Cell shapes on the surface of the \textit{Drosophila} wing imaginal disc

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\textbf{SUMMARY}

Antibodies that bind to antigens on the surfaces of imaginal disc cells can be used to visualize the cellular morphology of the disc exterior. Using this technique, we have examined wing imaginal discs at various times during the third larval instar, paying particular attention to those areas where the anteroposterior and dorsoventral compartment borders are located. We have been unable to detect any distinguishing feature in the shapes of the cells at the anteroposterior compartment border. However, there is a line of unusually shaped cells extending across the disc, from anterior to posterior and which, in the central region of the disc, takes the form of a shallow groove in the epithelium. A number of observations suggest that this line is coincident with the dorsoventral compartment border.

\textbf{INTRODUCTION}

One genetic technique that has proved particularly useful in studying the development of \textit{Drosophila} is the ability to describe cell lineages by producing, via somatic recombination, clones of cells homozygous for a recessive marker in a heterozygous background (Stern, 1936). The part of the fly most studied by clonal analysis has been the wing and adjacent thorax. It has been demonstrated that, very early in development, the cells which will give rise to the adult wing are subdivided into two (anterior and posterior) groups, defined by the observation that a clone originating in one never extends into the other (Garcia-Bellido, Ripoll & Morata, 1973, 1976). Furthermore, each of these developmental compartments makes a specific set of adult structures. There are indications that these compartments are further subdivided by other lineage restrictions at later times in development (Bryant, 1970; Garcia-Bellido & Merriam, 1971; Garcia-Bellido \textit{et al.} 1973, 1976).

Rather than marking the epidermal cells directly, the experiments referred to above have all utilized genetic markers for adult cuticular structures. During the larval stages, the cells which will secrete the wing cuticle are present in

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flattened sacs called imaginal discs. In a mature larva, the wing imaginal disc contains approximately 50000 cells, most of which are present in the convoluted columnar epithelium that makes up one side of the sac. (Imaginal disc morphology is reviewed by Poodry, 1980.) Most of the reverse side consists of a squamous epithelium known as the peripodial membrane. The disc is continuous with the larval hypoderm, to which it is connected by a thin stalk. Thus, the outside of the disc is the basal surface of the epithelium, and is covered by an extracellular basement membrane. This covering, combined with the poor optical properties of the columnar epithelium, make it very difficult to examine the general morphology of the cells of the disc.

We have recently been generating monoclonal antibodies against cell surface antigens of imaginal discs. After a disc has been fixed in formaldehyde, many of these antibodies penetrate the basement membrane around the disc and bind to the exposed surfaces of the cells, without penetrating very far between them (Fig. 1). Under simple conditions, some of these antibodies can be used to produce immunofluorescent images showing the basal outlines (i.e. on the exterior surface of the disc) of the epithelial cells. We have examined the gross morphology of the cells of the wing disc, paying particular attention to those areas where the compartment borders are located, based on fate mapping (Bryant, 1975) and recent clonal analysis studies (Wilcox, Brower & Smith, 1981; Brower, Lawrence & Wilcox, 1981).

**MATERIALS AND METHODS**

The production and general characteristics of the two antibodies used here have been described previously (DA.1B6, Brower, Smith & Wilcox, 1980; DK.1A4, Wilcox et al., 1981). To examine cell shapes, dissected imaginal discs were first fixed for 10 min at 4°C in 2% formaldehyde in *Drosophila* Ringer. After washing in Ringer, the discs were incubated for 45 min at 37°C in antibody DA.1B6 (typically about 8 μg/ml in PBS), washed in PBS, incubated for 30 min at 37°C in fluorescein-conjugated rabbit anti-mouse IgG (Miles-Yeda) diluted in PBS, and washed again in PBS. Discs to be treated with both antibodies were first incubated in antibody DK.1A4 (typically about 2-5 μg/ml in RPMI medium [Gibco]) at 37°C for 45 min, washed in RPMI medium, incubated in tetramethyl-rhodamine conjugated rabbit Fab'2 antimouse IgG (a gift of R. J. Morris) at 37°C for 30 min, washed in RPMI medium, and then fixed and treated as above. The discs were mounted on slides, slightly flattened with a coverslip, and examined with a Zeiss fluorescence microscope using epi-illumination.

