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Mechanisms of compartment formation: Evidence that non-proliferating cells do not play a critical role in defining the D/V lineage restriction in the developing wing of *Drosophila*

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SUMMARY

The dorsoventral (D/V) lineage boundary in the developing wing disc of *Drosophila* restricts growing cells to the prospective dorsal or ventral compartments of the wing blade. This restriction appears along the prospective margin of the wing some time during the middle to late stages of wing disc growth. It has been proposed that the restriction is established and maintained by the formation of a zone of non-proliferating cells that acts as a barrier between cells in the dorsal and ventral compartments (O'Brochta and Bryant, *Nature* 313, 138-141, 1985). In the adult, however, no group of barrier cells has been identified between the compartments. This study will show the following. (1) A group of cells does exist that lies between the dorsal and ventral rows of margin bristle precursors; these cells, which express *cut* in the late third instar wing disc, are thus in an ideal position to act as barrier cells. (2) This *cut*-expressing region is split into dorsal and ventral regions by the expression of the dorsal-specific gene *apterous*. (3) The D/V lineage restriction defined by marked dorsal and ventral clones lies in the middle of the *cut*-expressing region and is exactly congruent with the boundary of *apterous* expression. (4) No group of barrier cells is observed between dorsal and ventral clones. (5) Clones often run along the boundary for long distances, suggesting that they can grow along the D/V boundary without crossing it. These results thus do not support the existence of a groups of cells acting as a barrier between dorsal and ventral compartments. Nor do they support a critical role for division rates near the D/V boundary in establishing or maintaining the lineage restriction.

Key words: *Drosophila*, wing imaginal disc, cell lineage, compartments, *apterous*, LIM domain, transcription factor, *cut*, homeobox

INTRODUCTION

In the developing imaginal discs of *Drosophila*, a small number of lineage restrictions appear that prevent the mixing of cells in adjacent compartments. These restrictions are maintained during a period of extensive growth, despite the lack of any strictly reproducible patterns of descent within each compartment (see Postlethwait, 1978). Despite much study, however, the cellular mechanisms responsible for these restrictions are not fully understood. In this study, I will examine the mechanisms underlying the formation of one particular compartment boundary, the dorsoventral compartment boundary that appears within the wing imaginal disc.

The wing discs in *Drosophila* are set aside during embryonic development as a discrete anlage of 20-30 cells each (Bate and Martinez-Arias, 1991; Cohen et al., 1991, 1993). These grow throughout larval life, forming by late third instar sacs of approximately 52,000 epithelial cells (Garcia-Bellido and Merriam, 1971a). During metamorphosis the discs undergo a complicated series of morphological changes and differentiate to form precise patterns of adult tissue types (e.g. Waddington, 1940; Postlethwait, 1978).

Lineage tracing studies have shown that the fates of the imaginal discs cells are for the most part undetermined until the later stages of disc growth. For instance, the separation of sensory organ, wing vein and epithelial lineages does not occur until the middle to late stages of the third and final larval instar (Garcia-Bellido and Merriam, 1971b; Diaz-Benjumea et al., 1989). Nonetheless, early arising lineage restrictions do limit marked clones to particular compartments of the wing. In the wing blade, the existence of two lineage compartment boundaries has been demonstrated: the anteroposterior (A/P) (Garcia-Bellido et al., 1973, 1976), which lies just anterior to the fourth longitudinal vein, and the dorsoventral (D/V) (Bryant, 1970; Garcia-Bellido and Merriam, 1971a; Garcia-Bellido et al., 1973, 1976), which separates the prospective dorsal and ventral wing blade epithelia.

The A/P restriction is present in the disc anlage from very early in development (Garcia-Bellido et al., 1973, 1976), even before the lineages of the wing and leg disc anlage

have separated (Wieschaus and Gehring, 1976; Steiner, 1976). It is thought that the A/P boundary is established and maintained by stably inherited anterior and posterior states of 'selector' gene expression, which control in some manner the affinity of the cells in each compartment (Garcia-Bellido, 1975). The transcription factor encoded by *engrailed* (*en*) may act in this manner. *en* is expressed in the posterior of the disc anlage (Cohen et al., 1991) and late third instar wing disc (Kornberg et al., 1985; DiNardo et al., 1985; Brower, 1986; Blair, 1992a), and *en* mutations alter both the identity of posterior cells (Garcia-Bellido and Santamaria, 1972; Morata and Lawrence, 1975; Eberlein and Russell, 1983; Brower, 1984; Gubb, 1985) and their ability to obey the lineage restriction (Morata and Lawrence, 1975; Lawrence and Morata, 1976; Kornberg, 1981; Lawrence and Struhl, 1982). While some violations of compartment-specific *en* expression do occur, these only appear late in larval development and are apparently not sufficient to alter the A/P lineage restriction (Blair, 1992a). Region-specific genes other than *en*, such as *invected* (Coleman et al., 1987), *cubitus interruptus-D* (Eaton and Kornberg, 1990; Orenic et al., 1990; Blair, 1992a) and *hedgehog* (Lee et al., 1992), may also play a role in this process.

Unlike the A/P boundary, the D/V boundary appears only in the middle stages of larval development (Bryant, 1970; Garcia-Bellido and Merriam, 1971a; Garcia-Bellido et al., 1973, 1976; Morata and Lawrence, 1979). The mechanism by which the D/V lineage restriction is imposed upon the growing disc is the subject of some debate. In one view, the dorsal and ventral compartments correspond to two different regions of selector gene activity and cell affinity, much as is hypothesized for the anterior and posterior compartments; while the dorsal and ventral cell states must arise during the growth of the disc, they are from that point on stably inherited. It was recently found that the transcription factor encoded by *apterous* (*ap*) is expressed in what appears to be the dorsal region of the wing disc (Cohen et al., 1992) beginning in the middle of the second instar (Williams et al., 1993). As *ap* mutations can apparently alter D/V identity (Stevens and Brower, 1986), this is consistent with the existence of an A/P-like selector gene mechanism.

An alternative hypothesis, however, was proposed by O'Brochta and Bryant (1985), who showed that a zone of non-proliferating cells (ZNC) is formed along the prospective D/V boundary during the third instar. These authors suggested that this reduction in division rates was sufficient to separate the two compartments; that is, the ZNC acted as a barrier that lay between clones in the dorsal and ventral compartments. Thus, the A/P and D/V compartment boundaries might be the result of very different cellular mechanisms. The ZNC is first detected in early third instar as a stripe 1-2 cells wide; by late third instar, it has widened to 6-10 cells, presumably by recruiting adjacent cells (O'Brochta and Bryant, 1985). Subsequent workers have confirmed the existence of the ZNC at late third instar using BrdU incorporation (Schubiger and Palka, 1987; Hartenstein and Posakony, 1989; Usui and Kimura, 1992).

If the ZNC acted as a barrier that lay between dorsal and ventral compartments, one would expect the vast majority of dorsal and ventral clones to obey different compartment boundaries, one on the dorsal and one on the ventral side of the ZNC (see Discussion); such a result was predicted by O'Brochta and Bryant (1985) and Brower (1985). However, in adults only one D/V boundary is apparent. The most 'marginal' cells observed on the surface of the adult wing are the dorsal and ventral rows of margin bristles (Hartenstein and Posakony, 1989; though see below for discussion of Palka et al., 1979). These two rows lie within the dorsal and ventral compartments, respectively, and no barrier cells have been reported to lie between them in the adult (Bryant, 1970; Garcia-Bellido and Merriam, 1971a; Garcia-Bellido et al., 1976). The lack of any apparent barrier has been used to argue against the validity of the ZNC hypothesis (e.g. Garcia-Bellido and de Celis, 1992).

