Compartments in the Imaginal Wing Disc of Drosophila Melanogaster

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COMPARTMENTS IN THE IMAGINAL WING DISC OF DROSOPHILA MELANOGASTER

By

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B.S., University of Central Florida, 1979

THESIS

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INTRODUCTION

Like most Dipterans, Drosophila melanogaster exhibits a complex life cycle composed of embryonic, larval, pupal, and adult stages. Fertilization of the egg takes place as it passes down the oviduct. This is accomplished by sperm entering the egg through the micropylar opening. Sperm penetration activates the meiotic divisions of the female nucleus (Counce, 1973). The pronucleus will unite with the sperm pronucleus to form the zygote. Shortly after fertilization, the zygote nucleus begins to divide mitotically. Approximately twelve synchronous divisions occur producing nearly 6000 nuclei (Schubiger and Wood, 1977). The mitotic rate for these nuclei is believed to be around ten minutes per division. As the nuclei divide, most of them migrate toward the cortex of the egg stopping just beneath the plasma membrane (Counce, 1973). The nuclei which do remain in the center of the egg become the vitellophages, cells believed to be active in the metabolism of yolk (Poulson, 1950). The migration of the nuclei to the periphery of the embryo produces the syncytial blastoderm (Merriam, 1978). Cellular blastoderm formation is facilitated by the establishment of membranes around each nucleus (Sonnenblick, 1950). The first nuclei to be enveloped are the polar nuclei which migrate posteriorly through the periplasm. These will ultimately form the germ cells of the developing fruit fly (Fullilove and Jacobsen, 1978).
Following cellular blastoderm formation, the organizational events of gastrulation occur producing the ectodermal, mesodermal and endodermal germ layers. The ectoderm will give rise to the integumentary system, hypoderm, and central nervous system. The mesoderm produces the somatic and visceral musculature, the heart and circulatory system, as well as other connective tissues. The endoderm is primarily responsible for the formation of the midgut (Fullilove and Jacobson, 1978).

The larva hatches from the egg at around 22 hours after oviposition (Poulson, 1950). The larva feeds and grows through three larval stages known as instars. The late third-instar larva becomes sessile, secretes a thick chitinous pupal case and metamorphoses into an adult fly.

Early in embryogenesis, groups of hypodermal cells derived from the ectodermal germ layer are separated from all other larval cells (Gehring and Nothiger, 1973). These groups of cells differentiate to form structures known as imaginal discs. There are nine pair of imaginal discs and a single genital disc in Drosophila larvae (Crick and Lawrence, 1975). Each imaginal disc mitotically increases in cell number throughout larval life but remains functionally dormant. At the onset of pupation, when the concentration of ecdysone (a molting hormone) increases substantially, these discs differentiate into their adult cuticular structure.

The dorsal mesothoracic disc (or wing disc) is of particular interest to this study on compartmentalization. Previous work on
compartments was done using the adult derivatives of this disc. The wing disc gives rise to the wing blade, its musculature, the pleurae and half of the dorsal mesothorax (Bryant, 1978). It arises from approximately 12 primordial cells which are set aside during early embryogenesis (Garcia-Bellido and Merriam, 1969; Bryant, 1970). These primordial cells divide approximately ten times during larval growth producing approximately 50,000 cells in the late third instar wing disc (Garcia-Bellido and Merriam, 1971). After puparium formation, the wing blade is formed through an evagination of the wing pouch. The pouch evaginates along the wing margin analogous to an inflating balloon (Waddington, 1940). As the wing pouch continues to elongate, the dorsal and ventral wing surfaces contact one another. This contact is believed to occur through cellular processes extending from the imaginal cells into the interior of the evert wing pouch (Waddington, 1940). The wing veins, located between the wing surfaces, are believed to be formed where these cellular processes are not present. Shortly after eclosion (emergence from the pupal case), the wing inflates with fluid and distends caudally. The fluid is then withdrawn and the wing flattens.

Genetic determination is the central process governing development of all structures, including the wing. Determination is the initiation of a particular developmental pathway from many which cells are capable of following (Hadorn, 1965). One
could envision determination as being totipotent cells sequentially becoming more restricted or specialized in their responses to the biochemical environment that surrounds them. This concept is paramount to the theory of compartmentalization.

A compartment is a developmental unit that is derived from a group of cells or polyclone (Crick and Lawrence, 1975). It is produced by the stepwise separation of a broad group of cells into smaller groups through a restrictive process (Garcia-Bellido et al., 1976). Clonal analyses using induced somatic recombination have shown that compartments exist in the adult wing blade of the fruit fly (Bryant, 1970; Garcia-Bellido, 1973; 1976). A clonal restriction was found that separated the dorsal from the ventral surfaces. The anterior-posterior (A/P) compartments are believed to be established between 3 and 7 hours into development (Morata and Lawrence, 1977). The dorsal-ventral (D/V) compartments are believed to be established some time between 24 and 48 hours (Bryant, 1970; Garcia-Bellido, 1973; 1976).

