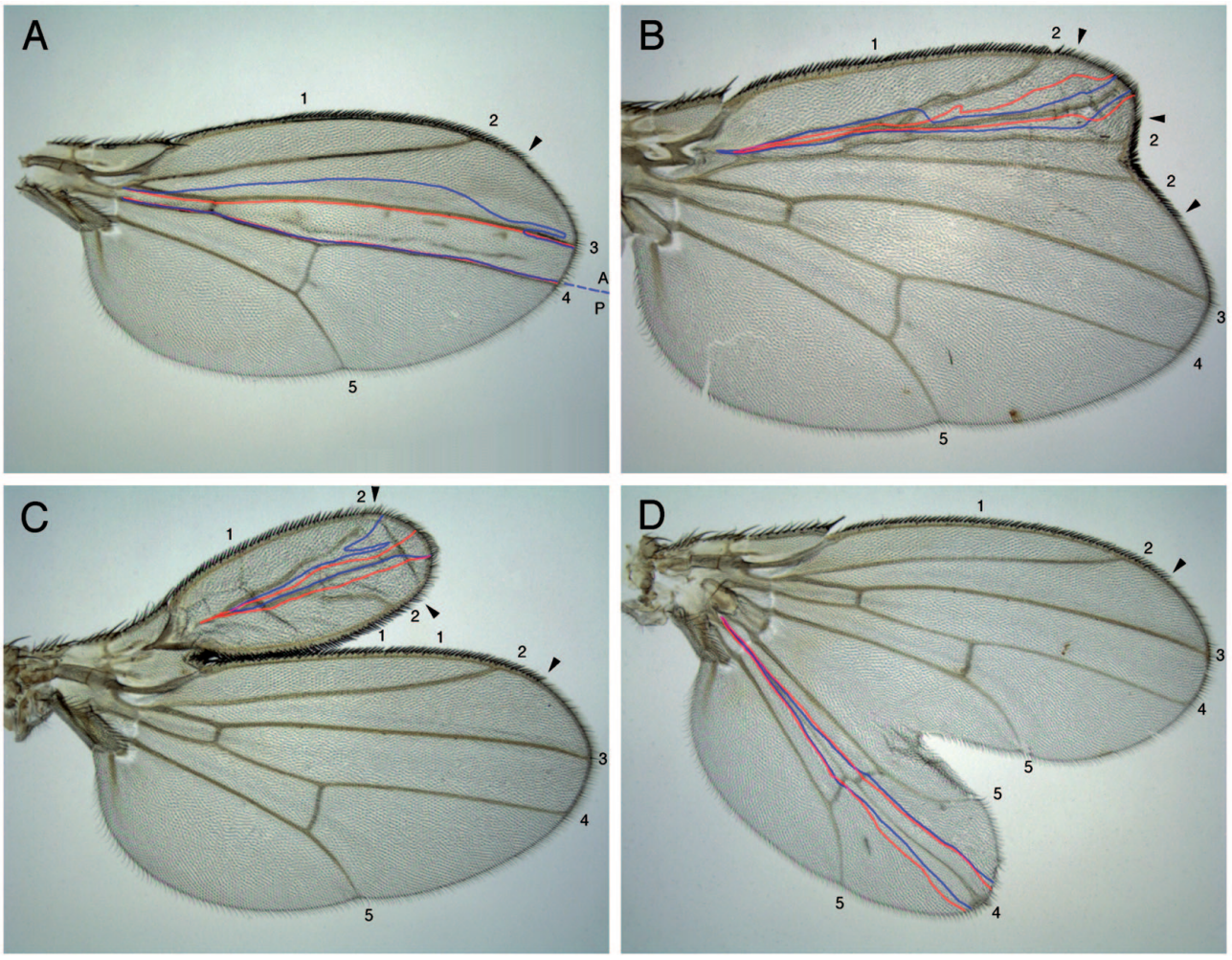


Fig. 5. Reorganized wing patterns associated with clones of *Tubα1>dpp* cells. Wings containing *Tubα1>dpp* clones that contribute to both surfaces of the wing (clone borders are outlined in blue ventrally and in red dorsally, veins are numbered along the wing margin, and the transition between triple and double row bristles in marked by arrowheads; cells in the clone were identified by the f^{36a} marker which is not visible at this magnification). (A) An anterior compartment clone positioned next to the compartment boundary (i.e., in the region in which *dpp* expression is normally induced by *hh*). This clone does not reorganize the pattern of neighboring tissue, but some cells within the clone differentiate extra vein tissue (visible between veins 3 and 4). (B) 2*2 pattern organized by a *Tubα1>dpp* clone which is positioned just anterior to the normal vein 2 in the anterior compartment. Note that the clone lies along the plane of symmetry of the duplicated anterior patterns. (C) A 12*21 double-anterior wing induced by an anterior *Tubα1>dpp* clone positioned anterior to the normal vein 1. (D) An m54*45m double-posterior wing organized by a *Tubα1>dpp* clone positioned posterior to the normal vein 5 in the posterior compartment.

double-anterior (1*1) or double-posterior (m*m) patterns and, in every such winglet, we observe a stripe of *Tubα1>dpp* cells along the plane of symmetry on both the dorsal and ventral surface (Fig. 6C,D). Moreover, we also find flies that form asymmetric anterior-posterior winglets exhibiting a 1*m pattern (Fig. 6B,E,F) as well as flies that form two winglets from a single wing primordium (e.g., Fig. 6F). In all cases in which two winglets are formed by a single primordium, double-anterior winglets arise anteriorly and double-posterior winglets arise posteriorly to the remaining winglet (e.g., Fig. 6F). Thus, ectopic *Tubα1>dpp* expression can suffice to organize both anterior and posterior wing pattern, even in the absence of endogenous *dpp* gene function. Moreover, clones arising in the anterior or posterior compartment at a distance from the boundary appear to organize double-anterior or double-posterior winglets, respectively, whereas clones arising in close proximity to the boundary appear to organize asymmetric anterior-posterior winglets composed of cells from both compartments. Thus, we infer that the type of pattern formed depends on the compartmental provenance of the responding cells and hence on the state of activity of the *en* gene.

We also note that double anterior and double posterior winglets generated by *Tubα1>dpp* clones in the absence of endogenous *dpp* activity appear to be less extensive than those organized by *Tubα1>dpp* clones in wild-type wings. For example, such clones in the anterior compartment form 1*1 rather than 12*21 patterns, while those in the posterior compartment form m*m rather than m5*5m or m54*45m patterns (compare Figs 5C and 6C, and Figs 5D and 6D). In principle this could be because *Tubα1>dpp* expression in otherwise wild-type wings can induce the expression of the endogenous *dpp* gene. However, as noted above, we cannot detect any dpp