However, it will be demonstrated in this study that the dorsal and ventral bristle precursors in the developing wing are in fact separated by an additional group of ZNC cells: the stripe of early arising *cut*-expressing cells (Jack et al., 1991; Blochlinger et al., 1993) which appears along the margin during the third instar. Although the early *cut*-expressing stripe was originally thought to correspond to a subset of the bristle precursors (Jack et al., 1991; though see Blochlinger et al., 1993), this study will show that *cut* expression defines a group of cells that lies between the dorsal and ventral bristle rows, even after the formation of bristle precursors is complete. This stripe of early *cut*-expressing cells is in an ideal position to act as a barrier separating the dorsal and ventral compartments.

Because the cells of the early *cut* stripe have not been identified in the adult, their position with respect to the D/V boundary has not been established by any of the studies to date. Therefore, in this study, I make use of new techniques that allow mitotic recombinant clones to be examined in the disc and developing wing at a cell-by-cell level of resolution (Blair, 1992a,b; Xu and Rubin, 1993). Since these techniques use antibodies to visualize marked clones, double staining can be used to co-localize clones and regions of gene expression.

The results show that barrier cells are not present between the dorsal and ventral compartments at late third instar. Rather, the lineage boundary defined by both dorsal and ventral clones lies in the center of the early *cut* stripe, and is exactly congruent with the boundary of *ap* expression. Nor does the behavior of clones support the existence of a mitotically quiescent 'trap' at the time the D/V boundary is established, as clones can apparently grow for long distances along the *ap* boundary without crossing it. These results thus do not support a critical role for division rates in establishing the D/V lineage restriction.

MATERIALS AND METHODS

Fly stocks

WG 1296 (Blair, 1992a) is a viable P[lArb] insertion at 3D on the X chromosome in a ry^{506} background, and was generated by the laboratory of Dr W.J. Gehring; it and $M(1)o^{sp}/FM6$ stocks were obtained from the *Drosophila* Stock Center in Bloomington. *neu-LacZ* (*A101*)/*TM3,Sb* is a lethal P[lArb] insertion into the *neu* locus (Boulianne et al., 1991) generated by the laboratory of Dr W.J.

Gehring. *ap-LacZ/CyO* is a hypomorphic enhancer trap insertion into the *ap* locus (Cohen et al., 1992), and was kindly provided by Dr S. Cohen. *5A-πM* is a viable insertion of a P[w^+ hs-*myc* epitope] element at 5A on the X chromosome (Xu and Rubin, 1993), and was kindly provided by Dr T. Xu. *WG 1296 M(1)o^{sp}/FM7* and *5A-πM M(1)o^{sp}/FM7* stocks were generated by recombination.

Mitotic recombination

Eggs were generated from the following virgin female × male crosses: + × *WG 1296*. *ap-LacZ/CyO* × *5A-πM*. *WG 1296 M(1)o^{sp}/FM7* × +. *5A-πM M(1)o^{sp}/FM7* × *ap-LacZ/CyO*. Eggs were collected in bottles for 4-6 hours. Larvae were reared at 25°C and irradiated at approximately 48 hours after egg laying (4 krad using a Sheperd Mark 1 ^{137}Cs gamma-ray source at a dose rate of 1.2 krad/minute). This dosage, though high when compared to the more standard 1 krad of X-irradiation, does not appear to affect the formation of the D/V restriction, and does not induce crossing of the A/P lineage boundary (Blair, 1992a); some slowing of subsequent development might, however, be expected. Female wandering third instar larvae were collected at 5 days AEL for non-*Minute* crosses, and 5 or 6 days AEL for *Minute* crosses; the 6 day collection biased the collection for the more slowly developing *marker Minute*/+ larvae. Approximately 40% of the discs examined contained a marked clone that lay within five cells of the D/V boundary.

Histology

πM larvae were heat-shocked by placing them in a moist dish floating in a 38°C water bath for one hour and then incubating at 25°C for 1-2 hours prior to dissection (Xu and Rubin, 1993).

Wandering third instar discs were dissected in saline and fixed for approximately 2 hours at 4°C in a Pipes-formaldehyde solution (Brower, 1986) as described previously (Blair, 1992a). Washes and antibody incubations were carried out at 4°C in PBS containing 0.3% Triton X-100.

For discs, anti-*cut* anti-β-gal double staining was performed using 1/2,000 clp-2 rabbit anti-*cut* (Blochlinger et al., 1988; kindly provided by Dr Y.N. Jan) and 1/200 mouse-anti-β-gal mAb (Promega) overnight. Anti-*myc* anti-β-gal double staining was performed using 1/400 mouse anti-*c-myc* mAb (Oncogene Science) and 1/10,000 rabbit anti-β-gal (kindly provided by Dr R. Holmgren) overnight. Both primary incubations were followed by 1.5 hours in 1/200 biotin-anti-mouse IgG (Vector) and 1/800 FITC-anti-rabbit IgG (U.S. Biochemicals), followed by 1.5 hours in 1/1600 RITC-streptavidin (Vector) and 1/800 FITC-anti-rabbit IgG.

For 10 hours AP and older stages, pupae were fixed overnight and dissected as described previously (Blair et al, 1992). Anti-*cut* anti-β-gal double-stained *neu-LacZ* wings were incubated overnight as above, but this was followed by 1.5 hours in 1/200 biotin-anti-rabbit IgG (Vector) and 1/800 FITC-anti-mouse IgG (U.S. Biochemicals), followed by 1.5 hours in 1/1600 RITC-streptavidin and 1/800 FITC-anti-mouse IgG. A few wings were also examined after staining with anti-β-gal alone (1/10,000), followed by a nickle-intensified Vector ABC-DAB protocol described previously (Blair et al., 1992).

Discs and wings were mounted and examined (Blair, 1992a) using a conventional fluorescence microscope to prescreen discs and a Bio-Rad confocal microscope for detailed examination and photographs. Fig. 4 was obtained by tracing photographs of anti-*c-myc* and anti-β-gal staining taken at the same apical focal plane; all clones for this figure were also examined at several focal planes to confirm the identity of boundary nuclei. As the induction of hs-*myc* epitope expression varied, discs were not included in which expression was too faint to allow the unambiguous identification of individual nuclei.

RESULTS

Cell types on the developing margin

In the following sections, the development of margin-specific cell types will be reviewed and additional information presented.

Bristle development

Like other insect bristles, the bristles of the anterior (sensory) and posterior (non-sensory) margin of the adult wing appear to descend from sensillar or posterior bristle mother cells (SMCs and PMCs, respectively). These cells arise from the wing disc epithelium during late larval and early pupal life. There are two waves of SMC and PMC differentiation; first to appear are the chemosensory SMCs, followed by the mechanosensory SMCs and the PMCs.

The chemosensory SMCs can be identified by their strong expression of a variety of enhancer trap lines (e.g. Ghysen and O'Kane, 1989; Huang et al., 1991; Blair et al. 1992). The earliest of these markers to appear is an insertion in the *neuralized* (*neu*) gene (the *neu-LacZ* (*A101)* enhancer trap; Boulianne et al., 1991), which identifies chemosensory SMCs beginning in the latter part of the third instar (Huang et al., 1991, Blair et al., 1992; Fig. 1A). Starting at approximately 1-3 hours after pupariation (AP) the cells go through a series of divisions to form the tormogen, trichogen, sheath and neuronal cells (Hartenstein and Posakony, 1989; Huang et al., 1991; Blair et al., 1992). *cut* is not expressed in these cells until 2-6 hours AP (Jack et al., 1991; Blochlinger et al., 1993).