For the generation of mutant *sdh* clones, *cn sdh*8 *bw/CyO* females were crossed to *M(2)c83a/bw* males (see Lindsley & Grell, 1968; Lawrence, 1981) and the progeny X-irradiated at 24-48 h after egg laying (AEL) with 1000 or 1500 R (see Lawrence & Morata, 1977). The third instar larvae were collected
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and heated in *Drosophila* Ringer solution at 47 °C for 12–15 min; this heating reduced the background staining in the *sdh* clones. Discs were then dissected from the larvae and incubated for 1.5–3 h in the staining solution. The staining solution (after Pearse, 1972) was made by combining 1 part 0.5 M succinate with 10 parts of a solution comprising: 100 mg nitro blue tetrazolium (Sigma Chemical Co., St Louis, U.S.A.), 5 ml 1 M tris HCl (pH 7.5), 10 ml 0.05 M MgCl₂, 65 mg NaN₃, 49 mg NaCN, and distilled water to 110 ml (final pH 7.7–2). Unirradiated *sdh/sdh* discs showed uniform staining under these conditions. After staining, the discs were dehydrated in two rinses of 100% ethanol and cleared by immersion in oil of cedar wood (BDH Chemicals Ltd., Poole, England). The discs were mounted in Euparal (GBI Ltd, Denton, England) and photographed using bright-field optics. When cell shapes were to be examined in discs containing *sdh* clones, the following alterations were made to the above scheme: the larvae were incubated at 47 °C for only about 5 min and, after staining, the discs were fixed and treated with antibody as before, without any dehydration. These alterations were necessary to allow the immunofluorescent visualization of cell morphology, but also resulted in higher levels of background staining in the *sdh* clones, often making it difficult to determine exactly where (i.e. between which two cells) the edge of the clone was.

RESULTS

General features

The overall disc morphology and patterns of cell shapes from wing discs dissected at various times during the third larval instar are illustrated in Figs. 2–12. In discs from late-third-instar larvae the cells of the central region of the columnar epithelium, which will make the adult wing blade (Bryant, 1975), are typically much smaller than the cells in more peripheral regions, which will make the more proximal adult structures (Fig. 11). This size discrepancy is not simply an artifact of the folding pattern of the disc (that is, these cells are not smaller only because we are looking at the inside edge of a curve as compared to an outside edge), for the central cells also look smaller if their apical surfaces are examined. The adepithelial cells lie in the deep pocket over the presumptive notum (Fig. 3). These cells are believed to be precursors of some of the adult muscles (Reed, Murphy & Fristrom, 1975). The antibody, which does not stain the adepithelial cells, generally fails to penetrate this layer sufficiently to show the outlines of the underlying epithelial cells (Fig. 2).

The central region of the reverse side of the wing disc is covered by the very thin, squamous cells of the peripodial membrane (Figs. 4, 12). These cells have a large exposed area, and are typically elongated in the long axis of the disc. The peripheral cells on this side of the disc look similar to the cells of the columnar epithelium.

The size difference between central and peripheral cells, on both sides of the
Fig. 1. Part of a wing imaginal disc which has been fixed, incubated in antibody, paraffin embedded and sectioned. (The ventral end of the disc is to the right; see Fig. 2.) The antibody does not penetrate far into the intercellular spaces, so that the fluorescence is confined to the external surface of the columnar epithelium (ce) and the peripodial membrane (pm). Thus, all subsequent immunofluorescence micrographs (except for Fig. 3 and 19) are images of the basal profiles of the disc cells. The arrowhead indicates the dorsoventral groove (see Figs. 10 and 20). × 380.

Fig. 2. Immunofluorescence image of a late-third-instar wing disc. This disc is flattened slightly, although the edges of the deep folds in the epithelium still show up as bright lines. A large pocket over the presumptive notum contains the adepithelial cells (ac), which do not stain with this antibody (DA.1B6) and prevent it from reaching the underlying epithelial cells. The black line indicates the approximate plane and limits of the section in Fig. 1. The four quadrants of the disc are indicated (AV = anterior ventral, PD = posterior dorsal, etc.), and are defined with respect to the regions of the adult wing and mesothorax that each will make. Unless stated otherwise, all subsequent figures are of this, the columnar epithelial side of the disc. × 190.

