**engrailed expression in the anterior lineage compartment of the developing wing blade of Drosophila**

SETH S. BLAIR

Department of Zoology, University of Wisconsin, Madison, WI 53706, USA

Summary

The developing wing of *Drosophila melanogaster* was examined at larval and pupal stages of development to determine whether the anterior-posterior lineage boundary, as identified by lineage restrictions, was congruent with the boundaries defined by the expression of posterior-specific (*engrailed*, *invected*), and anterior-specific (*cubitus interruptus-D*) genes. The lineage boundary was identified by marking mitotic recombinant clones, using an enhancer trap line with ubiquitous β-gal expression in imaginal tissues; clones of +/+ cells were identified by their lack of β-gal expression. Domains of gene expression were localized using antibodies and gene specific lacZ constructs. Surprisingly, it was found that *engrailed* expression extended a small distance into the anterior lineage compartment of the wing blade, as identified with anti-e/i/i/iv mAb, anti-en polyclonal antiserum, or an e/i-promoter-lacZ insert, ryxho25. This anterior expression was not present in early third instar discs, but appeared during subsequent larval and pupal development. In contrast, the expression of *cubitus interruptus-D*, as identified using the ci-DlacZ insert, appeared to be limited to the anterior lineage compartment. Thus, *en* expression is not limited to cells from the posterior lineage compartment, and *en* and ci-D activities can overlap in a region just anterior to the lineage compartment boundary in the developing wing. The lineage boundary could also be identified by a line of aligned cells in the prospective wing blade region of wandering third instar discs. A decapentaplegic-lacZ construct was expressed in a stripe several cells anterior to the lineage boundary, and did not define or overlap into the posterior lineage compartment.

Key words: segment polarity, cell lineage, homeotic mutations, Drosophila, gene expression, compartment, wing disc.

Introduction

Mosaic analysis has shown that, from the time of its formation, the anlage of each imaginal disc in *Drosophila* is formed from two lineally distinct groups of cells, the anterior and posterior (AP) lineage compartments (Garcia-Bellido et al., 1973, 1976; Crick and Lawrence, 1975; Steiner, 1976; Lawrence and Morata, 1977; Struhl, 1977). As each disc grows and differentiates, the cells of the two compartments do not intermingle. Rather, they remain as distinct regions within the disc, separated along a reliably localized, smooth boundary. As defined by marked clones resulting from mitotic recombination, this boundary is visible not only in resultant adult appendages, but also at larval stages of development (Brower et al., 1981; Kuhn et al., 1983).

Though the location of the AP boundary is reproducible from fly to fly, it does not correspond in adults to any distinct morphological feature. For instance, in the wing blade the AP boundary lies in the apparently featureless epithelium in the region between the third and fourth longitudinal veins (Garcia-Bellido et al., 1973, 1976). Rather, the AP lineage compartments are thought to correspond to, and be defined by, regions of differential gene activity established during embryonic development. In particular, the gene *engrailed* (*en*) is thought to be expressed in the posterior lineage compartment (Kornberg et al., 1985; DiNardo et al., 1985; Brower, 1986), and to play a critical role in defining or maintaining the posterior identity of these cells (Garcia-Bellido and Santamaria, 1972; Garcia-Bellido, 1975; Morata and Lawrence, 1975; Lawrence and Morata, 1976; Kornberg, 1981; Lawrence and Struhl, 1982; Brower, 1984). The highly homologous gene *invected* (*inv*) is also expressed in what appears to be the posterior compartment (Coleman et al., 1987), while the gene *cubitus interruptus-Dominant* (*ci-D*) is expressed in an anterior specific pattern (Orenic et al., 1990; Eaton and Kornberg, 1990). These genes are expressed within complementary, non-overlapping regions within each segment in the gastrulating embryo. The small number of cells forming the imaginal disc anlage (see Cohen et al., 1991) are thought to inherit and maintain this expression pattern, which in turn acts in some manner to control the identity and affinity of these cells during growth and differentiation.

However, a recent study examining the expression of
decapentaplegic (dpp) in the wing has called the posterior compartment-specific expression of en into question (Raftery et al., 1991). Indeed, while the posterior expression of en and inv, and the anterior expression of ci-D in imaginal discs have been well defined, their exact relationship to the lineage boundary as defined by clonal analysis has never been established. This point is critical, since it has been assumed that the ability to express en is clonally inherited; such inheritance is an important part of the “selector gene” model of compartmental identity (Garcia-Bellido, 1975; see Discussion).

In the present study, I have examined this question using a novel method for marking mitotic recombinant clones in developing imaginal tissue. Enhancer trap lines of Drosophila (Bellen et al., 1989; Bier et al., 1989; Wilson et al., 1989) were found that express the non-endogenous enzyme β-galactosidase (β-gal) ubiquitously in the developing imaginal discs, due to the presence of a single insertion of the β-gal coding sequence into a transcriptionally “neutral” location in the genome. By inducing mitotic recombination in flies heterozygous for this insert, clones of homozygotic cells could be identified by the presence or absence of the β-gal protein. In this way, the AP lineage boundary could be visualized in larval and pupal stages prior to the secretion of adult cuticle, and co-localized with the regions of gene expression.