Spatial and temporal relationships between cells within imaginal discs have been studied extensively in genetic mosaics. One technique involves gynandromorph analysis. The spontaneous loss of an unstable ring X-chromosome leads to the formation of a gynandromorph (Hinton, 1955), an XX/XO female/male mosaic. Both morphological and enzymatic markers can be used to differentiate the XX from XO tissue (Hotta and Benzer, 1972; Janning, 1978). Ideally, the loss of the ring X-chromosome would occur
after the first zygotic division, resulting in a XO nucleus associated with its XX counterpart (Hotta and Benzer, 1972). The adult gynandromorph would be phenotypically recognizable as a half male (XO), half female (XX) fly. The extent of the mosaicism depends upon the cleavage step at which the X-chromosome was lost. If it occurs later in blastulation the XO area will be smaller; if it occurs earlier, the XO area will be large. Sturtevant (1929), who pioneered gynandromorph fate mapping using Drosophila similans, showed that the embryo was genetically determined into segments during development. The major limiting factor of the gynandromorph technique regarding its usefulness for studies of compartmentalization is the time at which the mosaic is initiated. Mosiacs have been shown to originate anywhere from the first to the ninth cleavage division, while the embryo is still a syncytium (Zalokar et al., 1980). This is prior to compartmental boundary formation. Therefore, the mosaic cells could migrate through the presumptive boundaries before they were established.

X-ray induced mitotic recombination overcomes the limitation of the gynandromorph technique. Because clones can be generated at any developmental stage, they can be confined to compartments by inducing them some time after boundaries are known to be established. Recombination studies have shown that the imaginal primordial cells are coalesced in relation to the segments they will occupy rather than the type of organs they will form. In the mesothorax, for instance, the cells destined to become wing
and second leg are associated together very early in embryonic life (Wieschaus and Gehring, 1976). Within 3 hours after oviposition, these cells become competent (determined) for the specific anlagen they will form. Later, after blastoderm formation, the cells separate into their respective imaginal discs and further differentiate. It is here, in the post-blastoderm stage, that induced recombination studies have been useful in the study of compartmentalization (Merriam, 1978).

Because of the finite number of cell divisions taking place in an imaginal disc, induced mitotic recombination, like the gynandromorph technique, produces small clones following late irradiation times. Reduced clone size hinders the visualization of clonal restrictions because small clones would have to be fortuitously located near a boundary in order to provide any information on compartments. Probably the only meaningful interpretation of such clones would be through the use of a composite drawing of many small clones (Bryant, 1970). The discovery of a mutant known as Minute, proved to be beneficial in overcoming the drawback of small clone size (Morata and Ripoll, 1975). Minute (M) is a dominant mutation that is cell lethal in the homozygous or hemizygous condition (Lindsley and Grell, 1968). The Minute gene reduces mitotic rates and exhibits an increased frequency of mitotic recombination.

Morata and Ripoll (1975) produced clones of marked cells (multiple wing hair) (mwh), javelin (jv), by inducing somatic
recombination in \( \text{M/mwh } \text{jv} \) heterozygotes. Following the proper segregation of the recombinant chromosomes, two daughter cells (one \( \text{M/M} \), the other \( \text{mwh } \text{jv/mwh } \text{jv} \)) were produced. The \( \text{M/M} \) cell was eliminated (homozygous lethal) and the \( \text{mwh } \text{jv/mwh } \text{jv} \) cell and its progeny (a clone) proliferated at rates higher than the \( \text{M/mwh } \text{jv} \) background cells. This technique, known as the "Minute Technique" could produce clones much larger than those produced by conventional somatic recombination methods.

Garcia-Bellido and his co-workers (1973; 1976) used the Minute Technique to map the developmental compartments in the wing of \emph{Drosophila melanogaster}. They demonstrated that a clonal restriction existed between the anterior and posterior surfaces of the wing blade. Its location was signified by the straight line obedience of clones slightly anterior to the fourth wing vein. A second restriction was found to separate the dorsal and ventral wing surfaces. Its location was believed to be between the medial and ventral bristle rows and between the dorsal and ventral bristle rows of the anterior and distal wing margin respectively (Garcia-Bellido and Merriam, 1971; Garcia-Bellido \textit{et al.}, 1973; 1976).

Additional support for the existence of compartments in the wing comes from experiments using the homoeotic mutant engrailed (Lawrence and Morata, 1976; 1977). When the engrailed gene is homozygous, it produces a near mirror image of the anterior wing blade in the posterior position. This suggested that the anterior and posterior wing surfaces were genetically determined as separate
developmental units by the fact that the engrailed mutation recognized the A/P boundary.

The work of Garcia-Bellido and other scientists using similar techniques has relied on noticeable changes in the cuticular patterns of adult structures and has neglected to look at their larval precursors directly. This is primarily due to the lack of distinguishable characters needed to identify mitotic recombination among the imaginal cells. The advent of histochemical stains, however, enabled researchers to study the effects of homoeotic mutants with regard to compartmentalization (Kuhn and Cunningham, 1977a; 1977b; S prey et al., 1981). Such studies required that the cells within an imaginal disc display differential activities producing a characteristic pattern.