Fig. 3. Late-third-instar wing disc, fixed and stained with fluorescein-conjugated peanut agglutinin. This lectin binds to the adepithelial cells, but does not recognize the disc epithelium (compare with Fig. 2). × 130.
Fig. 4. The reverse side of a wing disc that is slightly younger than the one in Fig. 2. The large central cells are the squamous epithelium of the peripodial membrane (see Fig. 1). × 200.

Figs. 5 and 6. Columnar epithelium (Fig. 5) and peripodial membrane (Fig. 6) of wing discs from early third-instar larvae (about 72 h AEL). There is no obvious physical manifestation of the anteroposterior compartment boundary, which, based on studies of later discs, would be expected to run approximately vertically across the columnar epithelium. × 320.

disc, can be seen as early as 72 h AEL (very early in the third larval instar) (Figs. 5, 6). And, by 85 h, the columnar epithelium of the disc begins to take on its characteristic pattern of deep folds (Fig. 7).

The anteroposterior compartment border

The border between the anterior and posterior compartments is known to run approximately through the centre of the wing imaginal disc (Brower et al.)
Fig. 7. Ventral and central regions of a wing disc from a larva at about 85 h AEL. At this time the disc is beginning to take on its characteristic pattern of large folds, and the presumptive wing area, in the centre of the micrograph, is becoming a pouch. Again, no compartment borders can be detected. x 490.

Figs. 8 and 9. Wing discs from near the middle of the third instar. Fig. 8 shows a disc from a larva at 96 h AEL; the first suggestions of the dorsoventral discontinuity can be discerned between the arrowheads. Fig. 9 is a slightly later disc, in which the discontinuity is clearly present. In each case, the inset diagram shows the exact position of the discontinuity (dotted line). Fig. 8 × 490; Fig. 9 × 330.
Fig. 10. A higher magnification of part of the disc shown in Fig. 2. Although the central wing pouch is slightly out of focus, the discontinuity which appears to correspond to the dorsoventral compartment boundary can be followed between the arrowheads. The arrow indicates the region of cell alignment in the centre of the disc. (see Figs. 13–15). x 290.
Figs. 11 and 12. Cells of the columnar epithelium (Fig. 11) and peripodial membrane (Fig. 12) in the anterior ventral region of late-third-instar wing discs. In Fig. 11, note that the cells in the centre of the wing pouch (arrow) are smaller than those in the more peripheral areas. (The cells immediately surrounding the wing pouch have been distorted by the flattening of the disc which is necessary to see the central area.) The anteroposterior compartment border runs vertically approximately through and just above the arrow, and then to the left, reaching the disc margin somewhere near the arrowheads. However, no obvious morphological feature indicates the border. 11 × 470; 12 × 360.
Fig. 13. Central region of a late-third-instar wing disc, showing the area of cell alignment. At the dorsal (lower) end of the line there appears to be a relatively large cell; there is no distinct ventral terminus. Similar structures are not seen on the nearby folds. The anteroposterior compartment border runs vertically somewhere through this region of the disc. × 750.

Figs. 14 and 15. Central region of the late-third-instar wing disc containing a large \textit{sdh} clone in an \textit{sdh}+ background. Fig. 14 is a bright-field image, showing the \textit{sdh} clone (unstained). The boundary of the clone (and probably the anteroposterior compartment boundary) lies between the arrowheads. Fig. 15 is an immunofluorescent image of the same disc; the arrow in this figure is in the same location as the arrow in Fig. 14, and indicates the region of cell alignment. Since the clone includes and extends beyond it, the region of cell alignment cannot correspond to the compartment boundary. × 480.
We carefully examined this region of the disc, looking for any change in cell shape or other discontinuity that might indicate the border. As can be seen in Figs. 5–8, there is nothing distinctive in the morphology of the cells in this region of wing discs from early to mid-third-instar larvae.