Surprisingly, I have found that during late larval and pupal life a small region of apparent en expression appears in the anterior lineage compartment of the developing wing blade, and that this region of anterior expression overlaps the compartment-specific expression of ci-D. The implications of this finding for wing development will be discussed.

Materials and methods

Fly strains

Flies were reared at 25°C. Oregon R flies were used for wild type. The WG 1296 strain was obtained from the collection of P-element lines at the Drosophila Stock Center in Bloomington. WG 1296 is viable as a homozygote; the insertion has been localized to region 3D on the X chromosome (N. Swaminathan and E. Ruthison, personal communication). This line was used for marking clones because of the higher rate of mitotic recombination on the X chromosome (Stern, 1936; Garcia-Bellido, 1972; Ashburner, 1989). cf-Dlac (Eatton and Kornberg, 1990) flies were kindly provided by Dr T. M. Orenic, ryxho25/CyO (Hama et al., 1990) by Dr M. Schubiger, and BS3 0 (Blackman et al., 1991) by Dr M. Sanicola. ryxho25 homozygotes only occasionally survive as adults (Hama et al., 1990) at observed rates of approximately 1%; the great majority of larval and pupal wings from the ryxho25/CyO stock were presumed to be heterozygotes β-gal expression appeared unaltered in ryxho25/+ and cf-Dlac/+ flies.

Generation of mitotic recombinant clones

To obtain marked wild-type clones, virgin wild-type females were mated to WG 1296 or ryxho25 males. Eggs were collected for 2 to 3 days on fly food. The developing larvae were then irradiated using a gamma-ray source at 14 or 4 krad, 0-1 days after the collection ended, thus, each bottle contained larvae irradiated at a variety of stages. To provide an estimate of the age at irradiation, wandering larvae and white prepupae were collected at measured times after the irradiation. The size and frequency of clones generated depend upon the time of irradiation, clones being larger but less frequent in flies irradiated earlier in development. To generate large clones marking the AP boundary, larvae were collected approximately 72 hours after irradiation. The wandering third instar lasts from approximately 96-120 hours after egg-laying (AEL) (Ashburner, 1989); the time of irradiation was thus approximately 24-48 hours AEL. Small clones in ryxho25/+ wings were generated using 4 krad at approximately 48-72 hours AEL; larger clones from earlier irradiations were too rare to be useful. Anti-β-gal was used to detect clones; this avoids the problem of dye diffusion occasionally encountered using the Xgal-based enzyme activity assay.

Fixation

For anti-β-gal staining only, or staining coupled with FITC-phalloidin staining, tissue was fixed in PBS containing 4% EM grade formaldehyde (Polysciences). For anti-en/inv single and double labeling, discs and wings were fixed in either 4% formaldehyde-PBS, a Pipes-parafomaldehyde solution (Brower, 1986), or a modified Pipes-parafomaldehyde solution (Raftery et al., 1991); all three gave similar staining, though the latter two fixatives were more consistent. In some cases, 1-2 µg/ml Hoechst 33258 was added to the fixative. Dissected wandering third instar discs were fixed for 20-30 minutes at room temperature, while 24-36 hour AP pupae were partially removed from the pupal case and fixed overnight at 4°C prior to dissection of wings (Blair and Palka, 1989).

Antibody staining

All incubations were performed in PBS containing 0.3% Triton X-100 at 4°C. For labeling with anti-β-gal alone, Promega monoclonal mouse anti-β-gal was used. For double labeling, anti-en/inv supernatant or ascites fluid and rabbit anti-β-gal antiserum (kindly provided by Dr S. Carroll) were used. A mixture of mouse-anti-en/inv and rabbit anti-en polyclonal (DiNardo et al., 1985) was kindly provided by Dr J. P. Vincent.

The primary antibody concentrations used depended upon the flies being stained and the type of labeling utilized.

(1) For diaminobenzidine (DAB) staining, a nickle-intensified antibody protocol was used. After incubation overnight in primary antiserum (1/10,000 mouse anti-β-gal, 1/2,000 anti-en/inv ascites, 1/50 anti-en/inv supernatant, or 1/5 to 1/50 anti-en polyclonal), tissue was incubated for 1.5 hours in biotinylated anti-mouse or anti-rabbit IgG (1/200, Vector), washed and incubated for 1.5 hours in Vector A+B Elite solution (1/100 dilution from stocks). Tissue was washed into 0.1 M Tris (pH 7.65), and reacted in 0.5 mg/ml DAB (Sigma) in Tris containing 0.04% NiCl2 and 0.015% H2O2.

(2) Fluorescent double labeling with anti-en/inv and rabbit anti-β-gal was performed by preincubating the tissue in PBS-T containing 5% neonatal calf serum (Sigma), a primary incubation overnight with 1/500 anti-en/inv ascites and 1/200 rabbit anti-β-gal, a 1.5 hour secondary incubation in 1/1600 FITC anti-mouse IgG (US Biochemical) and 1/200 biotinylated anti-rabbit IgG (Vector), and a 1.5 hour tertiary incubation in 1/1600 FITC anti-mouse IgG and 1/1600 RITC-streptavidin (Vector). Equivalent staining could be achieved using 1/5 dilution of anti-en/inv supernatant.

