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Creating a *Drosophila* wing de novo, the role of *engrailed*, and the compartment border hypothesis

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SUMMARY

Anterior/posterior compartment borders bisect every *Drosophila* imaginal disc, and the *engrailed* gene is essential for their function. We analyzed the role of the *engrailed* and *invected* genes in wing discs by eliminating or increasing their activity. Removing *engrailed/invected* from posterior wing cells created two new compartments: an anterior compartment consisting of mutant cells and a posterior compartment that grew from neighboring cells. In some cases, these compartments formed a complete new wing. Increasing *engrailed* activity also affected patterning. These findings demonstrate that *engrailed* both directs the posterior compartment pathway and creates the compartment border. These findings also establish the compartment border as the pre-eminent organizational feature of disc growth and patterning.

Key words: *Drosophila*, *engrailed*, *invected*, compartments, clonal analysis, wing disc

INTRODUCTION

The segments of the insect epidermis are subdivided into developmental compartments (Garcia-Bellido et al., 1973; Lawrence, 1973). Initially there are two per segment, an anterior and a posterior compartment, and each compartment generates a precisely defined region. The compartments' boundaries present an absolute barrier to cell movements and so can be revealed as lines of cell lineage restriction, but they rarely reveal their position with any morphological landmarks. Compartment borders leave little direct evidence of their presence.

Garcia-Bellido proposed that compartmentalization involves a series of binary subdivisions that progressively restrict the developmental potential of imaginal disc cells (Garcia-Bellido, 1975). Although strong evidence exists today only for the A/P compartments present in every segment and for the dorsal and ventral compartments that subdivide the wing and haltere discs, these few partitions and the borders that they create nevertheless play a key role in disc organization and growth. The D/V subdivision is controlled by the *apterous (ap)* gene which is expressed by the dorsal wing and haltere cells (Blair, 1993; Diaz-Benjumea, 1993; Williams et al., 1993). The A/P subdivision is controlled by the *engrailed (en)* gene (Lawrence and Morata, 1976) which is expressed by the cells of the posterior compartments (Kornberg et al., 1985). Clues to the role of compartments can be gleaned from the phenotypes of *ap* and *en* mutants and from the properties of other genes that are expressed in compartment-specific patterns.

Loss of *ap* function changes dorsal cells into ventral cells, and the affected cells may join the ventral compartment (if they are close to the compartment border), or they may induce an ectopic border that separates them from their dorsal neighbors (Blair, 1993; Diaz-Benjumea, 1993). Such mutant clones lose two attributes of normal dorsal cells – their dorsal character and their recognition of the D/V compartment border as an impediment to movement. Although posterior compartment cells that lose *en* function change to anterior type and cross the A/P compartment border in an analogous manner, the transformations are incomplete. Somatic clones of lethal *en* alleles alter the pattern of veins in the posterior wing and make innervated anterior-type bristles along the posterior wing margin (Kornberg, 1981; Lawrence and Struhl, 1982), but these transformations are less severe than those caused by the viable en^1 allele and they do not produce extensive anterior patterns. Accounting for these partial transformations has been difficult, especially since these *en* mutations terminate EN protein translation prematurely and are apparent null alleles (Gustavson, 1993).

Other genes expressed in a manner that reflects A/P disc subdivision include, *hedgehog (hh)* and *invected (inv)*, both expressed in all posterior compartment cells (Coleman et al., 1987; Tabata et al., 1992), *cubitus interruptus (ci)*, expressed in all anterior compartment cells (Eaton and Kornberg, 1990), and *patched* (*ptc*; Phillips et al., 1990), *decapentaplegic* (dpp; Raftery et al., 1991) and *LF06* (S. Eaton and T.B.K., unpublished data), each expressed most prominently along the anterior side of the A/P border. These patterns of expression are functionally related. *Inv* is physically linked to *en* and both genes are apparently controlled by the same regulatory region (Gustavson, 1993). Directly or indirectly, *en* positively regulates *hh* and negatively regulates *ci*, *ptc* and *dpp* (Tabata

et al., 1992; Sanicola et al., 1995; Schwartz et al., 1995). HH protein can cross the A/P compartment border and effect the increased expression of *ptc*, *dpp*, *LF06* and other genes in anterior cells along the A/P compartment border (Tabata and Kornberg, 1994). It is believed that activation of *dpp* is of critical importance (Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Kojima et al., 1994), since it encodes a secreted transforming growth factor β-like protein (Padgett et al., 1987) that may act as a long range morphogen. Since the shape of the stripes of cells that express *dpp* and the other border-specific genes is directly related to the distribution of HH protein, which is itself determined by the shape of the compartment border, the control of gene expression in the anterior compartment that *en* effects through its regulation of *hh*, functionally links the two compartments and directly links the border with regulation of pattern and shape (Tabata and Kornberg, 1994).

The present study was initiated to understand better the functional relationship between *en* and other compartment-specific genes. Using mutations that abolish both *en* and *inv* activity, we found that *hh* expression declined while *dpp*, *ci*, and *ptc* expression increased in cells that lost *en* and *inv* function. The consequence is a complete anterior transformation, accompanied by formation of a new A/P compartment border and a new axis of A/P polarity. These remarkable transformations reveal a surprising plasticity and autonomy in compartment formation and function, and indicate that juxtaposition of anterior and posterior compartment cells alone provides sufficient information to create a new compartment border. When appropriately situated, such ectopic borders can initiate the formation of an appropriately patterned imaginal appendage.

MATERIALS AND METHODS

Imaginal disc staining

Standard protocols for immunofluorescence and immunohistochemistry were used as described previously (Tabata and Kornberg, 1994). Antibodies used were: rat anti-PTC (from R Johnson and M Scott); rabbit anti-DPP (from M. Hoffmann); mouse anti-MYC (from G. Ramsay and M. Bishop); rat anti-CI (unpublished and from R. Holmgren); rabbit anti-HH (Tabata and Kornberg, 1994) and mouse 4D9 monoclonal anti-EN/INV (Patel et al., 1989). Secondary reagents were from Jackson Immunologicals.

Generation of mutant clones

Clones of mutant cells were generated by FLP-mediated mitotic recombination as described by Golic (1991), and Xu and Rubin (1993). Genotypes were: *FRT43D Df* en^E*; FRT43D* en^{LA10}; *FRT43D* inv^{30} $en^{9\text{-}6}$; *FRT43D* ptc^{7m} $Dfen^E$, and *FRT43D* ptc^{6D} $Dfen^E$. *FRT43D* ptc^{7m} and *FRT43D* ptc^{6D} yielded similar results.

For clones in discs, *y hsp70-FLP/Y; FRT43D* en^{LA10}/+ flies were crossed with *w*;*FRT43D Myc45F, 47F*. For clones in wings, *y hsp70-FLP/Y; FRT43D (mutant)*/+ flies were crossed with *y w*;*FRT43D y*+. FLP-mediated recombination was induced by incubating second instar larvae in a water bath for 60 minutes at 37°C. In excess of 20,000 *Df* en^E wings with abnormal patterns were analyzed. For other genotypes, approximately 1,000 wings with abnormal patterns were analyzed.

Expression of EN protein by UAS/GAL4

EN protein was ectopically expressed by the UAS/GAL4 system developed by Brand and Perrimon (1993). The UAS-*en* construct was made by placing an *Eco*RI cDNA fragment containing the *en* ORF into pUAST. The UAS-*en* transgenic fly was crossed to GAL4-enhancer-trap lines 112-97 (a gift from J. N. Jan) and *en*-GAL4 (a gift from K. Yoffe and N. Perrimon) and grown at either 28°C (112-97) or 25°C (*en*-GAL4).