The precursors of the mechanosensory bristles appear later in development. Previous studies have shown that additional cells along the anterior margin faintly express *neu* during and after chemosensory SMC development; however, large, definitive mechanosensory SMCs cannot be identified at 5-6 hours AP (Huang et al., 1991; Blair et al., 1992). I have therefore re-examined later developmental stages using *neu-LacZ*. At some time between 8 and 10 hours AP large cells appeared which strongly expressed *neu*; these cells filled the gaps between the clusters of chemosensory cells in numbers and with a spacing appropriate for mechanosensory bristle cells (Fig. 1B). They appeared to begin dividing by 15 hours AP; this is consistent with previous work using BrdU incorporation to follow the timing of S-phase in bristle precursors (Hartenstein and Posakony, 1989).

Posterior margin bristle cells develop with a timing similar to that of the anterior margin mechanosensory bristles. Cells along the posterior margin stain faintly with *neu* as early as wandering third instar (Blair et al., 1992); however, strongly expressing, definitive PMCs do not appear until approximately 8-10 hours AP (Blair, 1992b), and apparently begin dividing at approximately 14-18 hours AP (Hartenstein and Posakony, 1989; Blair, 1992b).

cut-expressing cells

At middle and later stages of the third instar, a stripe of *cut*-expressing cells 3-5 cells wide appears along the prospective margin of the wing disc; the cells in this 'early *cut* stripe' are located between the dorsal and ventral rows of

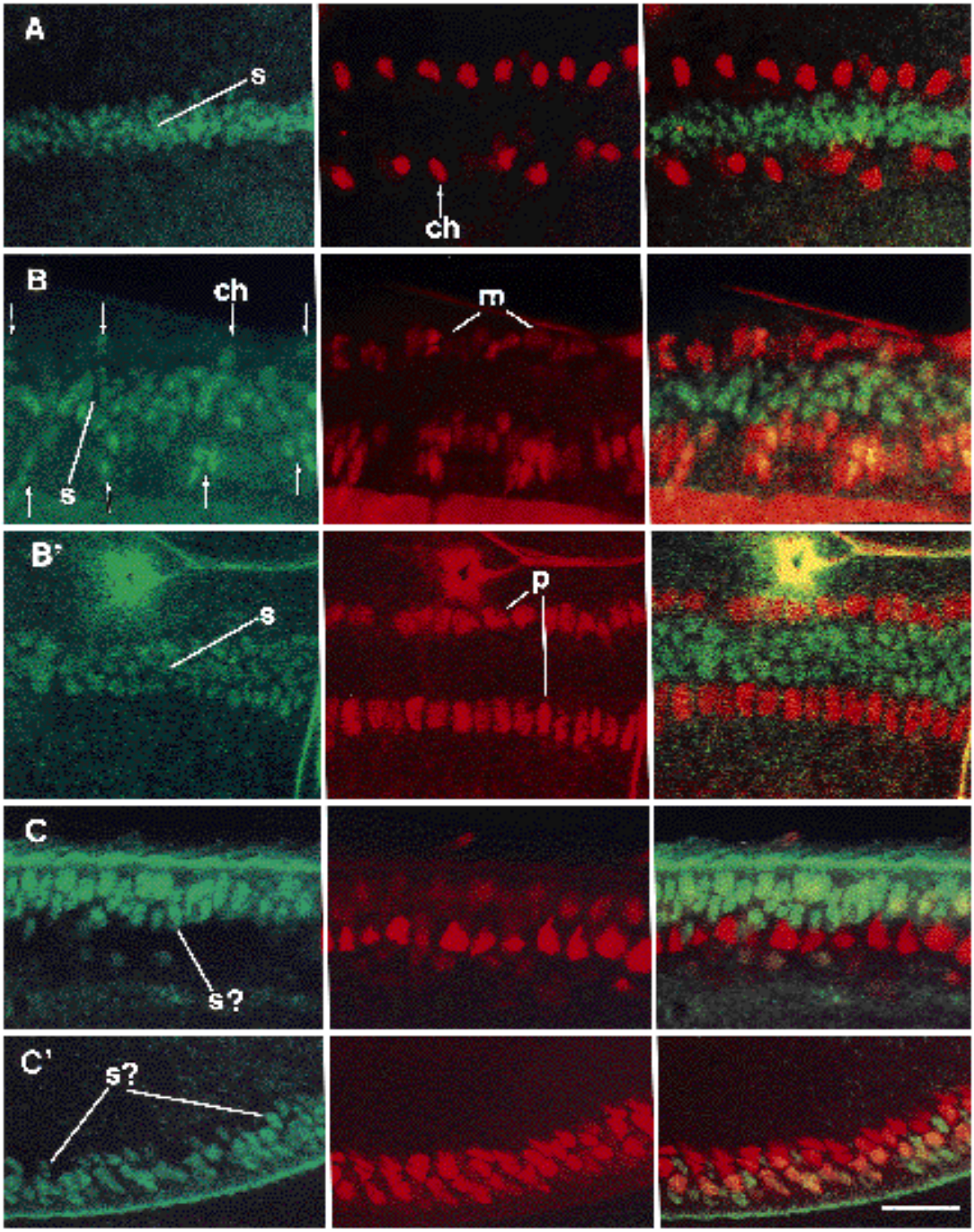

Fig. 1. Margin regions of *neu-LacZ* wing discs and pupal wings stained with anti-*cut* (green) and anti-β-gal (red); overlapping stain is yellow. Left panel is anti-*cut*, middle is anti-β-gal, and right is double image. Proximal is left, anterior is up. (A) Portion of prospective anterior margin in late third instar. Stripe of early *cut*-expressing cells (s) lies between the dorsal and ventral rows of chemosensory SMCs (ch), which do not express *cut* at this stage. (B,B′) Portions of anterior and posterior margin, respectively, at 12 hours AP. Periodic clusters of chemosensory cells express *cut*, but mechanoreceptor SMCs (m) and PMCs (p) do not. The stripe of *cut*-expressing cells is still visible between the dorsal and ventral rows of SMCs and PMCs. (C,C′) Side view of portion of anterior and posterior margins, respectively, at 34 hours AP. Cells are still visible that express *cut* but not *neu-LacZ* (s?); these may be cells from the early *cut*-expressing stripe. Because of the side view, confocal sections show only a small fraction of the *cut*-expressing cells. Scale bar, 25 μm.

chemosensory SMCs (Jack et al., 1991; Blochlinger et al., 1993; Fig. 1A), and lie within the margin domain of *wingless-LacZ* expression (unpublished data). Because *cut* is expressed in the precursors of peripheral sensilla (Blochlinger et al., 1988, 1993; Jack et al., 1991), these cells were originally thought to be the precursors of the mechanosensory and posterior bristles (Jack et al., 1991); this identification was further based on the position of these cells, and on the lack of non-bristle epithelial cells along the margin of adult wings (Hartenstein and Posakony, 1989). However, this view has been questioned (Blochlinger et al., 1993). The number of cells in the stripe is larger than can be accounted for by mechanosensory precursors. Moreover, *cut* is expressed in the stripe cells well before the time at which *neu*-expressing PMCs and mechanosensory SMCs appear (see above), while in other SMCs *cut* expression only appears after the expression of *neu* (Blochlinger et al., 1993; see Fig. 1).