(3) To label cell outlines in anti-β-gal-stained discs, the
procedure of Condic et al. (1991) was modified. Discs were incubated overnight with mouse anti-β-gal (1/200), and labeled using either a 3 hour incubation in RITC anti-mouse IgG (US Biochemical) for rxyho25, or for ci-D<sup>rec</sup> using 1.5 hours in 1/200 biotin anti-mouse IgG (Vector) followed by 1.5 hours in RITC streptavidin. Labeled discs were post-fixed for 30 minutes in PBS-formaldehyde containing a 1/10 dilution of FITC-phalloidin stock (3.3 μM in methanol; Molecular Probes). FITC-phalloidin labeling was also used on DAB-stained discs.

All tissue was mounted under coverslips in 80% glycerol-PBS, 0.5% propyl gallate and stored at -20°C. Wings were observed and photographed with a Nikon Microphot, a Zeiss Axioskop, or a Biorad confocal microscope. Wings and clones were also observed using a MTT video camera and Javelin monitor; clones for Fig 3 were traced onto transparencies directly from the monitor, and re-mapped onto a "generic" wing outline using an overhead projector.

Results

Marking clonal boundaries using β-gal marked clones
To mark mitotic recombinant clones in the developing wing, an enhancer trap line (WG 1296) was used that showed relatively uniform expression in imaginal tissues (Figs 1, 2, 6C). The line stained at all ages examined (mid-third instar through 36 hours AP), and stained other imaginal structures as well such as leg and eye-antennal discs (not shown). As in other P[Arb] lines, the β-gal is localized to the nuclei of the expressing cells, apparently due to targeting sequences in the construct (Bellen et al., 1989; Wilson et al., 1989).

Two types of clones (Stern, 1936) were expected from mitotic recombination in these flies: +/+ cells completely lacking β-gal expression and WG 1296/WG 1296 cells expressing twice the heterozygotic level of β-gal. In imaginal tissue, mitotic recombinant clones often, though not always, remain as contiguous patches of cells, and the two homozygotic clones produced by sister cells are commonly adjoining (Bryant, 1970; Garcia-Bellido and Merriam, 1971; Postlethwait, 1978). Here, such “twin-spots” were observed in all stages and discs examined (wing, haltere, leg and eye-antennal disc; also see Wolff and Ready, 1991, for an examination of eye development using this technique). After staining with anti-β-gal, +/+ patches of cells appeared as unstained regions (Figs 1, 2), while in ideal preparations the staining in WG 1296/WG 1296 nuclei was observably more intense than that in the surrounding tissue (see Figs 2C, 6C).

The fluorescent nuclear dye Hoechst 33258 could also be used to help visualize clones in DAB-stained wings (Fig. 2C-F). Because of the dark DAB reaction product, bright fluorescently stained nuclei were only visible in regions of tissue lacking β-gal expression. This is similar to the DAPI technique used extensively to observe staining patterns in embryos (Karr and Kornberg, 1989).

AP lineage boundary in pupal wings
To localize the AP boundary, large mitotic recombinant clones (see Materials and methods) were examined in pupal wings between 24 and 36 hours AP. At this stage of development, the dorsal and ventral epithelia have joined and a largely mature pattern of morphologically identifiable veins have formed (Waddington, 1940; Figs 1, 2, 3). These veins are slightly wider, relative to the rest of the wing, than those seen in the adult, but nonetheless provide reliable landmarks for the localization of boundaries. The adult cuticle is not secreted until some time after 36 hours AP (Waddington, 1940), after which antibody staining becomes problematic.

All +/+ clones that lay wholly or partially between L3 and L4 distal to the anterior cross vein (ACV) (see Table 1) are diagrammed in Fig. 1, and examples are shown in Fig. 2. Clones showed for the most part irregular outlines except along a distinct boundary 1-3 nuclei anterior to the anterior border of L4. As expected at the AP boundary, clones at this boundary formed a smooth, straight proximodistal line. Moreover, contiguous clones were never observed to cross this boundary. When visible, the adjacent twin-spot clone (β-gal/β-gal) always lay upon the same side of the boundary as did the unstained +/+ clone (see Fig. 2C). When the increased width of L4 in pupal wings relative to that observed in adults is taken into account, the localization of this boundary correlates well with the reported localization of the AP boundary in adults (Garcia-Bellido et al., 1973, 1976; Lawrence and Morata, 1977).

One concern in such clonal analysis is that the apparent single clones observed between L3 and L4 were in some cases fused clones from independent mitotic recombinant events (double hits). However, even at higher doses of irradiation (4 krad), no contiguous patches of marked cells were observed to cross the apparent AP boundary (Fig. 1C); if the rate of fused double hits was high, such patches should be common.

en and ci-D boundaries in pupal wings
Two different methods were used to mark the regions of en activity in pupal wings. The first was to stain with a monoclonal antibody (mAb) specific to en and inv (anti-en/inv; Patel et al., 1989) or with an anti-en polyclonal antiserum (DiNardo et al., 1985). Wings were stained at 28-32 hours AP, and boundaries identified relative to vein boundaries as noted above (polyclonal staining, Fig. 3A, A'); mAb staining, not shown). Staining levels at this age were relatively low; nonetheless, as in imaginal discs (Brower, 1986; see below), staining was much stronger in the posterior of the wing. Surprisingly, however, no distinct change in anti-en staining was observed at the apparent AP lineage boundary as defined above by clonal analysis. Rather, staining appeared to decline a small distance posterior to L3, well anterior of the AP boundary; much lower levels of stain were apparent anterior to this. Thus, it appears that en/inv-like antigens were being expressed in the anterior lineage compartment. Largely identical staining was observed using the mAb and the polyclonal antiserum. Since the polyclonal antiserum was gener-
A. Wandering 3rd instar