RESULTS

Invected contributes a non-essential and redundant function

En and *inv* are expressed in developmental programs that have virtually identical temporal and positional patterns, the proteins they encode have almost identical homeodomains (Coleman et al., 1987), and their effects on embryonic and imaginal development when ectopically expressed under a heat shock promoter are indistinguishable (Gustavson, 1993). Although many lethal mutations in *en* have been isolated, lethal *inv* mutants have not. Therefore, the role of *inv* has not been established, and as noted above, the phenotype of apparent null *en* alleles has curious aspects. To determine the phenotype of the null condition for *en* and *inv*, four mutant chromosomes were constructed (see Fig. 1). Two are deletions. One, *Df(2R)*en^E, lacks DNA between 9 kb upstream and approximately 50 kb downstream of the transcription unit. It removes the entire *en* transcription unit as well as the C-terminal three exons of *inv*. The second, *Df(2R)*inv^{30}, removes approximately 27 kb of the *inv* transcription unit, including the transcription start site, the first two exons and most of the second intron. Northern, in situ, and immunohistochemical analyses failed to detect any RNA or protein products of *inv* in inv^{30} mutant embryos or of *en* and *inv* in en^E mutant embryos (not shown). We conclude that inv^{30} is null for *inv* and that *Df* en^E is null for *en* and *inv*. *Df* en^E is an embryonic lethal; inv^{30} is viable and produces flies with a normal phenotype.

The inv^{30} deletion was used to construct a second chromosome lacking both *inv* and *en*. inv^{30} was mutagenized with EMS and chromosomes that failed to complement $Dfen^E$ were isolated. One of these, inv^{30} $en^{9\text{-}6}$, carries an pparent null *en* allele. We also characterized a mutant carrying the lethal en^{LA10} allele (Kornberg, 1981). en^{LA10} is an EMS-induced point mutation with an amber codon that terminates translation after residue 304. The truncated en^{LA10} peptide lacks a homeodomain and behaves as null (Gustavson, 1993).

en^E, inv^{30} $en^{9\text{-}6}$, and en^{LA10} animals fail to complete embryogenesis. Although the abnormal segmentation in these mutant embryos varies in severity, embryos lacking both *en* and *inv* function consistently had more extreme abnormalities than those lacking only *en* (see Fig. 1). Based on these and related observations (E. G. and T. B. K. unpublished data), we conclude that *inv* is not essential to embryonic development, but that it contributes an activity that is redundant to *en*.

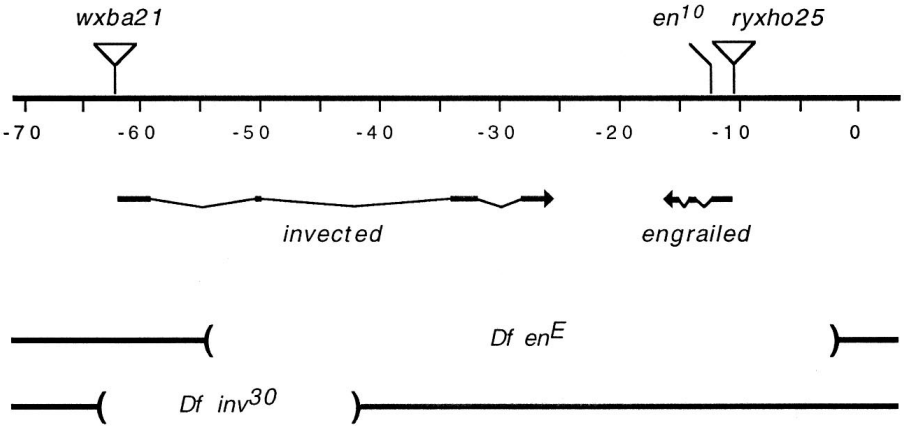
Cells that lose *en/inv* function undergo extensive genetic reprogramming

To investigate the regulatory role of *en/inv*, clones of cells homozygous for en^E were generated during the second instar using FLP-mediated recombination, and larval third instar wing discs were isolated. Some of the discs were abnormally shaped, and many of these had significant overgrowths that were associated with en^E *(en/inv*$^-$*)* clones. These discs were

analyzed with immunohistochemical probes for the anterior compartment-specific genes *ci*, *ptc* and *dpp*, and for the posterior compartment-specific genes *en*, *inv* and *hh*.

ci is normally expressed by all anterior compartment cells and is expressed most strongly by anterior cells that line the A/P compartment border (Fig. 2A; Slusarski et al., 1995). However, in discs with an en^E clone in the posterior compartment, *ci* was often mis-expressed. As shown in Fig. 2B, *en/inv*$^-$ cells in a large clone induced *ci*, and CI protein was most prominent along the edge of the clone. With respect to *ci* expression, the border of this clone resembles the A/P compartment border. This result is characteristic of all such large *en/inv*$^-$ clones, although in smaller clones, CI protein accumulation was less consistent (not shown). Many of these clonal patches were associated with apparent bulges of the disc epithelium, and neighboring regions with elevated levels of EN protein were frequently observed. These areas with elevated levels of EN protein may represent clonal twin spots (Fig. 2N).

ptc expression responds in a similar manner in *en/inv*$^-$ clones. Like *ci*, *ptc* is normally expressed by all anterior compartment cells, and the relative distribution of PTC protein is even more strongly biased toward the A/P compartment border where it accumulates in punctate structures (Fig. 2C). *ptc* was expressed ectopically by *en/inv*$^-$ cells in the posterior compartment, and PTC protein accumulated most prominently at the periphery of the clone (Fig. 2D). As with *ci*, such behavior was characteristic of large clones, but was less consistent in smaller ones. *dpp* is normally expressed by anterior compartment cells that lie along the A/P compartment border (Fig. 2E; Raftery et al., 1991). Large clones of posterior *en/inv*$^-$ cells induced *dpp* expression along their edge (Fig. 2G,H), and the pattern of *dpp* expression bears strong resemblance to the appearance of the normal A/P compartment border. Lastly, *hh* is normally expressed by all posterior compartment cells (Fig. 2I), and HH protein can be observed to be predominantly distributed diffusely throughout a sub-apical layer among the posterior cells. A small fraction accumulates at the apical surface of anterior compartment cells along the A/P compartment border (Fig. 2I; Tabata and Kornberg, 1994). As expected, HH protein was reduced or absent in large posterior *en/inv*$^-$ clones, except for its apical accumulation in punctate granules at the edge of the clones (Fig. 2K,L). These punctate accumulations were consistently observed, even in smaller clones, although the reduction in levels of HH protein was not a universal feature of small clones. These results are consistent with those of Sanicola et al. (1995) who characterized *dpp*, *ptc*, and *hh* expression in $Dfen^E$ clones with enhancer trap and *lacZ* fusion constructs.