The situation is similar in discs from late-third-instar larvae, with one notable exception. In an area near the centre of the disc a group of cells typically become aligned with their long axes running across one of the major folds (corresponding to line 3 of Bryant, 1975) in the epithelium (Figs. 10, 13). The dorsal end of this line of cells, but not the ventral end, has a distinct terminus (Fig. 13). In order to see if this discontinuity is an indicator of the place where the anteroposterior compartment border crosses the fold, the following experiment was performed. Larvae which were heterozygous for the cell-marker sdh and for a slow-growing Minute mutation were X-irradiated during the first larval instar to produce sdh Minute+ clones. Wing discs with large clones that appeared to follow the anteroposterior border near the centre of the disc were selected and fixed, and then stained with antibodies to show the cell shapes. (For details see Materials and Methods.) Although the boundaries of the clones often run close to the area of cell alignment, the two are certainly not always coincident. This is most evident in those discs which have anterior clones, which are capable of moving or pushing the compartment border toward the posterior edge of the disc (Brower et al. 1981). In these discs, the area of alignment shows no significant displacement, and can be separated from the compartment border by many cell diameters (Figs. 14, 15). In summary, there is no constant relationship between this morphological feature of the disc and the compartment border.

The dorsoventral compartment border

In late-third-instar wing discs there is another alteration in the normal morphology of the cells that often appears as an alignment of cell boundaries. This discontinuity runs in a line from near the posterior margin through the

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Figs. 16 and 17. Anterior and posterior area (corresponding to the arrowheads in Fig. 10) containing the discontinuity which separates the disc into dorsal and ventral regions. These micrographs are from the same late-third-instar disc, which has not been flattened as much as the one in Fig. 10. × 750.

Fig. 18. Bright-field micrograph of a late-third-instar wing disc with a large sdh clone in an sdh+ background. The dorsoventral groove (between the arrowheads) forms a boundary for the clone (white). × 250.

Figs. 19 and 20. Central region of a late-third-instar wing disc treated to show cell outlines (DA.1B6 antibody/fluorescein) and an antigen which disappears from ventral cells (DK.1A4 antibody/rhodamine). The limit of bright rhodamine fluorescence indicating the DK.1A4 antigen (Fig. 19) corresponds to the dorsoventral groove (Fig. 20). The arrows are in identical locations on the two micrographs. × 740.
wing pouch and then down across the anterior end of the large central fold (which corresponds to line 3 of Bryant, 1975) (Figs. 10, 16, 17). In the posterior part of the disc, the cells are not always aligned all the way to the posterior margin. In the wing pouch, the discontinuity can be seen to be a small groove in the epithelium (Figs. 1, 20). We cannot determine the anterior terminus of the line as after crossing the central fold it is obscured by the deep invagination between this fold and the anterior margin of the disc.

The time of appearance of this groove between the dorsal and ventral regions of the disc is around the middle of the third larval instar. Specifically, we have not been able to detect it unambiguously in discs dissected from larvae 85 ± 1 h AEL (Fig. 7), but we have seen what appear to be very early stages in the formation of the groove in discs from larvae at 96 ± 1 h AEL (Figs. 8, 9).

A number of observations suggest that this groove is a physical manifestation of the dorsoventral compartment border. Its general location (Bryant, 1975) and time of appearance (Morata & Lawrence, 1979) are both consistent with this hypothesis. Also, the path followed by the groove is similar to the path followed by many dorsally or ventrally restricted sdh clones (Wilcox et al. 1981). Moreover, even after the heat treatment and dehydration used to visualize these clones we can sometimes see that the clonal boundaries, in the wing pouch, are coincident with the groove (Fig. 18).

We previously reported an antigen, defined by the monoclonal antibody DK.1A4, which disappears from the cells in the ventral part of the wing disc during the third larval instar (Wilcox et al. 1981). A comparison of the dorsoventral boundary defined by this antigen with the groove shows that they are coincident (Figs. 19, 20).

**DISCUSSION**

Fluorescently labelled antibodies against cell surface molecules provide a convenient way of visualizing the outlines of the epithelial cells of *Drosophila* imaginal discs. Using this method, we have examined the surface of the wing imaginal disc, paying particular attention to those areas where the compartment borders are located. There is no overt morphological manifestation of the anteroposterior compartment border. The dorsoventral compartment border, however, is characterized by a distinct discontinuity in the normal pattern of cell outlines in the columnar epithelium. This discontinuity takes the form of an elongation of the cells parallel to the border in some regions and, in the wing pouch, it is seen to be a small groove in the epithelium.