B. 15 krad

C. 4 krad
Compartments in the fly wing

Fig. 1. (A) Diagram of wandering third instar wing disc and 30 hour AP pupal wing. Wing pouch region (prospective distal wing blade) in wing disc lies between dorsal (D) and ventral (V) distal-most folds. Dark line marks the position of the prospective anterior and posterior margins (AM and PM). T marks the approximate location of the prospective distal tip. Pupal wing shows approximate position of tissue corresponding to these markers. D and V now lie on opposite surfaces of the wing blade in similar proximodistal positions. Gray lines mark longitudinal veins (L1-L5) and the anterior and posterior cross veins (unlabeled). Box outlines detail for B and C. Wing disc and pupal wing not drawn to scale. (B,C) Outlines of +/+ patches (hatched areas) in irradiated WG 1296/+ pupal wings projected onto generalized wing diagrams; (B) 1.5 krad, (C) 4 krad. Patches from same wing shown on same diagram. All clones observed that lay wholly or partially between L3 and L4 distal to the anterior cross vein are shown, except for those from the experiment including Fig. 2D, which faded prior to scoring.

ated against a region of the en protein that contains little homology to inv (see Coleman et al., 1987), it is unlikely that the anterior compartment staining is due to inv activity alone (see Discussion).

A second method used to mark the region of en activity was to stain wings from a stock carrying an en promoter-lacZ construct, ryxho25 (Hama et al., 1990). In ryxho25/CyO (Fig. 3B,B') or ryxho25/+ (see Fig. 4) pupal wings, the pattern of expression detected with anti-β-gal was different from that observed above using anti-en; nonetheless, expression was also apparent in the anterior lineage compartment, as a region of faintly stained cells anterior to a region of more strongly stained cells. The strongly labeled cells appeared to define the posterior lineage compartment, while most or all of the faint cells were clearly within the anterior lineage compartment. The region of faint anterior staining was widest and more intense near the distal tip of the wing, occupying about two thirds of the region between L4 and L3. Finer, thinner regions were apparent further proximally, but could not be detected proximal to the anterior cross vein.

The anterior staining is not due to the diffusion of dye from posterior cells. In all other marking experiments, using either WG 1296 or ci-DPlac (see below), fixed β-gal as detected by antibodies remained strictly localized. Nor is the anterior stain due to processes from posterior cells. While the β-gal in ryxho25 wings at 30 hours AP was distributed throughout the cells, at earlier stages (24 hours AP), the stain became visible in epithelial nuclei, even in the region of faint anterior structures (not shown). Finally, small clones of +/+ cells could be generated late in the development of ryxho25/+ larvae (see Materials and methods). As above, +/+ clones could be identified by the lack of β-gal, but could not be identified in the region of the wing not expressing β-gal. These unstained patches were clearly clones, however, as they were not observed in unirradiated ryxho25/CyO flies. Clones in the region of faint anterior staining could extend to the posterior limits of faint staining (defining the AP lineage boundary) without any apparent disruption of staining in the adjacent, posterior cells (Fig. 4), while posterior clones adjacent to the lineage boundary did not disrupt anterior staining (not shown).

It has been reported that the expression of ci-D is limited to the apparent anterior compartment (Orenic et al., 1990; Eaton and Kornberg, 1990). Staining 30 hour AP wings from the ci-DPlac enhancer trap line (Eaton and Kornberg, 1990) showed that, unlike either anti-en or ryxho25 staining, ci-D activity corresponded approximately to the AP lineage boundary as defined above (Fig. 3C,C'). Staining in this line was not uniform throughout the anterior compartment, however, as levels of expression dropped slightly in a region from midway between L3 and L4 down to the AP lineage boundary. The region of fainter expression in ci-DPlac corresponded approximately to the region of faint anterior staining in ryxho25. Staining stopped completely 1-3 cells anterior to L4. Thus, it appears that the ci-DPlac more clearly defined the AP compartment boundary in pupal wings than did either anti-en or ryxho25, and overlapped the anterior expression of en/inv (compare with Fig. 3A,A'). No gap or region of reduced staining was observed in ryxho25/+/; ci-DPlac/+ flies (not shown).

Boundaries in third instar wing discs

The prospective wing blade region of the imaginal disc is shown in Fig. 1A. At this stage, the dorsal and ventral epithelia have not yet joined together (Waddington, 1940). Rather, they lie on opposite sides of the prospective anterior and posterior margin. The epithelium is highly folded; however, the wing "pouch" region, which lies between the two most distal dorsal and ventral folds, is fairly flat, and corresponds to the future distal portion of the wing blade (Bryant, 1978). From mapping using sensillum-specific enhancer traps, the region between the two distal folds will form the wing blade from about halfway along the wing blade.

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<th>Irradiation (krad)</th>
<th>Clones defining compartment boundary</th>
<th>L3-L4 clones*</th>
<th>Total clones†</th>
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*All clones partially or wholly located between L3 and L4 distal to the ACV.
†All clones distal to the approximate site of the convergence of L1 and L3.