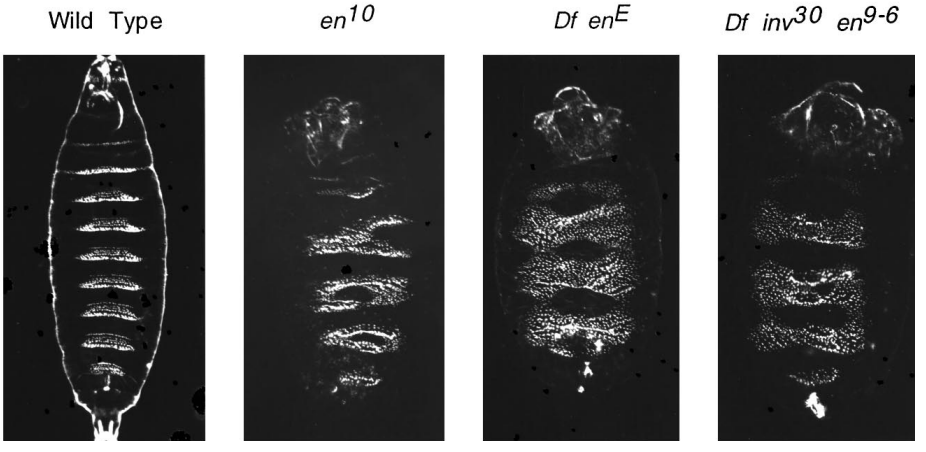


Fig. 1. Structure and phenotype of *en* and *inv* null alleles. (A) The *engrailed/invected* genomic region is depicted as a solid horizontal line, above which are indicated the locations of relevant mutations, and below which are indicated the number of kb proximal to the insertion associated with the en^1 allele (0). *ryxho25* and *wxba21* are inserted 250 bp and 6 bp upstream to the respective start sites of *engrailed* and *inv* transcription (Hama et al., 1990). Also shown are the *en* and *inv* transcription units, and the approximate structure of two deletions that were isolated as revertants of the P element insertions. (B) Photographs of ventral cuticles prepared from (in order from left to right) wild-type, en^{LA10}, $Df(en^E)$, and $Df(inv^{30})en^{9-6}$ embryos. Note the more extreme denticle lawn phenotype of the deficiency embryos.

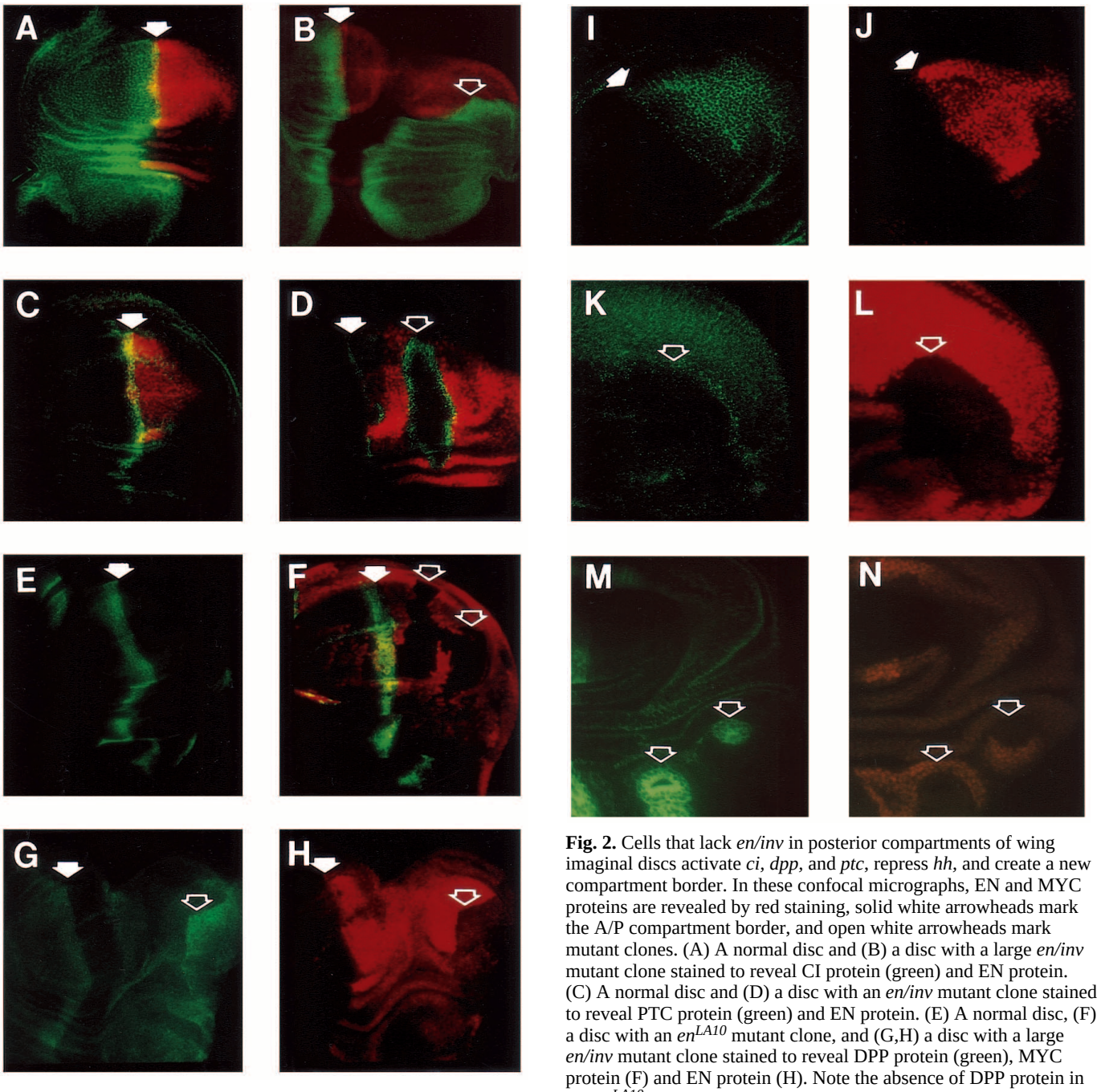


Fig. 2. Cells that lack *en/inv* in posterior compartments of wing imaginal discs activate *ci*, *dpp*, and *ptc*, repress *hh*, and create a new compartment border. In these confocal micrographs, EN and MYC proteins are revealed by red staining, solid white arrowheads mark the A/P compartment border, and open white arrowheads mark mutant clones. (A) A normal disc and (B) a disc with a large *en/inv* mutant clone stained to reveal CI protein (green) and EN protein. (C) A normal disc and (D) a disc with an *en/inv* mutant clone stained to reveal PTC protein (green) and EN protein. (E) A normal disc, (F) a disc with an en^{LA10} mutant clone, and (G,H) a disc with a large *en/inv* mutant clone stained to reveal DPP protein (green), MYC protein (F) and EN protein (H). Note the absence of DPP protein in the en^{LA10} mutant clone and the non-uniform distribution of DPP in the *en/inv* mutant clone. A normal disc (I,J) and a disc with an *en/inv* mutant clone (K,L) stained to reveal HH protein (green) and EN protein. (M) A disc with several small *en/inv* mutant clones stained to reveal CI protein and (N) EN protein. Note that the level of EN protein is higher among some of the cells adjacent to the clones, suggesting that these cells are the clonal twin spot. In addition, ectopic folds are present in the region of the clone and CI protein is absent from the center of the clones in this optical section. Adjacent optical sections reveal CI protein in the center of the clone.