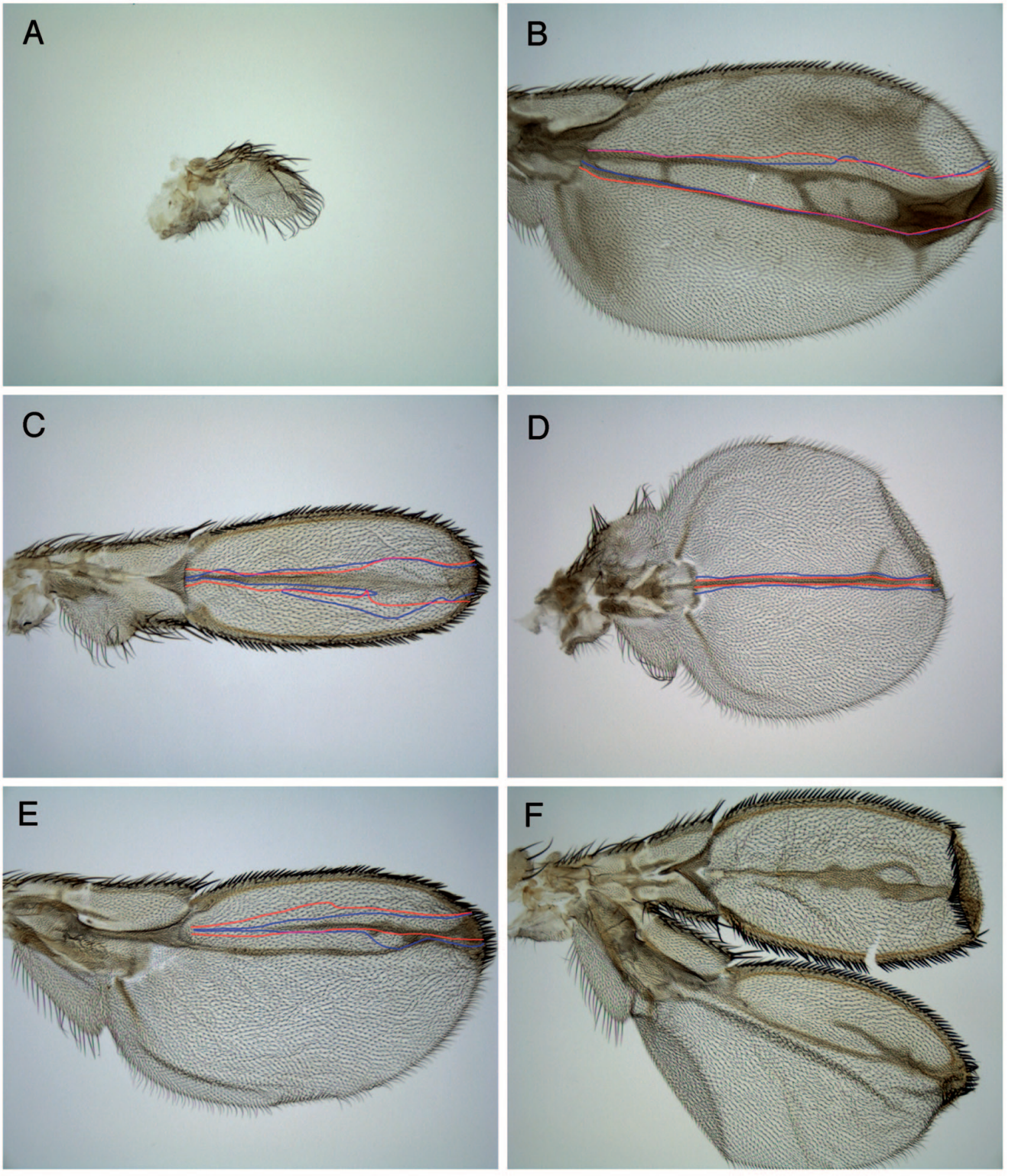


Fig. 6. Winglets organized by clones of *Tubα1>dpp* cells in *dpp* mutant wings. (A) 'No wing' phenotype associated with the dpp^{d8}/dpp^{d10} mutant genotype. This transheterozygous combination of *dpp* mutations selectively reduces normal *dpp* activity in the wing imaginal disc, blocking patterning and proliferation in both compartments. Only the most proximal anterior and proximal posterior margin structures are apparent. (B-F) Partial rescue of the no wing phenotype by clones of *Tubα1>dpp* cells. All such rescued wings have *Tubα1>dpp* clones that contribute to the dorsal and ventral surface of the wing and form stripes running down the middle of each winglet (clones outlined in red dorsally and blue ventrally). Symmetric double-anterior (1*1) and double-posterior (m*m) winglets are shown, respectively, in C and D. Asymmetric anterior-posterior (1*m) winglets are shown in B and E. Two winglets formed by a single disc are shown in F: note that the double anterior 1*1 winglet (top) is positioned anteriorly to the anterior-posterior (1*m) winglet below. A-F are shown at the same magnification (and at 1.5× the magnification in Figs 1 and 4).

protein expression associated with *Tubα1>dpp* clones; moreover *Tubα1>dpp* cells do not appear to induce expression of *dpp-lacZ* reporter genes (data not shown). Another possibility is that in dpp^+ discs, dpp protein secreted by *Tubα1>dpp* cells acts additively with endogenous dpp protein secreted by anterior cells along the compartment boundary.

In sum, ectopic expression of dpp can reorganize both anterior and posterior wing pattern, depending on the compartmental provenance of the responding cells. This result supports the proposal that hh organizes both anterior and posterior wing development by inducing anterior cells along the compartment boundary to secrete dpp. Moreover, it indicates that dpp specifies anterior or posterior wing patterning according to the state of *en* activity in responding cells. We also find that the level of ectopic dpp expression generated in these experiments is low relative to endogenous *dpp* expression and suffices to reorganize only those portions of each compartment which are positioned at a distance from the compartment boundary. As we discuss below, these and related findings provide suggestive evidence that dpp exerts its organizing influence by acting as gradient morphogen.