Table 1. Number of contiguous +/+ patches ("clones") in WG 1296/+ pupal wings
Fig. 2. Large recombinant clones of +/+ (++) cells in WG 1296/+ pupal wings (24-36 hours AP). Wings were stained with anti-β-gal and DAB; (A,B) ++ nuclei are unlabeled, and WG 1296/+ cells are dark; (C-F) wings have been counterstained with the fluorescent nuclear dye Hoechst 33258, and ++ nuclei are more brightly fluorescent. In C the double-dark WG 1296/WG 1296 cells (β) are visible. Clones extending between L3 (3) and L4 (4) define the AP compartment boundary (A/P) just anterior to L4. Clones in A, C, and E define the anterior compartment; clones in B, D, and F define the posterior compartment. A-D irradiated at 1.5 krad, E,F irradiated at 4 krad, note multiple clones in each wing. Wing in D not included in Fig 1. Scale bars, 50 μm.

The anti-β-gal staining obtained with the ryxho25 and ci-Dplac lines at wandering third instar was similar to that in pupal wings (Figs 5A,B, 6A,B). In ryxho25, a strongly stained posterior region was observed, as was a fainter region of anterior staining. As in pupal wings, the anterior stain was especially distinct near the prospective distal tip (Fig. 5A). The anterior staining was, however, fainter at this stage than in pupal wings, and often not visible in fluorescently labeled discs (e.g. Figs 6A, 8A). ci-Dplac wing discs showed, as in pupal wings, a simpler pattern of staining, being expressed in a sharply defined region in the anterior of the prospective wing blade (Figs 5B, 6B); the drop in ci-D expression observed in pupal wings was not visible in wandering third instar wing discs. As in pupal wings, no gap in staining was observed in ryxho25/+; ci-Dplac/+ wing discs (not shown); thus the two regions of staining either abut or overlap. If the regions of stain do not shift between larval and pupal stages, the strong posterior staining in ryxho25, and the stained anterior region in ci-Dplac, mark the posterior and anterior lineage compartments, respectively.

As shown previously (Brower, 1986; Patel et al., 1989), strong anti-en polyclonal or anti-en/inv mAb staining was limited to the posterior of the prospective
Compartments in the fly wing

Fig. 3. Regions of gene activity in pupal wings (28-32 hours AP). (A,A') Whole wing and detail of anti-en polyclonal staining. Strong staining extends well anterior of L4 (4) towards L3 (3). Faint staining is observed further anterior. × marks debrs. (B,B') Whole wing and detail of anti-β-gal staining in rxyho25/CyO. Note region of dark stain in posterior region, and region of faintly stained cells between L4 and L3. ? marks approximate site of AP lineage boundary, as defined in Fig. 1 and 2. (C,C') Whole wing and detail of anti-β-gal staining in ci-Dplac. Staining extends from anterior down to approximate site of the AP lineage boundary. Arrowhead marks region of slightly fainter staining from midway between L3 and L4 down to the AP boundary. Scale bars, 50 μm.

To compare the location of the putative AP lineage boundary with en expression, rxyho25 and ci-Dplac wandering third instar wing discs were double stained with anti-β-gal and anti-en/inv mAb. As in pupal wings, the boundaries marked in these double-stained wings were not congruent. In wandering third instar wing discs, the anti-en/inv staining clearly extended anterior to the region of intense β-gal expression in rxyho25 (Fig. 6A), and overlapped the region of expression in ci-Dplac/+ (Fig. 6B), especially near the prospective distal tip. This overlap could be observed both with conventional and confocal fluorescence microscopy.

To confirm that these boundaries are not shifting between wandering third instar and older pupal wings, the clone marking technique was used to define the AP lineage boundary directly in wandering third instar discs. WG 1296/+ larvae were irradiated as above (4 krad), and wandering third instar wing discs were double stained with anti-β-gal and anti-en/inv mAb. Since no contiguous patches of marked cells were observed to cross the AP boundary in pupal wings, even at the high dosage used, it was expected that the AP boundary would be clearly visible in wandering third instar discs. Of 12 +/- or WG 1296/WG 1296 clones observed in the region of the anti-en/inv boundary, all...
drew a smooth line posterior to the anterior-most region of anti-\textit{en/inv} expression (10 anterior, 2 posterior clones; e.g. Fig. 6C). Thus, the anti-\textit{en/inv} boundary is not congruent with the AP lineage boundary in wandering third instar wing discs.