Based upon the observed reprogramming of patterns of gene expression in posterior compartment cells that lose *en/inv* function, we conclude that large clones of *en/inv*$^-$ cells create an ectopic compartment border. With respect to the proteins that are expressed by border cells and that we examined, this ectopic border has all of the biochemical properties of a normal border. In addition, the discs with large *en/inv*$^-$ clones and ectopic borders have grossly abnormal shapes, indicating that these borders are also associated with repatterning and re-organizing the discs.

en/inv$^-$ clones transform posterior into anterior cells and duplicate both anterior and posterior structures

The adult wing of *Drosophila* is constructed of two sheets of epithelial cells which reproducibly decorate its various parts with an array of veins, bristles, and other structures. It is

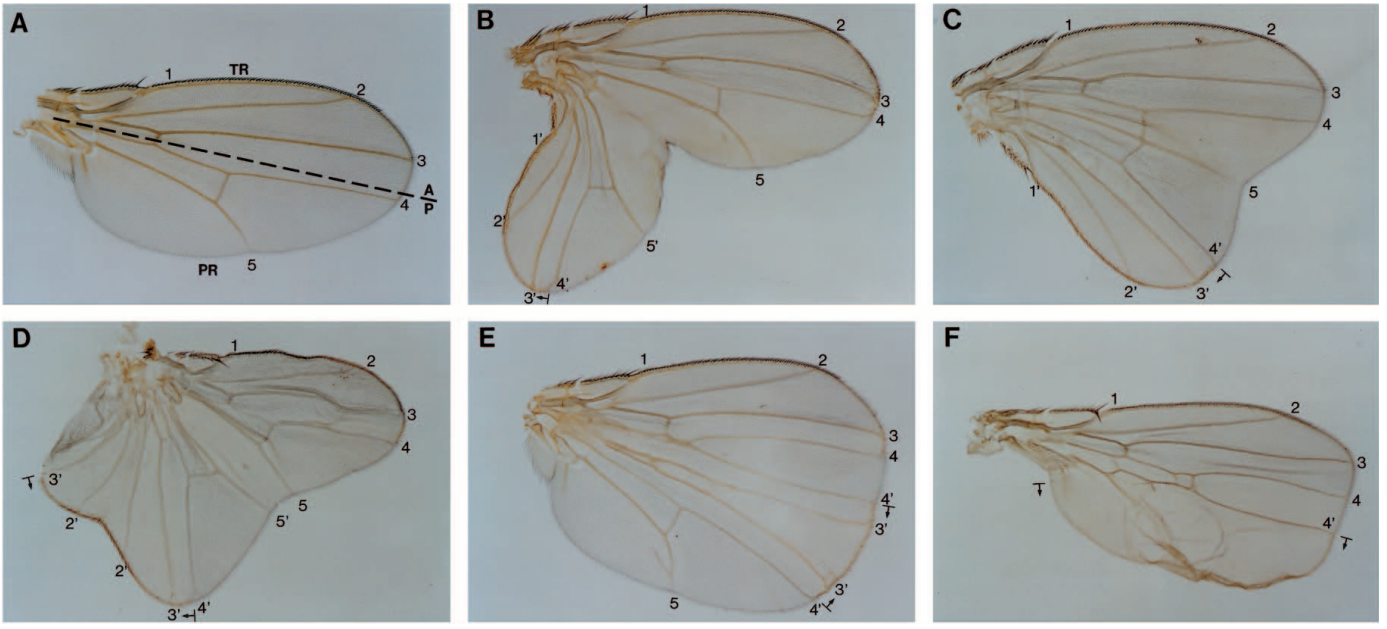


Fig. 3. Large clones of *en/inv*$^{-}$ cells in the wing cause extensive re-patterning. (A) A wild-type wing labeled to show the A/P compartment border (dashed line), the five wing veins, and the distinctive bristles present on the anterior (TR) and posterior (PR) wing margin. The other photographs are of wings with clones of homozygous *Df en*^{E} (B-E) and *en*^{LA10} cells (F). These clones are marked with *y*, and their position is indicated by the arrows. The clones in (B) and (C) duplicated tissue in the notum as well as the wing (not shown). Duplicated veins are indicated with primed numbers; their identity was established by their dorsal or ventral location and by the presence of campaniform sensillae. Note that in each wing, *y* 3rd and *y*$^{+}$ *4th* veins are present on either side of the clonal borders and that the ectopic border between these two veins establishes a new axis of anterior/posterior polarity.

broadly subdivided into A/P and D/V compartments, but the many characteristic structures distributed on its surface define multiple positions along its A/P, D/V, and P/D axes. In particular, the patterned rows of bristles along the wing margin, the veins, and the campaniform sensillae provided useful markers for this study (Fig. 3A).

en/inv^{-} clones induced by the FLP/FRT technique were marked by the loss of *y*$^{+}$ and were recognized in both the anterior and posterior compartments. As expected, *en/inv*$^{-}$ clones in the anterior compartment contributed to the formation of normal anterior patterns except in the region of the distal tip (see below). In contrast, clones in the posterior compartment were associated with pattern abnormalities which varied with the position and size of the clone. The most extreme pattern alterations involved clones that include the wing margin, several examples of which are shown in Fig. 3. The most striking example (Fig. 3B) has a clone along the wing margin posterior to the 5th vein that transformed the *en/inv*$^{-}$ cells to anterior. This clone generated an entire anterior compartment, complete with a row of anterior-type bristles along the wing margin, three veins, and appropriately placed campaniform sensillae; it also induced the neighboring cells, which are not part of the clone, to generate an entire new posterior compartment. The duplicated wing is an accurate mirror image of the original. Among the many wings with such duplications we recovered, 7 developed with sufficient normalcy (without bubbles or folds) to permit precise evaluation. These wings duplicated the posterior compartment to variable degrees, but in every wing, the original posterior compartment and the duplicated posterior compartment developed symmetrical sets of structures.

Posterior compartment *en/inv*$^{-}$ clones that reach the distal, but not the posterior wing margin, cause similar transformation of both tissue type and polarity. Fig. 3D,E show two wings with clones of this nature. In each, the *en/inv*$^{-}$ clone generated a mirror image duplication of anterior compartment patterns, but the extent of the duplication varied from small, containing two 3rd veins (Fig. 3E), to large, containing an almost complete anterior compartment containing several veins, with 4th veins adjacent to its edges (Fig. 3D). Each clone was flanked by 4th veins, a posterior structure. In normal wings, veins 3 and 4 are to either side of the compartment border. In wings with *en/inv*$^{-}$ clones containing duplicated anterior veins, the ectopic 3rd veins retained the D/V orientation and array of campaniform sensillae characteristic of normal 3rd veins. We suggest that these 3rd and 4th veins were produced adjacent to an ectopic compartment border and that the degree of duplication depended upon the position of the clone relative to the original A/P border. Many of the clones were not large enough to generate an organized pattern within the clone itself, but the flanking *en/inv*$^{+}$ tissue in the posterior compartment invariably generated mirror image duplications whose size and extent were also related to the location and size of the clone.