To resolve this issue, I have stained developing *neu-LacZ* wings with anti-β-gal and anti-*cut*. The results showed that some, and perhaps all, of the cells of the early *cut* stripe form a population which is distinct from the margin SMCs and

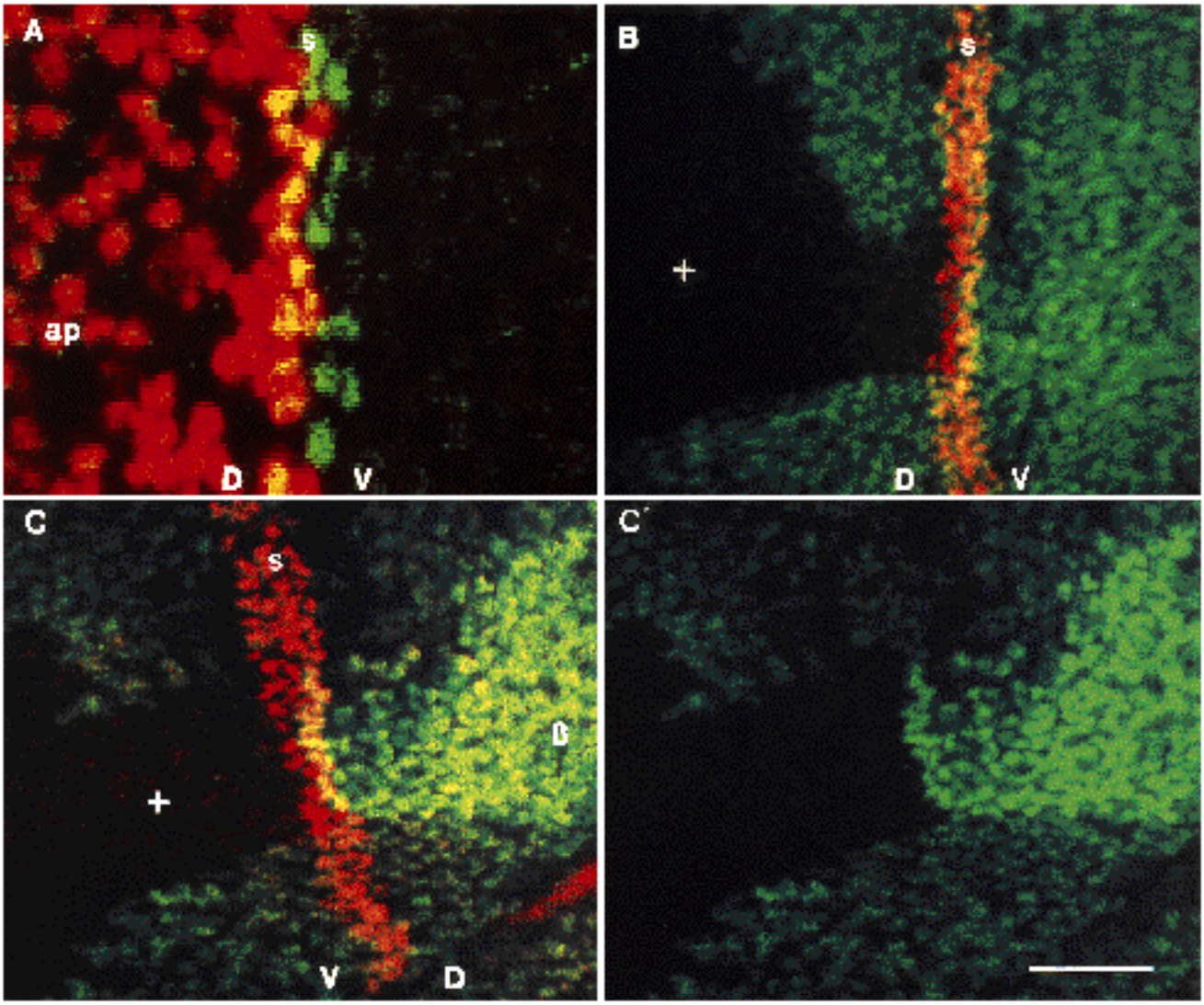

Fig. 2. Margin regions of late third instar wing discs. Dorsal (D) and ventral (V) are marked in each frame; proximal is up. (A) Detail of margin of *ap-LacZ* disc stained with anti-β-gal (red) and anti-*cut* (green); overlapping stain is yellow. Region of *ap-LacZ* expression partially overlaps early *cut*-expressing stripe, bisecting it into dorsal (expressing *ap*) and ventral (not expressing *ap*) regions. (B,C,C′) Clones in *WG 1296 M(1)o^{sp}*/+ discs, stained with anti-*cut* (red) and anti-β-gal (green); overlapping stain is yellow. (B) Large +/+ clone (+) in dorsal-anterior compartment of *WG 1296 M(1)o^{sp}*/+ wing disc. The clone is identified by its lack of β-gal expression and defines a straight D/V boundary in the middle of the early *cut*-expressing stripe (s). Smooth boundary at posterior of clone probably marks the A/P boundary. (C) Large ventral-anterior *M(1)o^{sp}*/+ (+) and dorsal-anterior *WG 1296 M(1)o^{sp}*/*WG 1296* (β) twin-spots. The *M(1)o^{sp}*/+ cells lack β-gal expression, while the *WG 1296 M(1)o^{sp}*/*WG 1296* cells express twice the heterozygotic level of β-gal. The D/V boundary defined by both clones bisects the early *cut*-expressing stripe. (C′) Same disc as C, showing anti-β-gal staining alone. Note lack of *WG 1296 M(1)o^{sp}*/+ barrier cells lying between the two marked clones. Scale bar, (A) 12 μm, (B,C,D) 25 μm.

PMCs. When chemosensory (Fig. 1A), mechanosensory (Fig. 1B), or posterior bristle (Fig. 1B′) mother cells first appeared they did not express detectable levels of *cut*. However, a stripe of *cut*-expressing cells was still visible at 12 hours AP, located between the dorsal and ventral rows of bristle precursors, even though the production of the bristle precursors appeared complete (Fig. 1B,B′). It is possible that the bristle precursors are derived from a subset of the early *cut*-expressing cells, as was suggested for chemosensory SMCs by Jack et al. (1991). However, this would require that these cells stop expressing *cut* as they begin to express *neu*, and re-express *cut* later in development.

The eventual fate of the non-bristle *cut*-expressing cells is unknown. Cells that express *cut* but not *neu* are present along the anterior (Fig. 1C) and posterior (Fig. 1C′) margins as late as 34 hours AP. At this time *neu* is expressed not only in bristle cells but also vein cells (Blair et al., 1992); non-sensillar *cut*-expressing cells thus form a population that is distinct from either sensillar or vein cells as defined by *neu* expression. While it has been reported that the most marginal portion of the wing margin lacks non-bristle epithelial cells (Hartenstein and Posakony, 1989), a few trichome-bearing cells are apparent between dorsal and ventral bristles in an end-on view of the anterior margin (see Fig. 2A in Palka et al., 1979). The number of these cells does not appear sufficient, however, to account for the entire early *cut* stripe.

apterous expression

ap is expressed in what appears to be the prospective dorsal region of the wing disc (Cohen et al., 1992), beginning in the middle of the second instar (Williams et al., 1993). As shown in Fig. 2A, *ap-LacZ* expression exactly bisects the early *cut* stripe along the margin. Thus, half the early *cut* stripe (and all the ventral SMCs and PMCs) lies outside the region of *ap* expression, while the other half of the *cut* stripe (and the dorsal SMCs and PMCs) lies within the region of *ap* expression.