Interestingly, anterior \textit{en} staining appears during the
Fig. 6. Fluorescent confocal images of double-stained wandering third instar wing discs. In all, left is anti-β-gal, center is anti-en/inv, right is double image. (A) rysho25/CyO; note region of anti-en/inv staining anterior to region of intense β-gal expression (anterior stain red in double image). (B) x-lymph/+; note region of overlap between regions of anti-en/inv staining and β-gal expression (overlap orange in double image). (C) Large anterior WG 1296/WG 1296 clone (brightly labeled) in WG 1296/+ disc. Pseudo-coloring used to increase contrast in left and center frames; yellower labeling is more intense. Left frame; clone (inside brackets) defines smooth line in prospective dorsal and ventral epithelia at the apparent AP boundary, but extends anterior to region of anti-en/inv staining, especially proximally and distally. Detail shows brighter image of clone, emphasizing proximal and distal limits; bright spots outside brackets due to noise from low-contrast image. Center frame; anti-en/inv stain, extending anterior to posterior boundary of clone (arrow). Focus and staining does not emphasize anterior staining, except near prospective distal tip (compare Fig. 5D). Right frame; double image without pseudo-coloring (anti-β-gal, red; anti-en/inv, green). Yellow shows overlap, especially obvious near prospective distal tip. Scale bar, 50 μm.
Fig. 7. *ryxho25/CyO* wing disc from early third instar (approximately 72 hours AEL). Top panel shows anti-\(\beta\)-gal stain, middle panel shows anti-\(en\)/inv mAb stain, and bottom panel shows double image. Note congruence of the boundaries of anti-\(\beta\)-gal and anti-\(en\)/inv staining. Scale bar, 25 \(\mu m\).

Fig. 8. (A, B) Fluorescent confocal images of apical surface of prospective distal region of *ryxho25/CyO* wandering third instar wing disc, stained with anti-\(\beta\)-gal to define AP compartment boundary and FITC-phalloidin to visualize cell outlines. Line of aligned cells marked by arrowheads. (A) Double image, showing congruence of aligned cells to region of \(\beta\)-gal expression. Anterior \(\beta\)-gal expression is too faint to be visible. (B) Single image, showing cell outlines only. Scale bar, 25 \(\mu m\).
course of the third larval instar. In early third instar
ryxho25 wing discs (approximately 72 hours after egg
laying, AEL), the boundaries of β-gal expression and
anti-en/inv mAb staining appeared completely con-
gruent (Fig. 7). By middle third instar (approximately
96 hours AEL, pre-wandering), anterior en expression
was apparent (not shown), extending past the boundary
of intense staining in rxyho25 in a manner similar to that
seen in wandering (late) third instar discs. Thus,
anticor en expression only appeared during the last
stages of wing disc growth. This provides a potential
explanation for the difference observed between anti-en
staining and the expression of β-gal in rxyho25 (see
Discussion).

**Aligned cells at the AP boundary**

In late third instar wing discs, the level of anti-en/inv
mAb and anti-en polyclonal staining drops slightly
along a line through the middle of the prospective wing
blade, except near the distal tip (Fig. 5C,D). The
location of this line corresponded at least approxi-
mately to the AP boundary, as defined by ci-Dpale,
the region of strong staining in rxyho25, and clonal analysis
(not shown).

A boundary of morphologically aligned cells has been
previously observed in this region along the apical
surface near the prospective distal tip, and may
correspond to the AP lineage boundary (D. Fristrom,
personal communication; this feature is distinct from
the furrowed cells observed further proximally and
basally along the folds by Brower et al. (1982), which do
not always mark the AP boundary). To localize the
aligned cells, rxyho25 wing discs were double stained
with anti-β-gal, to visualize the AP boundary, and with
FITC-phalloidin, which marks cell outlines (Condic et
al., 1991). A typical boundary is shown in Fig. 8. While
most cells in the epithelium were polygonal in shape,
cells at this boundary were more rectangular, and
formed an irregular but still distinctly smoother line of
apposition with adjacent cells. In some discs, this line
was harder to detect, or visible in only a portion of the
prospective wing blade; differences in deformation
during mounting may in part account for this variability.

When the boundary was visible, however, the limits of
intense β-gal expression in rxyho25 exactly defined the
cells on one side of the boundary (Fig. 8A); the line
formed by these aligned cells thus marks the AP lineage
boundary.

The cytoplasmic localization of β-gal in rxyho25
made it possible to resolve β-gal expression on the
apical surface of the epithelium. Unfortunately, stain-
ing with anti-en/inv, anti-en polyclonal, and the β-gal in
the WG 1296 and ci-Dpale lines, is largely nuclear. The
nuclei at this stage lie some distance from the apical
surface, making it difficult to assign particular nuclei to
particular apical cell outlines. Thus, though the limits of
ci-D expression, and the decrease in anti-en/inv and
anti-en polyclonal staining, both appeared to corre-
spond approximately to the region of aligned cells (not
shown), this could not be determined at a cell-by-cell
level of resolution. It was clear, however, that anti-en
staining extended anterior to this line.

dpp and the AP lineage boundary
decapentaplegic (dpp) is a gene that is expressed in a
stripe near the AP lineage boundary of imaginal discs.
In a recent study, a small amount of overlap was
observed between the regions of expression of en, as
detected with anti-en/inv mAb, and dpp, as detected
with dpp-lacZ constructs, in wandering third instar discs
(Raftery et al., 1991). To determine whether dpp was
being expressed in the posterior lineage compartment,
or in the region of anterior en expression, rxyho25/
BS3.0 wandering third instar wing discs were stained
with anti-β-gal; BS3.0 is a dpp-lacZ construct which
appears to give complete dpp staining in the prospective
wing blade (Blackman et al., 1991). As shown in Fig.
9A, a substantial gap was observed between the regions
of intense rxyho25 activity and the stripe of dpp activity.
Thus, the region of dpp activity does not appear to
extend as far posterior as the putative AP lineage
boundary, as defined by rxyho25. This was confirmed
by staining BS3.0 wings at 30 hours AP; the stripe of
dpp expression extended from L3 down to a line
halfway between L3 and L4 (Fig. 9B), well anterior of
the lineage boundary as defined by clonal analysis. The
overlap observed by Raftery et al. (1991) is therefore
with anterior en-expressing cells.