These phenotypes of *en/inv*$^{-}$ clones are different and significantly more extreme than those of *en* mutants that have been described previously (Morata and Lawrence, 1975; Lawrence and Morata, 1976; Kornberg, 1981; Lawrence and Struhl, 1982). To determine whether these differences can be attributed to the loss of *inv* function in the *Dfen*^{E} clones, clones of *en*^{LA10} and *inv*^{30} *en*$^{9\text{-}6}$ cells were also generated. Wings were recovered with *inv*^{30} *en*$^{9\text{-}6}$ clones that were associated with

anterior transformations and mirror image duplications (not shown) and that were similar in character to the $Dfen^{E}$ clones described above. In contrast, en^{LA10} clones caused pattern abnormalities in the posterior compartment without extensive transformation to anterior and without polarity reversal or extensive ectopic growth. Even a large en^{LA10} clone that includes a majority of the posterior wing margin did not cause significant transformation (Fig. 3F). Such en^{LA10} clones did not induce high levels of *dpp* expression (Fig. 2F). These results suggest that loss of *en* function alone is not sufficient to create a new compartment border or to generate an ectopic anterior compartment; rather, the combined loss of *en* and *inv* function is required to effect a complete transformation of posterior cells to anterior, and apparently as a consequence, to generate a new compartment border.

patched is required to limit expression of *decapentaplegic* to the vicinity of the A/P compartment border

A model that accounts for the compartment-specific patterns of *ptc* and *dpp* expression postulates that in the posterior compartment, *en* represses both genes, and that in the anterior compartment, *ptc* represses *dpp* except for the region of the compartment border where *ptc* is antagonized by HH (Capdevila and Guerrero, 1994; Tabata and Kornberg, 1994; Sanicola et al., 1995). In support of this model, Capdevila et al. (1994) found that *dpp* is derepressed in anterior wing disc cells in *ptc* mutant clones that had been induced by X rays. To compare the phenotypes of *ptc* mutant clones to *en/inv*$^{-}$ clones, and to determine whether *ptc* is required to produce the pattern duplications associated with *en/inv*$^{-}$ clones, we induced *ptc* mutant clones and *ptc en/inv* double mutant clones. We observed that clones produced large outgrowths in the anterior compartment that can be described as mirror image duplications of anterior wing, and that the extent of these duplications varied with the position of the clone (Fig. 4A-D). In these wings, *ptc* and *ptc en/inv* mutant clones produced no veins but were flanked by ectopic 3rd veins. These partially duplicated wings are similar to the wings produced by two other types of clones that induce ectopic *dpp* expression - PKA mutant clones (reviewed by Perrimon, 1995) and *hh*-expressing clones (Basler and Struhl, 1994). We interpret the similarities between these four types of clones as indicating that the pattern duplications are caused by ectopic expression of *dpp* (Fig. 4G). We conclude that *ptc* is needed to restrict *dpp* expression to the region of the compartment border. We suggest that the absence of vein and bristle patterns in the *ptc* clones may be due to the uniform expression of *dpp* in these clones (Fig. 4G), in contrast to the non-uniform *dpp* expression in *en/inv*$^{-}$ clones (Fig. 2G).

One conspicuous difference between the *ptc* mutant and *ptc en/inv*$^{-}$ clones was noted at the wing margin. *en* was derepressed in anterior *ptc* clones (Fig. 4H), in agreement with previous reports (Couso, 1991), and although *ptc en/inv*$^{-}$ cells produced chemosensory bristles in this region (Fig. 4F), *ptc* mutant clones did not (Fig. 4E). This observation is consistent with the expression of *en* at the distal wing margin just anterior to the A/P border in late third instar wing discs (Blair, 1992), and to the suggestion that *en* has a role in blocking the differentiation of chemosensory bristles in this region (Hidalgo, 1995; Jiang and Struhl, 1995).

No abnormal posterior compartment structures were observed in wings with *ptc* mutant clones. In contrast, pattern outgrowths that consisted of both mutant and non-mutant cells were found in posterior compartments with *ptc en/inv* double mutant clones (not shown). These outgrowths are similar to outgrowths commonly associated with *en/inv*$^{-}$ clones, although the frequency and size of posterior *ptc en/inv* clones was significantly lower.

Regulating *engrailed* expression is critical to both anterior and posterior compartments

Posterior compartment cells that lose *en* and *inv* function transform into anterior cells, suggesting that the combination of *en* and *inv* activities provides a posterior identity. If so, ectopic activation of *en* expression in anterior cells should induce a complementary transformation. To investigate the consequence of ectopic *en* expression, the GAL4-UAS system (Brand and Perrimon, 1993) was used to express *en* ectopically. A strain that expresses GAL4 in both compartments of the wing disc (112-97; Fig. 5D) was crossed with a strain carrying a UAS-regulated *en* cDNA. The flies with both the GAL4 and UAS-*en* constructs developed abnormal wings and halteres. Fig. 5A shows an example, with some of the medial triple row bristles along the anterior wing margin transformed to posterior-type, overgrowth in the anterior wing, and abnormal venation throughout. Ectopic *en* expression in the anterior compartments of third instar wing discs induced the synthesis of HH protein in patches and DPP protein rather broadly (Fig. 5E,F). Posterior compartments had normal patterns of HH protein, but the distribution of EN was reduced to patches (Fig. 5E,F). These effects on anterior wings are similar to transformations previously described for vg^{W} mutants (Williams et al., 1990). vg^{W} wing and haltere discs ectopically express *inv* in their anterior compartments, and transform anterior to posterior (J. Bell, personal communication). Although the effects of ectopic *en* expression in the anterior compartment can be interpreted as transformation toward posterior, the abnormalities in the posterior compartment were unexpected.

The effects of GAL4-driven *en* expression in the posterior compartments are a direct consequence of *en* overexpression and are not a non-autonomous consequence of genetic reprogramming in the anterior compartment. In the presence of a construct that expresses GAL4 in posterior compartments only (*en*-GAL4; Fig. 5I), UAS-*en* reduced EN protein in third instar wing discs to sparse patches and induced ectopic expression of *ci* and *dpp* in some areas of these posterior compartments (Fig. 5K,L). Although many of the animals carrying both the *en*-GAL4 and UAS-*en* genes died prior to eclosion, surviving flies had wings with normal patterns of veins and bristles in the anterior compartment, but in the posterior compartment, veins were abnormal and anterior-type triple row bristles lined the posterior margin. Some wings had marked outgrowths (Fig. 5G), while others had anterior transformations that were not accompanied by outgrowths (Fig. 5H). We assume that in these wings, posterior cells had transformed to anterior due to their reduced levels of EN protein, and that the variability of the outgrowth phenotype is related to inconsistent activation of *dpp* in affected regions. The reason for reduced *en* function in these posterior compartments is unknown, although the reduction of both EN protein (Fig. 5K,L) and GAL4 protein (Fig. 5J) suggest that a negative autoregulatory mechanism

might be at play. Perhaps elevated *en* expression in younger discs triggered a negative regulatory response. Regardless of the underlying mechanism, these dramatic transformations suggest that the non-uniform pattern in which *en* is normally expressed in the posterior compartment has functional significance.

We note one additional phenotype in flies that over-produce EN protein. In 112-97GAL4/UAS-*en* flies, rare partial transformations of haltere to wing were observed (Fig. 5C). This phenotype recalls similar transformations in vg^{W} mutants (Williams et al., 1990), in *Ultrabithorax (Ubx)* mutants, and in the *Ubx-like* alleles of *RpII*, the gene encoding one of the RNA polymerase II subunits (Mortin and Lefevre, 1981). Since EN protein is thought to reduce *Ubx* expression in the posterior compartments of wild-type halteres, the *Ubx*-like phenotype of *en* over-expressing flies may be a consequence of direct repression by elevated levels of EN protein. Alternatively, it could reflect an indirect effect on RNA polymerase II function through squelching, perhaps by sequestering an essential accessory protein.