The location of the D/V lineage boundary

Two different strains of *marker*-containing flies were used to identify mitotic recombinant clones. When examining *cut* expression, the *WG 1296* enhancer trap was used (Blair, 1992a,b). As shown previously, *WG 1296* expresses β-gal ubiquitously in imaginal disc nuclei. After inducing mitotic recombination in *WG 1296*/+ larvae, anti-β-gal was used to identify +/+ or *WG 1296*/*WG 1296* clones and anti-*cut* to identify the early *cut* stripe. Unstained +/+ clones were easily identified; in the best preparations, *WG 1296*/*WG 1296* clones could also be distinguished from *WG 1296*/+ nuclei.

Because the only marker available for *ap* activity is an *ap-LacZ* enhancer trap (Cohen et al., 1992), *LacZ*-based clone markers could not be used to co-localize the *ap* and D/V boundaries. Therefore an X chromosome insertion of the π*M* marker developed by Xu and Rubin (1993) was used. This construct expresses a short c-*myc* epitope ubiquitously in disc nuclei after heat-shock treatment, and can be detected with an anti-*myc* mAb. As with the *LacZ* marker, in most cases both +/+ and π*M*/π*M* nuclei could be identified in π*M*/+; *ap-LacZ*/+ wings. Mitotic recombination is more common on the X than on the second chromosome (Garcia-Bellido, 1972), and thus *ap-LacZ* expression was normal in most discs examined. However, in a few discs, gaps and abnormalities in the *ap-LacZ* expression were observed, apparently due to the production of *ap-LacZ*/*ap-LacZ* and +/+ twin spots. The *ap-LacZ* enhancer trap is a strong *ap* hypomorph (Cohen et al., 1992); since *ap* may have a role in controlling dorsoventral identity (Stevens and Brower, 1986), discs with abnormal *ap-LacZ* expression were not included in the data pool.

Clone boundaries in *Minute* wings

In order to maximize the size of clones, I used the *Minute* technique. *WG 1296 M(1)o^{sp}* and π*M M(1)o^{sp}* stocks were created and mitotic recombination was induced in either *WG 1296 M(1)o^{sp}*/+ or π*M M(1)o^{sp}*/+; *ap-LacZ*/+ larvae. In such larvae, +/+ clones grow faster than the surrounding *marker Minute*/+ cells, defining larger regions of the D/V boundary, while *marker Minute*/*marker Minute* cells die (Morata and Ripoll, 1975). To guarantee large clones, larvae were irradiated at approximately 48 hours AEL. This is prior to the time at which the D/V boundary is established in *M(1)o^{sp}*/+ flies (Garcia-Bellido et al., 1976; Morata and Lawrence, 1979), and thus some clones violated the compartment boundary. However, such clones were easily identified as they extended long distances into both compartments.

The majority of clones observed near the prospective wing margin defined a long smooth boundary along the margin. This apparent D/V boundary bisected the early *cut* stripe (e.g. Fig. 2B), and followed cell-for-cell the boundary of *ap-LacZ* expression (Table 1; Fig. 3C). Fig. 4 shows all those clones in π*M M(1)o^{sp}*/+; *ap-LacZ*/+ wings that lay within five cells of the *ap* boundary. The congruence between the lineage restriction and the *ap* boundary was observed for both dorsal and ventral clones. Even clones that violated the boundary often followed the *ap* boundary for a portion of their length. No evidence for any barrier cells either within or adjacent to the *ap-LacZ* boundary was found. The majority of clones extended for ten or more cells along the *ap* boundary; the longest clones extended along the boundary for over 25 cells.

In the cases described above, only +/+ clones were observed, as expected since the sister *marker Minute*/*marker Minute* cell dies. However, in a few cases, both +/+ and adjacent *marker*/*marker* clones were found (e.g. Fig. 2C); these cases are almost certainly the result of recombination between the *Minute* and *marker* loci, yielding *Minute*/+ and *marker Minute*/*marker* sister cells. The behavior of such clones is similar to that of large marked clones in non-*Minute* wings, which will be described in the following section.

Clone boundaries in non-*Minute* wings

In additional studies, clones were generated in non-*Minute* wings; marked clones thus grew at the same rate as surrounding cells. As expected, such clones did not always define the compartment boundaries for large distances, presumably because marked clones could not overgrow adjacent unmarked cells. Therefore, to maximize the size of the clones, larvae were irradiated at approximately 48 hours

AEL; again, this generated a mixture of large clones, which either crossed or obeyed the D/V restriction. As expected from the faster development of non-*Minute* wings, the proportion of crossing clones was much smaller (Table 1). Crossing clones could be distinguished as above.

The results were similar to those observed using the *Minute* technique. Several large +/+ or *marker*/*marker* clones were observed that appeared to obey a smooth lineage restriction in the region of the margin. This smooth outline was quite distinct from the more irregular clone outlines observed in other regions of the wing disc. In the majority of cases examined, the apparent D/V boundary lay in the middle of the early *cut* stripe, but followed exactly the boundary of *ap-LacZ* expression (Table 1).

The most striking cases were ones in which *marker*/*marker* and +/+ clones faced each other across the apparent D/V boundary (six cases). These clones were most likely the descendents of a single recombination event whose daughter cells were separated by the D/V restriction. They are unlikely to be the product of separate mitotic recombination events that happened to lie close to one another. If such events were common, similar facing clones should have been observed across the A/P boundary, and this was not observed in non-*Minute* discs (not shown). [1 pair of +/+ clones was observed in *Minute*/+ discs which faced across the A/P boundary (Fig. 4); this difference between *Minute* and non-*Minute* discs was probably due to the overgrowth caused by using the *Minute* technique.] Again, the clone boundary either lay in the middle of the early *cut* stripe (Fig. 2C,C′) or followed the boundary of *ap-LacZ* expression (Fig. 3A,B). No region of intervening *marker*/+ cells could be distinguished between the +/+ and *marker*/*marker* cells.

DISCUSSION

In this study, it was shown that the dorsal and ventral compartments are not separated by a lineally distinct region of barrier cells. A stripe of early arising *cut*-expressing cells does appear in this region (Jack et al., 1991; Blochlinger et al., 1993), and was shown to lie between the dorsal and ventral rows of margin bristle precursors. However, the D/V lineage boundary lies in the middle of this early *cut* stripe. Moreover, clones on the dorsal and ventral side define the same D/V compartment boundary, which is congruent with the boundary defined by the expression of *ap-LacZ*. The implication of these findings for various models of D/V boundary formation will be discussed in the following sections.

Table 1. Number of marked clones near *ap-LacZ* boundary

	Obey *ap-LacZ*			
	Dorsal	Ventral	Violate	Do not reach
+/+ in π*M5A M(1)o*^{sp}/+; *ap-LacZ*/+ discs	6	5	10	2
+/+ or π*M5A*/π*M5A* in π*M5A*/+; *ap-LacZ*/+ discs	24	15	10	2

Clones scored only if within 5 cells of *ap-LacZ* boundary.

Differential affinity?

The results of the clonal analysis are consistent with the hypothesis that the dorsal and ventral compartments correspond to two different regions of cell affinity. The congruence of the region of *ap* expression with the dorsal compartment also suggests that differential gene activity might play a role in defining this difference in affinity. *ap* encodes a homeobox gene containing a LIM domain, suggesting that it acts as a transcription factor (Cohen et al., 1992; Bourgouin et al., 1992). A direct role for *ap* in defining D/V identity, in terms of the types of genes expressed, is suggested by the phenotype of viable *ap* alleles (Stevens and Brower, 1987). A similar effect upon the formation of the lineage restriction has not been reported. However, *Polycomb* mutations can cause such D/V boundary violations as well as changes in D/V cell identity (Tiong and Russell, 1990). Since the *Polycomb* product is thought to act by maintaining patterns of gene expression (reviewed in Epstein, 1992), its mutant phenotype is consistent with the existence of other genes critical for D/V identity and affinity.