**Discussion**

In this study, a novel method for identifying mitotic
recombinant clones was used to identify the AP lineage
boundary in wandering third instar and pupal wings.
Surprisingly, it was found that the AP lineage boundary
defined by these clones was not congruent with the
region of staining identified with anti-en/inv mAb (Patel
et al., 1989), anti-en polyclonal (DiNardo et al., 1985),
or in the region of anterior en expression,
ryxyho25/BS3.0 (Hama et al., 1990) in wandering third instar or pupal wings.
Rather, in the prospective distal wing blade, a region of
en expression appeared in the anterior compartment
during mid-third instar, extending anterior to the true
AP lineage boundary. This differed from the activity of
ci-D, which, as identified using the ci-Dpale enhancer
trap (Eaton and Kornberg, 1991), was expressed in the
anterior compartment and appeared to define the AP
lineage boundary at all the stages observed. Thus,
contrary to expectations, the ability to express en-like
activity is not limited to cells derived from the posterior
lineage compartment, and en and ci-D can be expressed
in overlapping regions of tissue.

The similar staining patterns observed using the anti-
en/inv mAb and the anti-en polyclonal serum make it
unlikely that anterior compartment staining was due
to the anterior expression of inv protein rather than
en. The mAb should detect both en and inv, since
it was generated against the highly homologous homeo-
box regions of these proteins (Patel et al., 1989).
However, the polyclonal serum was generated against a
portion of the \( en \) protein which shares little homology to \( inv \), and embryonic staining is lost in \( en \) null mutations (DiNardo et al., 1985).

The anterior expression of \( \beta \)-gal in wings containing the \( en \) promoter-\( lacZ \) construct (\( rxyho25 \)) also argues for the expression of \( en \) in the anterior lineage compartment. It should be pointed out, however, that the details of the staining patterns observed using anti-\( en \) differed from that of the promoter construct; these different assays therefore cannot be used interchangeably in all tissues, as has been previously assumed. While all showed staining in the anterior lineage compartment, \( \beta \)-gal expression showed a dramatic, if incomplete, reduction anterior to the lineage compartment boundary, while the reduction in anti-\( en \)/\( inv \) mAb or anti-\( en \) polyclonal staining was not nearly so distinct in wing discs, and was undetectable in later pupal wings. It is possible that this is due to differences in the transcription of \( en \) and the construct. The promoter construct is a complicated P-element chimera containing 5.7 kb of \( en \) upstream DNA, which has reintegrated near the \( en \) transcription start site and disrupts the function of the \( en \) gene (Hama et al., 1990). Since the upstream DNA is insufficient to drive \( en \)-like expression in discs at other insertion sites (\( en \) mutations define over 70 kb of DNA; Drees et al., 1987), it appears that the construct is acting more as an \( en \) enhancer or promoter trap. As such, \( \beta \)-gal expression in this line may represent partial regulatory activity of the locus.

However, the late development of anterior \( en \) expression during the third instar provides a more likely explanation for the difference in staining. Assuming that the \( \beta \)-gal protein is more stable than \( en \), the accumulation of \( \beta \)-gal in the posterior compartment during early larval development would tend to intensify the compartment-specific staining relative to the later-arising anterior expression. Thus, even after anterior expression had appeared, the construct would provide an historically weighted record of transcription, while the more rapidly metabolized \( en \) protein would be more sensitive to current patterns of transcription. If this explanation is correct, the region of intense \( \beta \)-gal expression in \( rxyho25 \) should accurately define the posterior lineage compartment. The location of intense \( rxyho25 \) expression does appear to mark the boundary in pupal wings and, in wandering third instar discs, it exactly defines a boundary of cell alignment (D. Fristrom, personal communication; this study) in the prospective wing blade region, such as might be expected at the boundary between cells of differing affinities.

Region of overlapping gene activity

One intriguing feature of these results is the region of overlapping \( en \) and \( ci-D \) expression. The overlap defines a narrow stripe of cells largely or entirely anterior to the lineage compartment boundary, and corresponds approximately to regions of reduced \( \beta \)-gal expression in the \( en \)-promoter-\( lacZ \) and \( ci-D \)-enhancer trap lines. This may represent mutual inhibition of expression between the two genes. Derepression of \( ci-D \) has been previously noted in \( en \) mutant embryos and wing discs, although \( ci-D \) mutations have no effect upon \( en \) expression in embryos (Eaton and Kornberg, 1990).

In the developing wing disc, the region surrounding the AP boundary is the domain of expression of several genes critical for normal development (reviewed by Wilkins and Gubb, 1991). \( decapentaplegic \) (\( dpp \)) and \( patched \) (\( ptc \)) are expressed exclusively or predomi-
nantly in this region (Posakony et al., 1991; Raftery et al., 1991; Phillips et al., 1990). Moreover, wide-ranging morphological defects result if clones lacking these genes are induced just anterior to the AP boundary, but not elsewhere (Posakony et al., 1991; Phillips et al., 1990). One possible cue for the localized expression and function of these genes might be the overlap in en/inv and ci-D expression observed in the present study.