DISCUSSION

en, ci, dpp, ptc, and *hh* are part of a network of genes that organizes and patterns imaginal discs. Under their direction, anterior and posterior compartments acquire distinct identities and adopt separate developmental pathways. The studies reported here examine the interactions among these genes and demonstrate conditions that lead to the creation of ectopic compartment borders. The graded and polarized response to these borders provides evidence for a central organizing role of the A/P compartment border in appendage development, and requires a re-appraisal of the roles of *en* and of compartment borders.

The role of *engrailed* in wing disc development

The observations that en^{1} mutants replace some posterior structures with mirror image anterior counterparts and that *en* is only required in posterior compartment cells (Garcia-Bellido and Santamaria, 1972; Morata and Lawrence, 1975) led to the proposal that *en* is a homeotic selector gene that endows cells with a posterior identity (Garcia-Bellido, 1975). However, the observed replacements were incomplete, suggesting only partially reduced *engrailed* function in the mutants (Kornberg, 1981; Lawrence and Struhl, 1982). In this study, we show that posterior cells that lose both *en* and *inv* function can develop a complete anterior compartment (Fig. 3B). This indicates that *inv* contributes to establishing the posterior developmental pathway and that the combined activity of both *en* and *inv* constitute the complete homeotic selector gene function of posterior cells. The posterior-type transformations of anterior cells that express *en* ectopically provide supporting evidence for this homeotic role (Fig. 5A). The homeotic selector function of *en/inv* is cell autonomous, and it presumably describes the action of EN protein in controlling the set of cytodifferentiation genes that generate the patterns of the posterior compartments.

We have characterized numerous *en* alleles in somatic clones induced by X rays (Z. A. and T. B. K., unpublished data; Kornberg, 1981). This group includes alleles that truncate the protein (e.g. en^{4LA}, en^{LA7}, en^{LA10}, en^{11}), alleles with chromosomal breakpoints in the *en* regulatory domain (e.g. en^{X49} and en^{C2}), deletions of the *en* region (e.g. *Df* en^{E}, and *Df* en^{F}), as well as another with an undetermined lesion (e.g. en^{l50}). Although the ways in which these alleles perturb posterior development differ, certain phenotypes were shared by all. All were viable in both compartments, although some such as *Df* en^{E} reduced viability of posterior clones. All clones in the posterior compartment showed some degree of abnormality, involving transformation to anterior, misshapen veins, unusually extensive growth, or crossing the compartment border. In contrast, anterior clones developed normally, except at the distal wing margin where clones produced socketed bristles. This requirement for *en* at the wing margin is manifested in most of these alleles, and has been noted previously for *Df* en^{E} (Hidalgo, 1995). It presumably reflects the expression of *en* in the anterior compartment near the A/P border in late third instar discs (Blair, 1992), and its relevance to the principal roles that *en* plays during earlier development in orchestrating the growth of both the anterior and posterior compartments is unclear.

Some alleles, such as en^{1} and *Df* en^{E}, generated more extreme phenotypes, and we postulate that these alleles had mutations that affected both *en* and *inv*. en^{1} has a transposable element inserted in the region of the *en/inv* imaginal enhancer (C.-N. Chen and T. B. K., unpublished data). en^{1} may reduce expression of *en* and *inv* by partially inactivating their imaginal enhancer. Alternatively, the phenotype of the *en*-GAL4/UAS-*en* flies (Fig. 5) suggests that en^{1} might reduce *en* function by hyper-activating the enhancer.

Curiously, the severity of *Df* en^{E} clones depended upon the method used to induce them. Clones induced by X rays transform to anterior, can be associated with outgrowths at the wing margin, and can cross the A/P compartment border. However, duplications such as those illustrated in Fig. 3 were not observed. We do not understand why the clones induced by X rays and FLP/FRT yielded such dramatically different phenotypes. The explanation may be related to a requirement for clones to be of sufficient size to generate a viable, patterned unit, and the high frequency of clones that are induced with the FLP/FRT system brings a higher probability that multiple clones can coalesce to reach that threshold at a time when the disc retains sufficient plasticity. Clones that do not reach this threshold may be incapable of functioning normally, or they may be lost. Indeed, small FLP/FRT-induced clones were observed to form bulges that suggests a sorting out process (Fig. 2M,N). Such behavior could account for the observed short-fall in X ray-induced clones, and a minimum size requirement to establish an autonomous region in the disc would be an intriguing property relevant to the creation of autonomous fields.

The different phenotypes of imaginal cells that lack *en/inv* or *en* indicate that *en* mutant cells retain *inv* function. In contrast, we have observed that in embryos, *en* mutants lose both *en* and *inv* expression, ectopic expression of *en* induces *inv* expression, and ectopic expression of *inv* induces *en* expression (Gustavson, 1993). We rationalize these observations by suggesting that the role that *en* serves in embryos to maintain expression of both *inv* and itself is not retained in discs, and that the putative autoregulatory mechanism that has been proposed to maintain *en* expression in embryos (Moazed and O'Farrell, 1992) is supplanted by another in imaginal cells.

The compartment border hypothesis

In addition to its cell autonomous homeotic selector function, *en* has a role that is not cell autonomous and that is related to its indirect regulation of genes on the anterior side of the A/P border. In *Df en^{E}* clones, *ptc*, *ci*, and *dpp* were activated and *hh* expression declined (Fig. 2; Sanicola et al., 1995). This result demonstrates that *en* negatively regulates *ptc*, *ci* and *dpp* while positively regulating *hh* in posterior cells. In large posterior *Df en^{E}* clones, the boundary between *en*-expressing and mutant cells assumes properties of an A/P compartment border. As with the normal A/P border, discrete particles of HH protein and elevated levels of CI, PTC, and DPP lined the border in discs, and wing veins 3 and 4 were produced adjacent to this boundary in the adult. These veins are normally produced adjacent to the compartment border. These ectopic borders formed at the periphery of the clones; if the clone included the posterior margin, the border formed on the anterior side, but if the clone did not reach the margin, borders formed on both its anterior and posterior sides. Borders form wherever *en*-expressing and non-expressing cells are juxtaposed. This result supports a model that compartment borders are created as a consequence of the apposition of *en*-expressing and non-expressing cells (Kornberg, 1981). The patterns generated by these *Df en^{E}* clones reveal the pre-eminent role of the compartment border in organizing the imaginal disc, and suggest, perhaps, that the significance of compartments is both the step-wise sequestration of cells into successively more restricted units as well as the formation of the compartment border itself. In this context, it is relevant to note that the only *Df en^{E}* clones we found that were associated with pattern duplications were ones that reached the wing margin and the D/V compartment border. This correlation supports the proposal