Many of the clones that obeyed the D/V restriction in this study appear to have extended for long distances along the boundary (see below for further discussion of this point). This apparent growth along the boundary at first glance appears inconsistent with the presence of the ZNC, as cell division should not occur in this region. One possible explanation is that some reduced level of cell division occurs in the ZNC after it is formed. Another explanation is that the D/V affinity difference appears before the formation of the ZNC. Unfortunately, the exact time at which the D/V boundary appears has not been determined, due to technical limitations of the mitotic recombination technique (for discussion, see O'Brochta and Bryant, 1985; Brower, 1985); various authors have placed its appearance at different times between late first instar and mid-third instar (Bryant, 1970; Garcia-Bellido and Merriam, 1971a; Garcia-Bellido et al., 1976; Morata and Lawrence, 1979; O'Brochta and Bryant, 1985; Brower et al., 1985; Brower, 1985). It should be pointed out, however, that *ap* is expressed in what appears to be a dorsally restricted fashion beginning at mid-second instar (Williams et al., 1993), while the ZNC has not been detected before early third instar (O'Brochta and Bryant, 1985).

The ZNC as a barrier?

The simplest way of viewing the ZNC hypothesis is to predict that the ZNC prevents cells from crossing by acting as a barrier to clone expansion (Fig. 5A), and that the D/V boundary is in fact two boundaries, one on the dorsal and one on the ventral side of the ZNC (O'Brochta and Bryant, 1985; Brower, 1985). This model also requires that cells outside the original ZNC are prevented from entering it, despite any growth advantage that they might have. In most cases, growing clones would come to abut the ZNC from either the dorsal or ventral side, and the ZNC would not be included in either dorsal or ventral clones. This should be especially common for +/+ clones in *Minute*/+ wings, as

such clones might push intervening cells aside before reaching the ZNC barrier, even if they started growing some distance from the ZNC. In rare cases dorsal or ventral clones would extend into the ZNC, but only if the ZNC 'captured' the edge of a clone as the ZNC was being formed (Fig. 5A, bottom clone). Thus, the model predicts that most clones

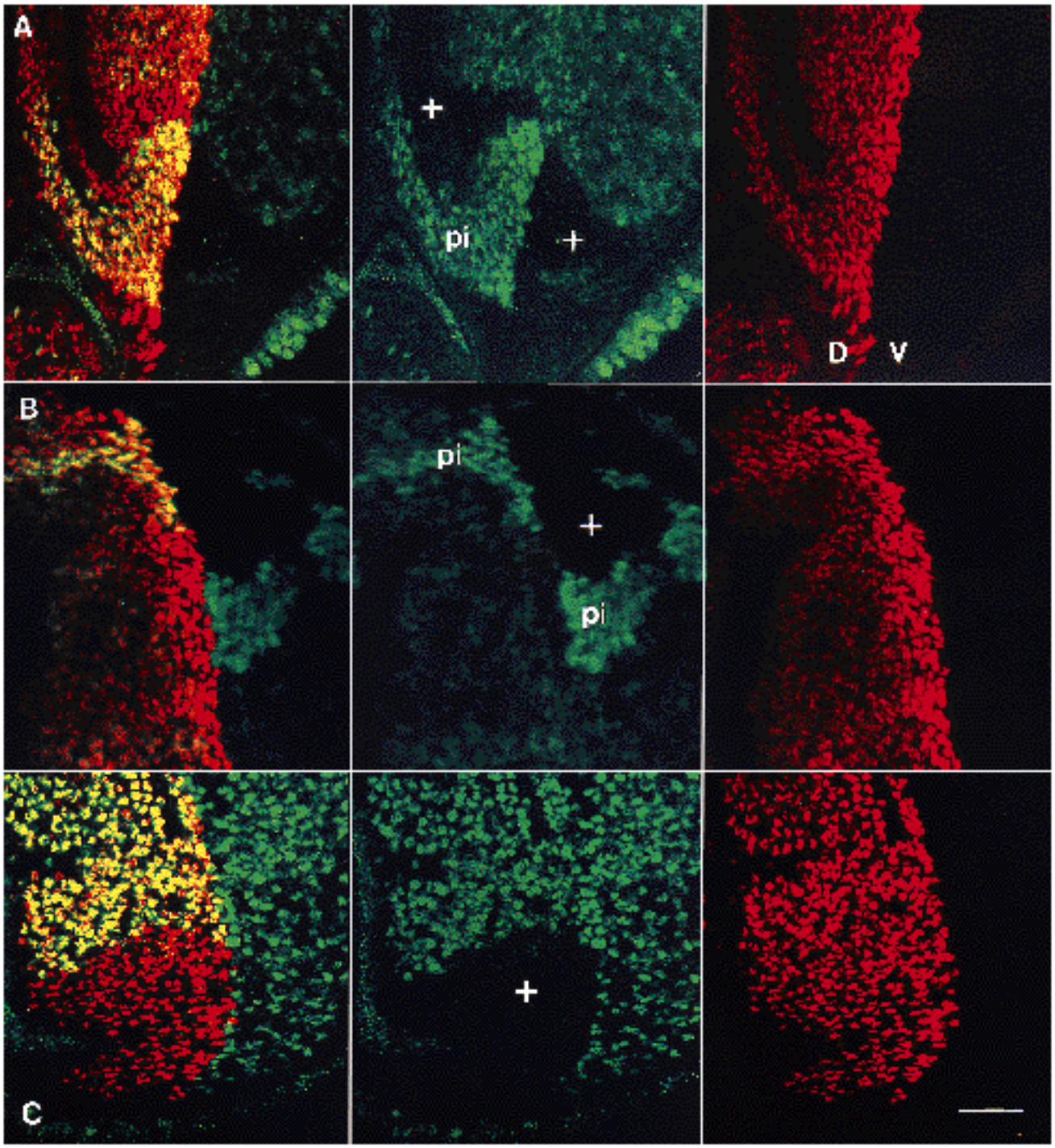

Fig. 3. Margin regions of late third instar wing discs containing *ap-LacZ* and π*M* markers, stained with anti-β-gal to identify *ap* (red) and anti-*myc* to identify π*M* marked clones (green). Cells lacking the π*M* marker do not stain with anti-*myc*, while clones with two copies of π*M* stain at twice the heterozygotic level. Left panel is double image showing overlapping expression in yellow. (A,B). +/+; *ap-LacZ*/+ (+) and π*M*/π*M*; *ap-LacZ*/+ (pi) clones in π*M*/+; *ap-LacZ*/+ disc. The D/V boundaries defined by dorsal and ventral clones follows exactly the boundary of *ap-LacZ* expression. (C) dorsal-posterior +/+; *ap-LacZ*/+ clone (+) in π*M M(1)o*^{sp}/+; *ap-LacZ*/+ disc. The D/V boundary defined by the clone follows exactly the boundary of *ap-LacZ* expression. Scale bar, 25 μm.