The results of recent studies make this simple scenario unlikely, however. ptc expression overlaps the region of β-gal expression in the en-promoter-lacZ construct in wing discs (Phillips et al., 1990); this overlap extends into what appears to be the region of strong β-gal expression as defined in the present study, which lies within the posterior lineage compartment. Some overlap is also observed between the regions of dpp-lacZ construct expression and anti-en/inv binding (Raftery et al., 1991). However, the overlap is much smaller than that observed in the present study between en and ci-D, even in the same region at the same developmental stage. In fact, a gap of several cells width was observed in the present study between the AP lineage boundary, despite the previously observed overlap with anti-en/inv staining. In fact, none of these boundaries in gene activity appear to be precisely congruent; there are several boundaries of gene expression lying near but not necessarily coincident with the AP lineage boundary.

A role for anterior en expression?

en expression is thought not only to mark the AP lineage boundary, but also to play a functional role in the establishment and maintenance of that boundary, acting as a "selector" gene specifying posterior identity (Garcia-Bellido, 1975). Not only do some mutant alleles of en induce partial duplication of anterior structures in the posterior of the wing (Garcia-Bellido and Santamaria, 1972; Morata and Lawrence, 1975; Eberlein and Russell, 1983; Brower, 1984; Gubb, 1985), but they also disrupt the formation of the AP lineage boundary (Morata and Lawrence, 1975; Lawrence and Morata, 1976; Kornberg, 1981; Lawrence and Struhl, 1982). Moreover, en activity only appears to be required within the posterior lineage compartment. Anterior clones homozygous for lethal en alleles can develop normally, form apparently normal anterior structures, and fail to disrupt the AP boundary, even if they lie adjacent to it (Kornberg, 1981; Lawrence and Struhl, 1982).

This raises an interesting question. If en activity is the critical arbiter of posterior identity, what prevents en-expressing cells in the anterior compartment from mixing with cells in the posterior?

One likely explanation is that the anterior compartment activity arises too late in imaginal disc development to affect clonal restrictions. Anterior en expression was not observed until mid-third instar, yet by late third instar most of the growth of the disc had already taken place (Bryant, 1987; Schubiger and Palka, 1987). As long as neighbor relations were maintained during subsequent development, there might be little opportunity for mixing between posterior and anterior en-expressing cells. However, it should be pointed out that cell rearrangements have been suggested to take place during disc eversion (Fristrom, 1976; though see Condic et al., 1991); it is hard to see how such rearrangements could fail to disrupt the AP boundary without some means of controlling mixing between the compartments. The alignment of cells (D. Fristrom, personal communication; this study) at the lineage boundary is also extremely suggestive of a difference in the affinities of anterior and posterior cells, at a time (wandering third instar) well after anterior en expression has appeared.

Thus, it seems more likely that the late expression of en is in some manner insufficient to alter the identity or affinities of anterior cells. Cells in older discs might no longer be dependent upon en levels for the control of cell affinities. However, clonal analysis (Garcia-Bellido and Santamaria, 1972) and temperature-shift experiments (Lawrence and Morata, 1976) show that posterior cells are dependent upon en activity for their identity, in terms of the structures formed, into late larval and even early pupal stages. Alternatively, it is possible that en must be present for some critical period to alter cell identity, or that a threshold amount of en might be required. Even by late third instar, anterior staining with anti-en does appear somewhat lower than expression in the posterior compartment (except near the distal tip), and the anterior staining with the en-promoter construct is extremely faint. Finally, the recent discovery of anterior-specific genes, such as ci-D (Orenic et al., 1990; Eaton and Kornberg, 1990), provides another possible mechanism, since the expression of such genes might counteract the effects of late anterior en expression upon cell identity.

The role of anterior en expression, however, remains unclear. While such expression might be useful for defining domains of gene activity in the wing, it does not appear to be essential for normal morphological development. If anterior en expression does have a role, therefore, it is either redundant, or the developing wing can in some manner regulate after its loss.

Ubiquitously expressing enhancer trap lines as a method for marking mosaic clones

To identify recombinant clones in the past investigators have had to rely upon available, conveniently located marker mutations (reviewed by Postlethwait, 1978; Lawrence et al., 1986; Ashburner, 1989). Most of these markers are cuticular in nature, severely restricting the stages and tissues that can be examined. Enzyme marker mutations have been used to identify clones in soft tissue that is not secreting cuticle (i.e. Brower et al., 1981; 1982; Kuhn et al., 1983), but these are not always compatible with other types of assays. The use of enhancer trap flies (Bier et al., 1989; Bellen et al., 1989;
Wilson et al., (1989) with fairly uniform expression of β-gal in developing imaginal tissues solves many of these problems. In this study, this method was used for examining the lineage of wild-type cells in the developing wing (see also Wolff and Ready, 1991). It should be pointed out, however, that as with cuticular markers, this method can be used as a means of marking clones of mutant cells, by inducing mitotic recombination in β-gal/mutant flies. In favorable material, mutant clones can be detected at a variety of developmental stages (Blair, 1991; Rulifson and Blair, 1991). As more ubiquitously expressed enhancer trap lines become isolated and the location of the inserts mapped, it is hoped that a family of such markers will be available for studies of mutations throughout the genome.

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References


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