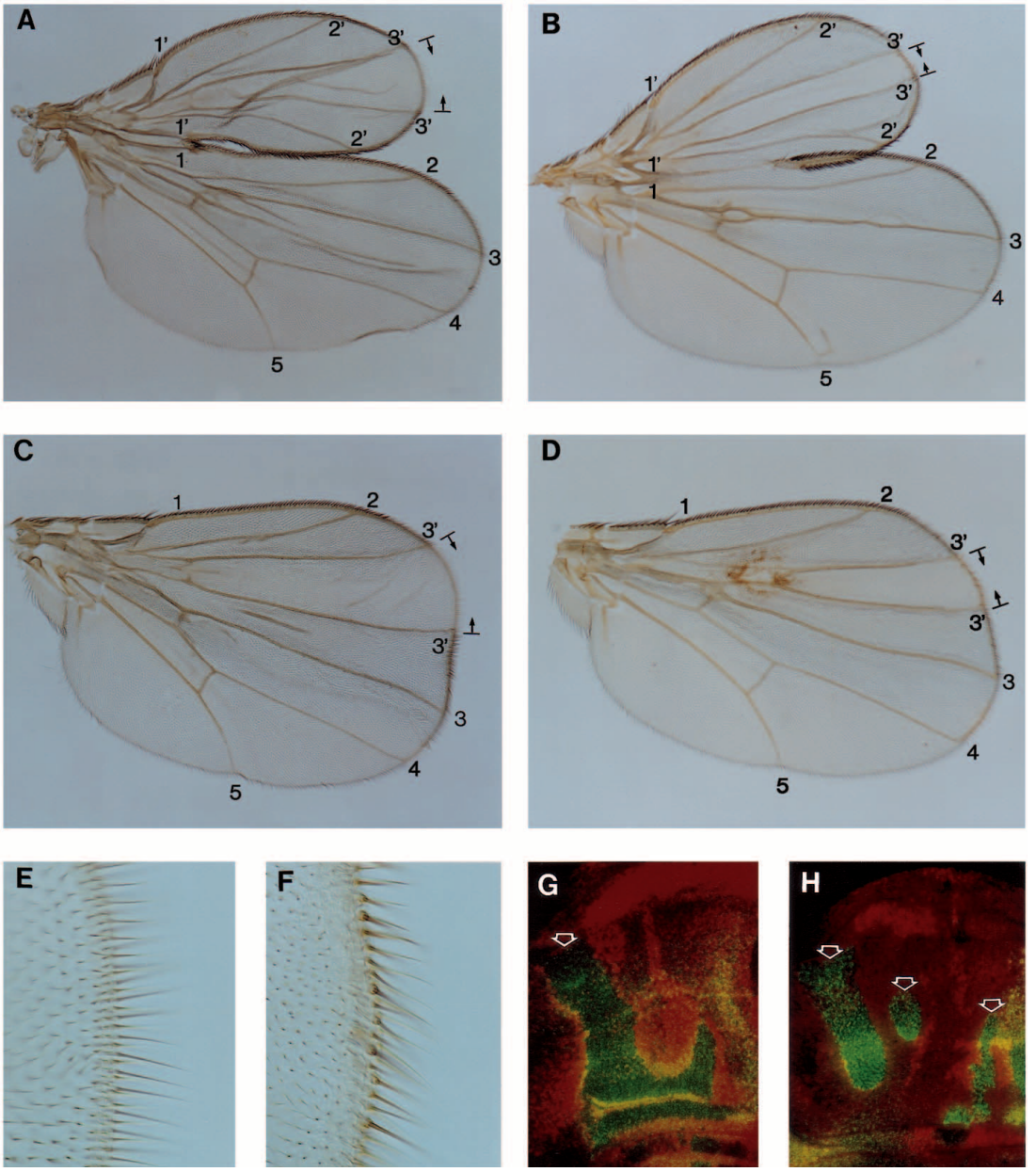


Fig. 4. Clones of *ptc* mutant cells induce growth of anterior tissue. Wings with *ptc* (A,C) and *ptc en/inv* (B, D) mutant clones in the anterior compartment induce neighboring cells to duplicate anterior structures. Arrows mark the clones of mutant cells and the primed numbers indicate the duplicated veins. Note that veins were not produced by mutant cells. Higher magnification views of the region of the distal wing margin that include the *ptc* (E) and *ptc en/inv* (F) mutant clones reveal that *en* mutant cells produced socketed bristles at the margin whereas the *en*$^{+}$ cells did not. Immunohistochemical staining reveals the position of *ptc* clones (white arrowheads, green patches) in the anterior wing blade region of third instar discs. Anti-MYC antibody (red) stains the non-mutant cells, while anti-DPP antibody (G) and anti-EN antibody (H) stained the mutant cells (green). Note the uniform level of DPP protein in the clone.

that the intersection of A/P and D/V borders is critical to proximal/distal axis formation (Gelbart, 1989; Campbell et al., 1993; Tabata and Kornberg, 1994).

To a remarkable degree, normal patterns can be produced by cells on both sides of ectopic compartment borders. Results obtained in this study and in others suggest that the influence of such borders correlates with their expression of DPP protein. The importance of DPP protein in imaginal development has been elegantly revealed by studies demonstrating that appendages cannot develop without *dpp* function (Spencer et al., 1982) and that a second P/D axis is generated upon ectopic induction of DPP protein synthesis (reviewed by Perrimon, 1995). We found that normal-type patterns developed within and adjacent to a clone only if ectopic DPP protein distribution was localized and graded as it is at normal A/P borders. Relevant examples were found at the edges of large *Df en*^{E}