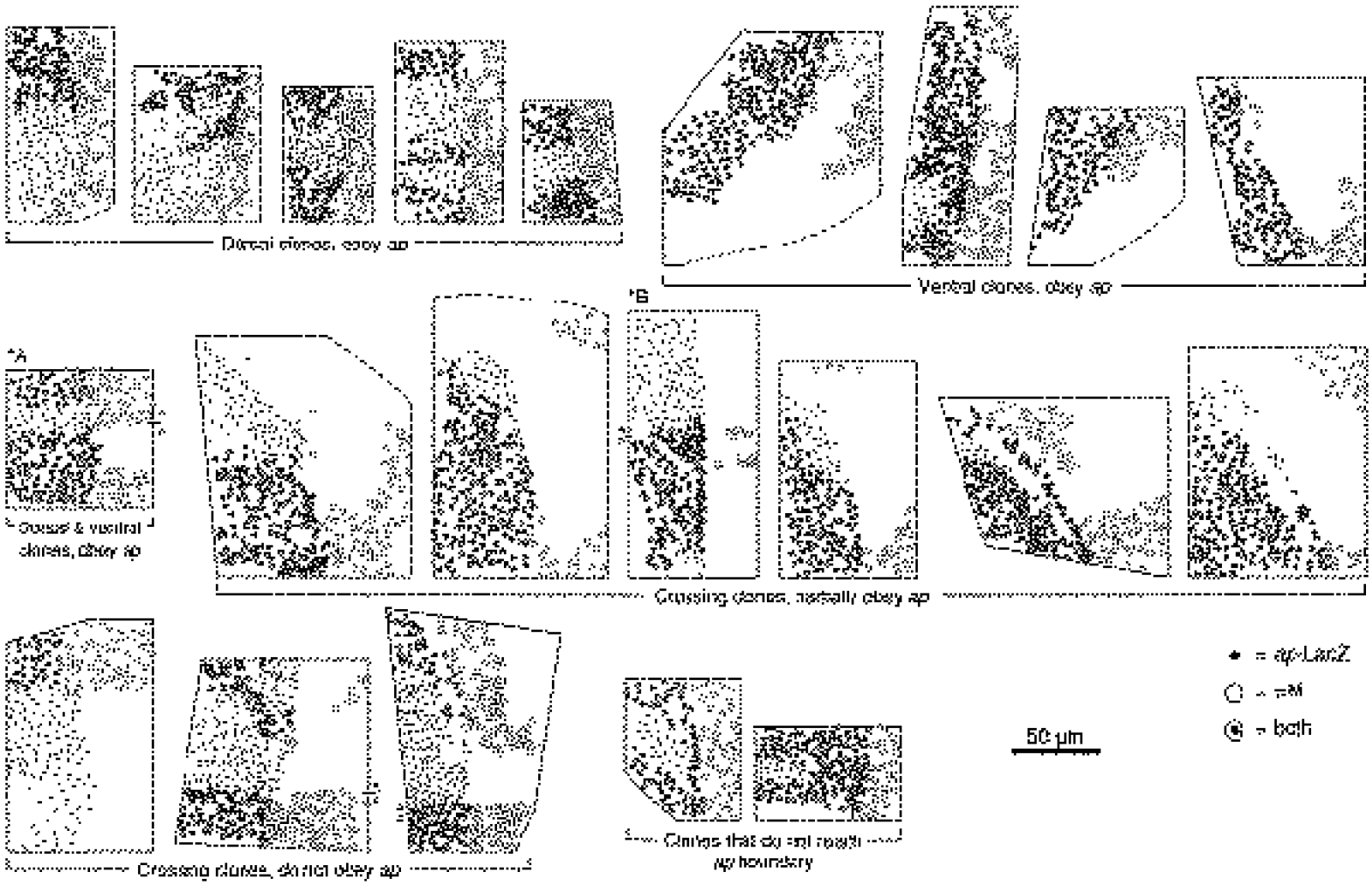

Fig. 4. Diagrammatic representation of marked clones in $\pi M\,M(1)o^{sp}$/+; *ap-LacZ*/+ wing discs. This figure shows all clones observed that lay within five nuclei of the *ap-LacZ* boundary in the wing blade, excluding those discs with abnormalities in *ap-LacZ* expression (see text). Regions of interest in each disc are shown in boxes; dotted lines represent limits of prospective wing blade region of discs. Dots represent *ap-LacZ*-expressing nuclei; open circles represent *πM*-expressing nuclei; filled circles represent nuclei that express both. When obvious, the A/P boundary is marked. All dorsal and ventral clones that obeyed an apparent D/V boundary followed exactly the boundary of *ap* expression. Some, but not all, of the clones that crossed the D/V boundary also partially obeyed the *ap* boundary, while others did not. Only two small clones were found that approached but did not define the *ap* boundary. In two cases clones were found in both the anterior and posterior compartments. Because clones generated by mitotic recombination do not cross the A/P boundary (Garcia-Bellido et al., 1976), these clones were assumed to be due to separate recombination events. In *A, both clones followed the *ap* boundary. In *B, the posterior clone crossed the *ap* boundary but partially obeyed it, while the anterior clone crossed the *ap* boundary without obeying it.

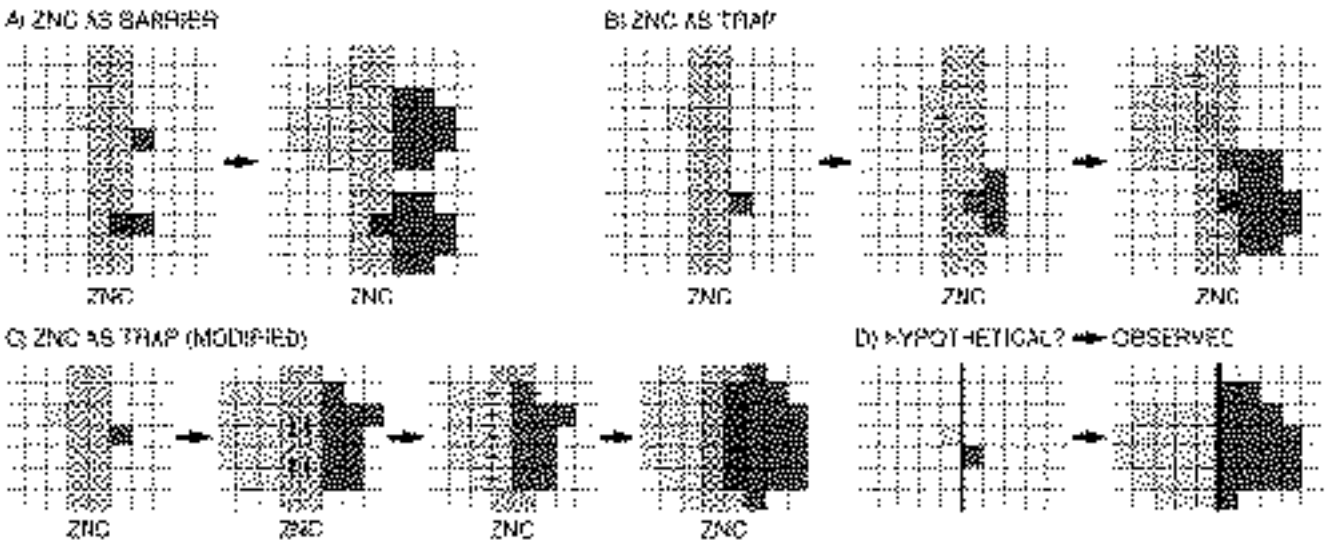

Fig. 5. Models of D/V compartment boundary formation. Shaded squares represent marked cells, dotted squares represent cells in the ZNC. (A) ZNC as barrier. Cells in the ZNC do not divide and cells are prevented from entering and exiting the ZNC. Two top clones were formed outside the ZNC after it formed, and thus define two separate lineage boundaries on either side of the ZNC. Bottom clone was 'captured' by the ZNC as it formed; portion of clone outside the ZNC grew and partially defined one side of the ZNC, while the portion inside the ZNC did not grow. (B) ZNC as trap. Cells in the ZNC do not divide, but cells outside the ZNC can occasionally enter the ZNC; upon doing so they stop dividing and cannot exit the ZNC. As clones expand the lineage boundary will contain a mixture of marked and unmarked cells. (C) ZNC as trap (modified). Cells in the ZNC do not divide, while cells outside the ZNC invade the margin along a long front after the ZNC breaks. The D/V boundary thus can contain long stretches of marked cells. This model requires that the invasion occurs in such a way that cells do not cross the D/V boundary, and that the cells cease dividing once in this region. (D) Hypothetical origin of observed twin spots that face each other along the D/V boundary. *marker*/*marker* and +/+ sister cells are separated by the formation of the D/V boundary (bold line) and grow, defining the same D/V boundary.

will define two different borders, one for dorsal and one for ventral clones.