DISCUSSION

Selector genes, compartments and limb development

Compartments have been proposed to play at least two roles: (i) to define distinct cell populations within which certain 'selector' genes control developmental fate (the compartment hypothesis; Crick and Lawrence, 1975; Garcia-Bellido, 1975; Lawrence and Morata, 1976), and (ii) to define interactive cell populations that generate organizing signals along the boundaries between them (the boundary hypothesis; Meinhardt, 1983; see also Crick and Lawrence, 1975). Our analysis of *en*, *hh* and *dpp* function in the wing provides evidence that anterior and posterior compartments perform both roles during limb development, and that they do so because of the consequences of the different states of *en* activity in each compartment.

First, our results confirm and extend previous findings (Morata and Lawrence, 1975; Lawrence and Morata, 1976; Lawrence and Struhl, 1982; Morata et al., 1983; Busturia and Morata, 1988) that *en* activity in posterior cells directs them not to intermix with anterior cells and hence subdivides each limb primordium into adjacent but immiscible cell populations. This is most clearly illustrated by our finding that ectopic *en* expression in anterior cells causes these cells to form circular patches that minimize contact with surrounding anterior cells or to cross the compartment boundary and mix with posterior cells on the other side (Figs 1, 2; see also Busturia and Morata, 1988). Conversely, we also observe that loss of endogenous *en* activity in posterior cells can lead to their minimizing contact with surrounding posterior cells, extending previous observations that such clones cross into the anterior compartment or are eliminated from the surrounding *en*-expressing epithelium (Lawrence and Struhl, 1982; Morata et al., 1983).

Second, we show that the state of *en* activity (whether 'on' or 'off') also dictates whether wing cells secrete *hh* protein or can respond to *hh* protein by secreting *dpp* (Figs 2, 3). Because *en* encodes a homeodomain protein implicated as a transcriptional repressor (Jaynes and O'Farrell, 1990; Han and Manley, 1993), *en* protein could directly bind targets in *dpp* and block transcription. This possibility is supported by the recent finding of en binding sites in the *dpp* gene which appear to mediate transcriptional repression of *dpp* in the posterior compartment (Sanicola et al., 1995). Similarly, it may bind and repress the transcription of other genes expressed only by anterior cells and involved in mediating their ability to respond to secreted hh protein (e.g., *patched*, *cubitus interruptus*^{D} (ci^D); reviewed in Hooper and Scott, 1992). The role of *en* in driving *hh* transcription is more complex because the initial transcription of *hh* in posterior cells is not dependent on *en* (Lee et al., 1992; Mohler and Vani, 1992; Tabata et al., 1992; Tashiro et al., 1993). Furthermore, although *hh* transcription in embryos subsequently becomes *en* dependent, the prevalent view is that this reflects a non-autonomous requirement for *en* function mediated by *en*-dependent wg signaling from neighboring cells (e.g., Perrimon, 1995). For the imaginal wing disc, our data establish that *en* activity is both necessary and sufficient to drive *hh* expression (Figs 2, 3) and indicate that the state of *en* gene activity autonomously dictates the transcriptional activity of the *hh* gene. Accordingly, we argue that once embryonic cells become heritably commited to either express or not express *en*, their descendants will be hh signalers or hh responders for the remainder of development.

Third, as a consequence of the ability of *en* to control transcription of downstream genes such as *hh*, *dpp*, *ptc* and ci^D, the selective activity of *en* in posterior cells creates an inductive interface wherever populations of anterior and posterior cells abut. The importance of this interaction is illustrated by experiments in which *en* is ectopically activated in anterior compartment cells (Figs 1, 2). Under these circumstances, the creation of an ectopic interface between *en*-expressing and non-*en*-expressing cells causes cell pattern to be reorganized on both sides of the interface. This is dramatically illustrated in Fig. 1D by the formation of both posterior (vein 4) and anterior (vein 3) pattern elements on opposite sides of the border of a *Tub>en* clone. Thus, the different epigenetic states of posterior and anterior cells allows them to interact across the compartment boundary to elicit signals that can organize growth and patterning in both compartments.

Finally, we show that cells expressing dpp can organize both anterior and posterior wing pattern (Figs 4, 5). Although ectopic dpp expression has previously been shown to cause the formation of supernumerary anterior or posterior wing patterns (Capdevila and Guerrero, 1994) these studies did not address the question of whether the ectopic dpp-expressing cells exert a long-range organizing influence on surrounding, non-expressing cells. Here we report that ectopic dpp-expressing cells are indeed able to exert a long-range influence over surrounding non-dpp-expressing cells. Moreover, we have obtained evidence that the compartmental provenance of the responding cells determines whether they respond by forming anterior or posterior pattern. We therefore infer that the state of *en* activity (i.e., whether 'on' or 'off') governs not only the abilities of wing cells to intermix and to induce organizing signals such as dpp, but also their capacity to respond to these signals in distinct ways, thereby generating different cellular patterns.