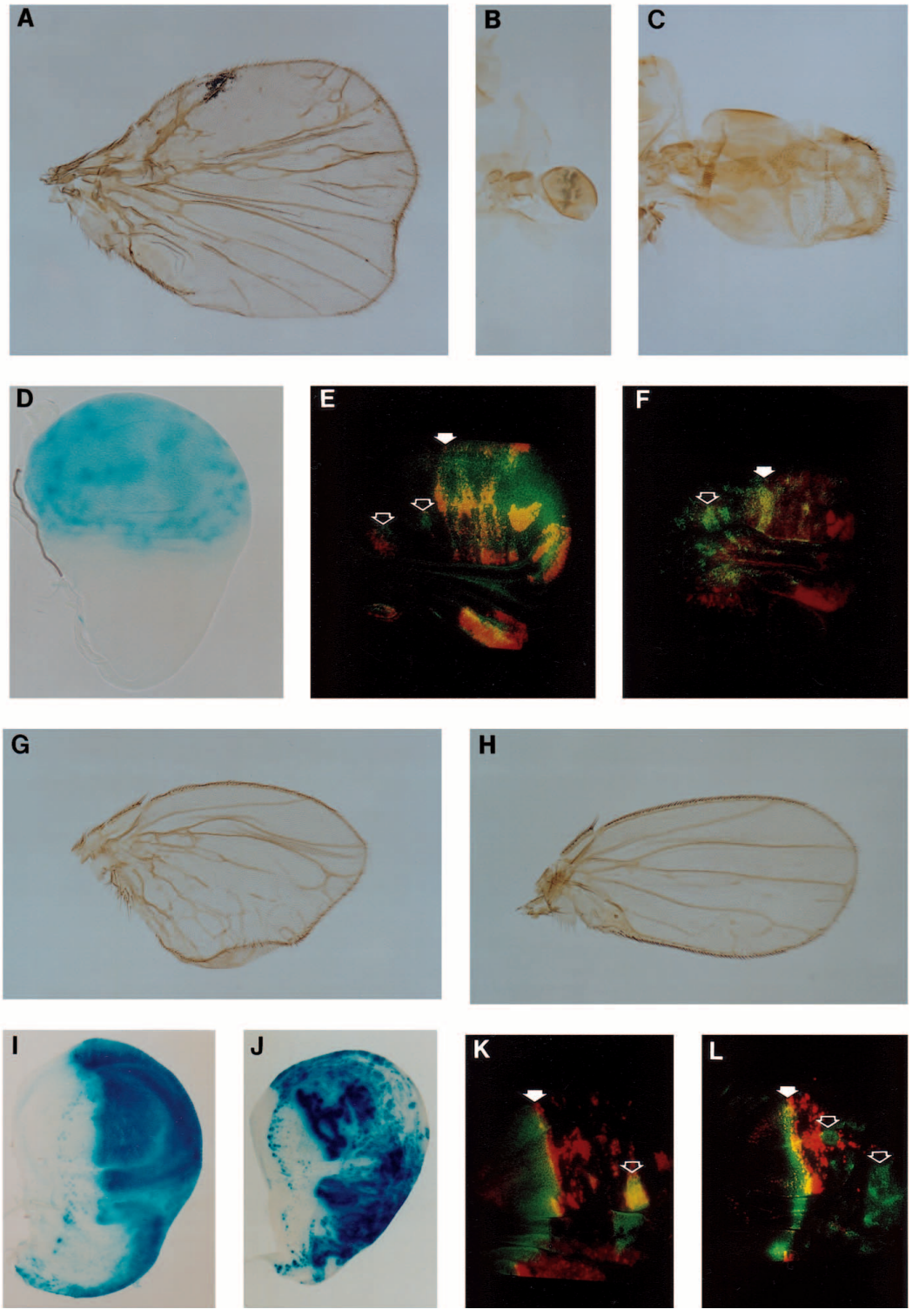


Fig. 5. Ectopic expression of *en* transforms both anterior and posterior wing compartments. 112-97-GAL4/UAS-*en* developed wings that were abnormally shaped, that partially replaced anterior wing margin bristles with posterior-type bristles, and that partially replaced posterior wing margin bristles with anterior-type bristles (A). The distribution of EN protein (red) in wing discs (E,F), of HH protein (E; green) and of DPP protein (F; green) was abnormal. Solid white arrowheads mark the A/P compartment border, and open white arrowhead mark the ectopically induced protein. Halteres of these flies (C) were larger than normal (B), and were transformed toward wing. β-galactosidase activity in 112-97 GAL4/UAS-*lacZ* wing discs indicates that this strain expresses GAL4 throughout the wing primordia (D). Wings from some *en*-GAL4/UAS-*en* flies were significantly enlarged, but all transformed posterior to anterior (G,H). The distribution of EN protein (red; K,L) and of CI (K; green) and DPP (L; green) proteins was patchy in the posterior compartment. β-galactosidase activity was uniform in the posterior compartment of *en*-GAL4/UAS-*lacZ* discs (I), but was patchy in *en*-GAL4/UAS-*en*/UAS-*lacZ* discs (J).

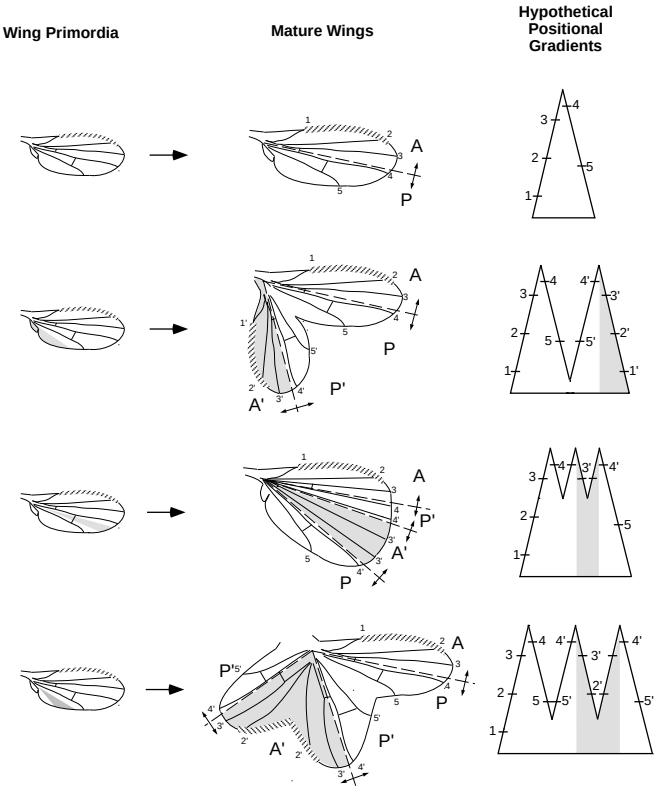


Fig. 6. *engrailed/invected* mutant clones reorganize the wing. The theoretical immature wing primordium (left), the adult wing (center), and the gradient of positional value (or morphogen) that is presumed to direct wing patterning (right) are illustrated. Top drawings are for a normal wing. Lower drawings are for the wings pictured in Fig. 3B,E,D. Positions of the *Df en^{E}* clones in the wings are presumed to be present in their respective primordia as indicated by shaded areas. Compartment borders, dashed lines; veins, numbered solid lines; duplicated veins, primed numbers; polarity at the compartment borders, arrows. The highest positional value (depicted as the highest point) has been assigned to the A/P border, since it presumably serves as the organizing center. In each wing, the boundary between the wild-type posterior compartment and the mutant clone creates a new A/P border, and each boundary is flanked by 3rd and 4th veins. Regions flanked by two gradients grow under the influence of both and produce mirror image duplications.

clones (Fig. 2G). In contrast, patterns that developed in response to patchy or uniform DPP protein distribution were disorganized. Examples include *ptc* clones which only produced normal patterns adjacent to mutant cells (Fig. 4G), and wings of *en*-GAL4/ and 112-97GAL4/UAS-*en* flies, in which normal patterns were disrupted in affected regions (Fig. 5A). It bears mention that it has yet to be demonstrated that ectopic expression of *dpp* is sufficient to create an entire new compartment, and there are other genes expressed in *dpp*-like patterns (S. Eaton, B. Yoshinaga, T. T. and T. B. K., unpublished data). For convenience, we have referred to *dpp* as representing the set of genes that are expressed along the A/P border and that may serve as morphogens.

Appropriately patterned *dpp* expression is important to cells on both sides of the compartment border. Fig. 3B illustrates a wing in which a *Df en^{E}* clone generated a normal anterior compartment. This clone also induced neighboring cells to develop a normal posterior compartment. Therefore, the influence that posterior cells exert on anterior-specific gene expression through *en*-dependent regulation of *hh* ultimately returns to pattern the posterior compartment. This conclusion illustrates the symmetry of the mechanisms that pattern the anterior and posterior compartments, involving *ptc* and *en* repression as well as *hh*-dependent induction across the compartment border. This conclusion is also consistent with the inability of posterior compartment cells to grow in the absence of *hh* (Basler and Struhl, 1994) and with the pattern duplications associated with ectopic expression of *dpp* (Capdevila and Guerrero, 1994). We account for the complex regulatory loop by the two separable *en* functions: its non-autonomous function mediated through *hh*, and its autonomous function of establishing compartment identity by controlling posterior determining genes.

Finally, we note that the capacity of wings to almost double in size (Fig. 3B; this fly also duplicated a portion of its notum, not shown) was not accompanied by developmental delay. This indicates that disc growth is subject to strict negative regulation, and that discs have the capacity to grow more than they normally do. We also note that every wing we recovered that had an ectopic compartment border was reorganized by intercalation of a mirror image duplication between the ectopic border and the original border. The continuity and directionality of these patterns suggests the existence of an instructive gradient, and we adopt the format of Lawrence and Morata (1994) to represent such a hypothetical gradient in these wings (Fig. 6).

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