This study shows, however, that the D/V boundary defined by dorsal and ventral clones is the same. This result is in partial agreement with the study of Kuhn et al. (1983), who found only one strong lineage restriction boundary along the prospective margin of third instar discs. These authors also described the existence of a second 'partial' lineage restriction dorsal to the 'main' D/V boundary. The present study does not support the existence of such a partial restriction. Even if a partial restriction exists, however, it would not be sufficient to establish the D/V lineage boundary.

The ZNC as a trap?

Another way of viewing the ZNC hypothesis is to suggest that the ZNC acts not as a barrier but as a trap. That is, cells can occasionally enter the ZNC, but once in that region they stop dividing and are therefore prevented from growing across the ZNC (Fig. 5B). This model requires that cells in the ZNC be prevented from crossing and exiting the ZNC during any cell rearrangements that might occur along the margin.

A difficulty with such a model is that it is hard to see how it could generate clones that defined the D/V boundary for long stretches without any violations. As shown in Fig. 4, such clones were common in this study; most extended for at least 10 and as many as 25 cells along the boundary. It is extremely unlikely that these clones reached anything near this size prior to the formation of the boundary. In that case all but a very few clones should have been split by the boundary, while in fact the majority of clones obeyed it. Even more dramatic clones were generated using earlier irradiations by Wilcox et al. (1981); while the *ap* marker was not available for that study, some clones appeared to have extended along almost the entire anterior or posterior D/V boundary in third instar discs.

Once in the ZNC cells should stop dividing, and any division along the margin would imply the existence of some lineage restriction mechanism other than the rate of division. However, if one allows occasional incursions into the ZNC and limited growth within it, the boundary next to a clone should contain a mixture of labeled and unlabeled cells (Fig. 5B); this was not observed.

A modified trap model can be constructed that takes this into account by assuming that the cells of the ZNC are prone to displacement by adjacent cells. As the wing grows, the region of 'original' ZNC cells might occasionally break, and a number of adjacent cells could then fill the gap along a broad front (Fig. 5C). This would explain the common occurrence of long stretches of marked cells along the D/V boundary. In other respects, however, this model is less attractive. If the clones of Wilcox et al. (1981) mark almost half the *ap* boundary, as appears likely from their figures, the invasion required would be impossibly large. The invasion would also have to occur in such a way that dorsal and ventral cells did not intermix; this seems unlikely without some additional mechanism. And, once the invasion

was complete, the cells at the prospective D/V boundary would have to immediately stop dividing, as otherwise crossings would be common after one of these invasions.

A consideration of the apparent twin spots that face each other along the D/V boundary presents a further difficulty for this model. It is unlikely that much of the growth in these clones occurred before the formation of the boundary, as this would require the boundary to have split the clones in a very precise position. This event should be rare, yet only two cases were observed in which apparent twin spots had both crossed the boundary prior to its formation. Yet if these clones originated from immediately adjacent cells (see Results), it is hard to see why in all five cases both clones had extended for long distances along the margin. In the modified trap model, such growth would require that all or a portion of both clones lay outside the ZNC, divided to form a broad front of cells, and then invaded the ZNC in the same place as the facing clone. It seems unlikely that this should occur in all of the observed clones.

Alternative mechanisms?

The discussion above is not meant to imply that the differential affinity hypothesis is the only possible explanation for the D/V boundary. Indeed, as is also true for the A/P restriction there is as yet no molecular explanation for the proposed compartment-specific differences in affinity. The dorsal and ventral compartments do differ in their expression of the PS-1 and PS-2 antigens (Wilcox et al., 1981; Brower et al., 1984). These antigens were shown to be different integrin α subunits (Leptin et al., 1987), suggesting a possible role in cell adhesion. However, this difference appears only during the middle stages of the third instar (Wilcox et al., 1981; Brower et al., 1985), and even by late third instar the D/V differences are not exact (Brower, 1984; Brower et al., 1984). This is well after the time at which *ap* is localized (Williams et al., 1993). Clonal analysis also indicates that the loss of the integrin β subunit shared by the PS-1 and PS-2 α subunits (MacKrell et al., 1988; Leptin et al., 1989) does not cause cells to cross the D/V boundary (Brower and Jaffe, 1989).

Attempts to demonstrate compartment-specific differences in cell affinity directly have as well met with only limited success. While some regional differences in cell sorting were claimed by Garcia-Bellido (1966), these findings were called into question in more recent studies. After using carefully tested cell dissociation techniques, no A/P differences in cell sorting (Fausto-Sterling and Hsieh, 1987) or adhesion (Fehon et al., 1987) were found. A D/V difference in cell adhesion was demonstrated (Fehon et al., 1987), as ventral cells bound more strongly to ventral than dorsal cells. However, as dorsal cells also preferred binding to ventral cells, it is not clear whether this preference could provide the basis for segregation.

It is possible that some behavior of cells in the boundary region, other than their reduced rate of division, plays a special role in the formation of the lineage restriction. For instance, divisions or cell rearrangements in this region might be preferentially oriented along the margin, preventing growth across the boundary. However, examination of clones generated late in development indicates that many cells grow away from, rather than along, the margin (unpublished observations; also see Bryant, 1970).

Cell death along the compartment boundary could provide another mechanism for restricting lineages. Clonal analysis does not support this hypothesis, since the size of marked clones along the compartment boundaries is not significantly lower than elsewhere in the wing blade (Postlethwait, 1978). However, some cell death was observed throughout the prospective wing blade using the dye exclusion technique, especially at the molt from second to third instar (Williams et al., 1993). While cell death has not been reported along the D/V boundary, neither clonal analysis nor dye exclusion may be sensitive enough to detect rapid and localized death.

It may also be that an affinity difference does exist, but it is limited to the boundary region itself rather than being distributed throughout the whole compartment. This could explain why assays for differential cell affinity in whole compartments have been largely unsuccessful. Only those cells facing non-like cells might express this difference; in effect, one compartment would 'induce' differential affinity in neighboring cells in the other compartment. Bands of cells have been reported near compartment boundaries that appear to differ in their ability to pass injected dyes (wing disc boundaries: Weir and Lo, 1982, 1984 - though see Fraser and Bryant, 1985; *Oncopeltus* segment boundaries: Blennerhassett and Caveney, 1984). Differences in gene expression have also been observed: several genes are known to be differentially transcribed on the anterior side of the A/P boundary (Phillips et al., 1990; Blackman et al., 1991; Blair, 1992a) and in the margin cells straddling the D/V boundary (Williams et al., 1993; this study).

At the A/P boundary the *hedgehog* gene product could play a role in transcompartment induction. In the wing disc, *hedgehog* expression is limited to the region of *engrailed* expression; *hedgehog* encodes a secreted product (Lee et al., 1992) which is thought to play an inducing role in the embryo (reviewed in Hooper and Scott, 1992). Thus, the formation of special boundary regions by trans-compartment induction seems plausible. However, any role for such boundary cells in maintaining lineage restrictions is at this point strictly hypothetical.

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