Subcompartmentalization of the wing and patterning along the proximodistal axis

In our prior analysis of ectopic *hh*-expressing cells (Basler and Struhl, 1994), we found that the ability of these cells to organize supernumerary double-anterior wings depends on their contributing to both the dorsal and ventral surfaces of the wing, which are themselves compartments established during larval development (Garcia-Bellido et al., 1973, 1976). In the present study, we have obtained similar findings for clones of ectopic *en*-expressing and ectopic *dpp*-expressing cells. These findings reinforce our proposal (Basler and Struhl, 1994) that the organization of cell proliferation and patterning along the proximodistal axis of the wing may require two signals: one, *dpp*, induced along the anteroposterior compartment boundary; the other induced by interactions between cells on opposite sides of the dorsoventral compartment boundary (Diaz-Benjumea and Cohen, 1993; Blair et al., 1994; Irvine and Wieschaus, 1994; Tabata and Kornberg, 1994; Williams et al., 1994). These two signals would normally be generated together in the vicinity of cells destined to give rise to the distal tip of the adult wing, consistent with the notion (Campbell et al., 1993) that they would define a distal 'organizer' governing growth and patterning along the proximodistal axis.

Are hh and dpp morphogens or inducers?

hh and dpp each belong to extensive families of signaling molecules which have potent organizing activities in animal development. Depending on the organism and developmental process examined, they have been described variously as inducers or morphogens. Accordingly, there is considerable controversy about how they exert their organizing influence – in particular, whether they do so by initiating or furthering a chain of inductive interactions, or by a gradient mechanism in which different concentrations dictate different cellular outputs.

In the context of the developing *Drosophila* wing, our present results provide strong support for the hypothesis that the long-range organizing activity of hh is mediated indirectly, through the short-range induction of dpp (Basler and Struhl, 1994). In the case of the anterior compartment, we previously demonstrated that hh is both necessary and sufficient to induce dpp expression in anterior wing cells and to organize anterior wing pattern. Here we extend these findings by showing that dpp expression can exert a similar long-range organizing influence on anterior pattern. Hence, the long-range organizing activity of hh can be attributed to its ability to induce dpp. The argument that dpp mediates the long-range organizing activity of hh is further strengthened by recent studies of the involvement of protein kinase A (PKA) in hh signal transduction (Jiang and Struhl, 1995; Lepage et al, 1995; Li et al., 1995; Pan and Rubin, 1995). These studies have shown that the loss of PKA activity mimics the reception of the hh signal and causes long-range reorganizations of anterior wing pattern. Moreover, they have estabished that these reorganizations result from ectopic expression of dpp by PKA-deficient cells.

The situation in the posterior compartment provides an additional line of evidence that hh organizes wing development by inducing dpp. Our present results show that *en* gene activity in posterior cells renders them refractory to hh signaling, even though they express the *hh* gene and are presumably exposed to secreted hh protein. Nevertheless, growth and patterning of this compartment depends critically on *hh* gene activity (Basler and Struhl, 1994; Hidalgo, 1994) as well as on dpp expression in neighboring anterior cells across the compartment boundary (Posakony et al., 1990). Because *en* activity blocks the ability of posterior cells to respond to hh protein, it follows that hh must organize posterior compartment development indirectly by inducing dpp expression in neighboring anterior cells. This inference is further supported here by our demonstration that ectopic dpp expression in the posterior compartment can organize symmetric double-posterior wings (Fig. 4D). Thus, as in the anterior compartment, the long-range organizing role of hh in the posterior compartment can be attributed solely to the induction of dpp in anterior cells neighboring the compartment boundary.