Although often considered as a two-dimensional sheet, the wing imaginal disc is in reality a three-dimensional sac. Therefore, a compartment boundary that bisects the columnar epithelium must also be continuous across the peripodial membrane or along the margin of the disc. Unfortunately, we did not see any morphological features on the peripodial side of the disc that would indicate where these boundaries lie.
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The segment boundaries in *Oncopeltus* larvae are characterized by an elongation of cells in an axis parallel to the boundary (Lawrence, 1973; Lawrence & Green, 1975). Like the segments in the *Oncopeltus* abdomen, the anterior and posterior compartments of the *Drosophila* wing imaginal disc are contiguous developing units, with separate cell lineages (reviewed by Lawrence, 1981b). However, in the disc, we have not been able to detect a change in cell shape, or any other morphological aberration, at the anteroposterior compartment border.

As to the significance of the small area of cell alignment which crosses the large fold near the centre of the disc, we can only speculate. The fact that its position apparently bears no fixed relationship to the position of the anteroposterior compartment border suggests that it is not directly related to the formation of any particular adult structure. This is not especially surprising, as it appears that the gross morphology of the disc is also unchanged by quite large changes in the position of the compartment border (Brower et al. 1981). If there is a particular function for this region, it is not specific to the wing, for we have observed a similar area of cell alignment across the homologous fold in the haltere imaginal disc.

Using the scanning electron microscope, Reinhardt, Hodgkin & Bryant (1977) observed a furrow running anteriorly to posteriorly across the wing pouch in the late third-instar disc and they suggested that this was the incipient wing margin. This almost certainly corresponds to the groove that we observe by immunofluorescence, and a number of observations suggest that it, and its more proximal continuations, indicate the dorsoventral compartment border. The early indications of the groove can be seen at about 96 h AEL, after the time that the dorsal and ventral compartments are established (probably near or slightly after 72 h AEL; Morata & Lawrence, 1979). The cells on either side of the groove have different biochemical properties, as evidenced by the loss from the ventral cells of the antigen recognized by antibody DK.1A4 (Wilcox et al. 1981). This biochemical difference is detectable at about 87 h AEL, again after the establishment of the dorsal and ventral compartments. Finally, using the *sdh* cell marker, we have observed large clones in the disc which appear to be restricted to the dorsal or ventral compartments, and the boundaries of these clones can sometimes be seen to coincide with the groove.

With the available evidence, we cannot say whether the DK.1A4 antigen is causally related to either the lineage restriction across, or the morphological manifestations of, the dorsoventral compartment border. As noted above, the time when antigen loss from the ventral cells is first detectable does not correlate precisely with the appearance of other dorsoventral differences. However, the significance of these temporal differences is difficult to judge, for the loss of the antigen from the ventral cells is very gradual, and the sensitivity of the cells to differences in antigen concentration may be greater or less than the sensitivity of our immunofluorescence techniques. It is interesting that there appears to be
some correlation between how sharply the DK.1A4 immunofluorescence decreases at the border and how easily we can see a morphological difference in the cells there. For example, both at the anterior end of the major fold in the disc and in the wing pouch, the border is clearly defined by both of the aforementioned criteria. Between these areas and near the posterior margin of the disc it is sometimes very difficult to say precisely (i.e. within one or two cell diameters) where the border lies by either criterion.

It is interesting that there are no overt morphological features which distinguish the anteroposterior border in the wing imaginal disc. Thus, there is no obvious barrier to prevent these two groups of cells from mixing with one another, supporting the hypothesis that the observed lineage restriction between the compartments results from some intrinsic cellular property. More direct evidence for an intrinsic compartmental difference can be found in studies of the *engrailed* gene, which apparently functions only in the cells of the posterior compartment and is required to maintain the integrity of the compartment border (Morata & Lawrence, 1975).

In contrast to the anteroposterior compartment boundary, the dorsoventral boundary, in the wing blade (where the lineage restriction is best documented and where the concentration difference of DK.1A4 antigen is sharpest; Bryant, 1970; Garcia-Bellido & Merriam, 1971; Garcia-Bellido et al. 1973, 1976; Wilcox et al. 1981), is characterized by a morphological entity: the wing margin. We have shown here that the dorsoventral border is also marked by a discontinuity in the morphology of cells of the wing imaginal disc. It seems possible, then, that this groove or furrow, which represents the incipient wing margin, may present a barrier which results in the observed lineage restriction. Although we cannot yet answer this question, the data indicate that it may be premature to assume that the anteroposterior and dorsoventral compartmentalizations are the result of analogous events.

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