Thus, for hh, the available evidence provides a strong argument that most or all of its organizing activities in the developing wing are due to its activity as a short-range inducer. We note that our experiments do not rule out the possibility that cell patterning in the vicinity of the compartment boundary may depend on other short-range responses to hh aside from dpp induction. Indeed, hh appears to trigger several other responses in anterior cells close to the boundary such as the expression of the reporter genes *LF06* and *P1531* and enhanced transcription of the gene *ptc* (Tabata and Kornberg, 1994; Capdevila and Guerrero, 1994). However, there is presently no evidence to suggest that different concentrations of hh protein elicit distinct short-range responses involved in organizing cell pattern. Our interpretation of hh as a short-range inducer contrasts with the recent proposal that hh exerts a long-range organizing influence on the dorsal epidermis of the larva by acting as a concentration-dependent morphogen (Heemskirk and DiNardo, 1994). However, we note that dpp is normally expressed in cells contributing to the dorsal larval epidermis (Jackson and Hoffmann, 1994) and, hence, may be mediating the organizing influence of hh in this tissue as well.

In contrast to hh, our results with dpp provide evidence that appears to favor a morphogen interpretation. Specifically, we observe that levels of ectopic dpp expression that are sufficiently low to fall beneath our level of detection are 'neutral' when generated close to the compartment boundary, the source of endogenous dpp protein, but can reorganize wing pattern when generated at a distance from the boundary in either compartment. Moreover, in the absence of endogenous dpp activity, these low levels of ectopic dpp expression organize only those portions of anterior or posterior compartment pattern that are normally formed at a distance from the compartment boundary. As noted previously, (Tickle et al., 1975; Tickle, 1981; Struhl and Basler, 1993), the ability of low levels of a putative signaling molecule to organize such subdomains of the normal pattern favors models involving gradient rather than sequential inductive mechanisms.

One criticism of this type of evidence is that hh signaling may induce the expression of molecules other than dpp, and these molecules may act in concert with dpp or modulate its activity to specify other elements of wing pattern closer to the compartment boundary. According to this hypothesis, the restricted organizing activities of ectopic dpp would be due to the absence of these other factors rather than to the limited amounts of ectopic expression that we have been able to generate experimentally. However, we have observed that

posterior cells in dpp^{d8}/dpp^{d10} wing discs appear to express *hh* normally (as assayed by the expression of the hh^{P30} reporter gene; data not shown). Hence, other responses to hh signaling aside from the induction of dpp should be normal. Nevertheless, *Tubα1>dpp* cells appear to have a similarly modest organizing activity in these discs, irrespective of whether they are expressed close to the compartment boundary, or at distance (Fig. 6). Consequently, the restricted organizing ability of *Tubα1>dpp* cells positioned at a distance from the compartment boundary does not appear to be due to the absence of other factors that are normally induced along the boundary by hh.

Another line of evidence that argues in favor of a gradient mechanism is our observation that the organizing potency of *Tubα1>dpp*-expressing cells is enhanced by the presence of endogenous *dpp* gene function (compare Figs 5 and 6). Although we do not know the mechanism by which this enhancement occurs, the fact that it occurs suggests that different concentrations of dpp protein can trigger different responses.

The key attribute of a morphogen, as distinct from an inducer, is that a morphogen organizes pattern by dictating distinct cellular responses at different concentrations. Hence, if *dpp* functions as a morphogen, we would expect that progressive increases in the level of ectopic *dpp* expression would correlate with corresponding increases in organizing potency. Such experiments have been performed for the dorsoventral organizing activity of *dpp* in early embryos and support the conclusion that *dpp* functions as a morphogen in this context (Ferguson and Anderson, 1992; see also Wharton et al., 1993). It remains to be seen whether equivalent experiments with *dpp* in the imaginal discs will provide further support for a gradient mechanism during limb development.

We thank T. Kornberg for communicating results and providing the $Df(2R)en^{E}$ mutation prior to publication, Y. Chen and M. Brunner for help with generating clones of $Df(2R)en^{E}$ cells, M. Brunner, A. Nakanishi and R. Perez for technical assistance, P. Beachy, R. Blackman, T. Kornberg, T. Xu and the Bloomington stock center for fly stocks, N. Brown for the CD2 coding sequence, N. Patel for the en antibody, V. Corces for the *f* genomic DNA, and Y. Chen, J. Dubnau, I. Greenwald, E. Hafen, T. Jessell, J. Jiang, P. A. Lawrence and A. Tomlinson for discussion and comments on the manuscript. K. B. is supported by the Swiss National Science Foundation and the Kanton of Zürich, and G. S. is an Investigator of the Howard Hughes Medical Institute.

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(Accepted 1 May 1995)