Janning (1973) found that the histochemical stain specific for the enzyme aldehyde oxidase (AO) produced such a pattern within the imaginal wing pouch. Since its discovery, AO has been used extensively in all types of insect larval and imaginal tissues. One of the first groups to use AO with regard to compartments in the imaginal wing was Kuhn and Cunningham (1977a). Initially with the homoeotic mutant, engrailed, they demonstrated that the AO positive and negative regions in the imaginal wing pouch closely correlated with the anterior and posterior compartments found by Garcia-Bellido et al. (1973; 1976). In a normal wing disc stained for AO, the anterior pouch is completely AO positive. The posterior wing pouch is AO negative with the exception of the AO positive
band which bisects it. When the engrailed mutation was present, the anterior and posterior wing pouch were almost completely AO positive. The increased AO activity in the posterior wing pouch was due to its transformation to an anterior wing pouch.

Further evidence of compartments came from their study of the AO pattern in homoeotic bithorax (bx), postbithorax (pbx) mutants (Kuhn and Cunningham, 1977b). The bithorax mutation transforms the anterior dorsal metathoracic or haltere disc into anterior wing (Lewis, 1963). The postbithorax mutation transforms posterior haltere into posterior wing. The AO staining pattern for the bx transformation showed that its anterior portion was AO positive and resembled the staining pattern of the anterior wing disc (Kuhn and Cunningham, 1977b). The staining pattern of the pbx mutation resulted in the posterior haltere displaying the same band of activity found bisecting the posterior wing disc. Next they stained a haltere disc that had both bx and pbx mutations; the AO pattern was identical to that found in the wing disc. This showed that the determination of compartments is controlled by individual genes and appears as separate units relative to AO activity.

The purpose of this study was to localize the boundaries between developmental compartments in the late third instar imaginal wing pouch of Drosophila melanogaster using clonal analysis.
Materials and Methods

Drosophila melanogaster strains used.

In this experiment, \textit{M(1)oS}/FM6 females were mated with \textit{y v f mal} males in half pint milk bottles containing a modified Drosophila medium (Lewis, 1960) at 25°C. The Minute (1) of Spencer is characterized by short, narrow integumentary bristles, an increase in the frequency of somatic crossing-over and a delay in the rate of development (Lindsley and Grell, 1968). This Minute has been described as a moderate Minute. The severity of the Minute condition is determined by the ease of distinguishing the mutant from wild-type characters. Figure 1A shows the AO staining pattern in a wing disc with the genotype \textit{M(1)oS}/mal. The presumptive dorsal and ventral surfaces of the anterior half of the wing pouch are AO⁺ while the surfaces in the posterior half do not show significant AO activity. An AO⁺ presumptive wing margin bisects the anterior and posterior wing pouch, separating the dorsal and ventral disc surfaces. The Minute is a dominant mutation which is lethal in either the homozygous or the hemizygous condition.

In the \textit{y v f mal} flies, the recessive maroon-like (mal) gene inhibits the enzymatic activity of AO in both the homozygous and hemizygous condition (Courtright, 1967). It can be seen in Fig. 1B that there is a lack of AO activity in the mal wing disc even after prolonged staining.
Figure 1. The aldehyde oxidase (AO) pattern found in M(1)osp/mal late third-instar wing discs is shown in A (X80). Pictured in B is a mal/mal mature wing disc lacking AO activity (X80). ab, anterior dark band; AC, anterior compartment; cs, "Central Spot"; D, dorsal wing surface; ib, interband; PC, posterior compartment; pb, posterior dark band; V, ventral wing surface; wm, presumptive wing margin.

Figure 2. AO⁻ clones showing the A/P boundary are pictured in A, B, and C (X80). The clone in A is confined to the presumptive dorsal wing surface stopping at the A/P border. In B the clone is restricted to the presumptive ventral surface showing the A/P band. Pictured in C, the A/P border can be seen in both the dorsal and ventral surfaces as a result of the clone extending into these areas. Pictured in D, E, and F are AO⁻ clones stopping at the posterior edge of the "Central Spot", within the presumptive wing margin (X80). This edge marks the A/P border within the margin from the posterior compartment. A/P, anterior-posterior boundary; cl, AO⁻ clone; pcs, posterior "Central Spot".
Irradiation procedure

Minute females were allowed to oviposit for 8 hours in a 5 cm diameter millipore filter plate (filled with modified Drosophila medium). To prevent the flies from escaping during the egg laying period, empty half pint bottles were inverted and placed over the millipore filter plates. The larvae were then irradiated at 72 hours after oviposition with 1000R (156 rpm, 100 kV, 5 mA) of X-rays through a 2 mm aluminum filter, focusing distance 20 cm. After irradiation the contents of the plates were placed in milk bottles containing fresh medium. M(1)osp/sp/mal late third-instar larvae were selected for analysis by gonad morphology and the presence of black mouth-parts. Because hemizygous Minute males are not viable, only heterozygous Minute females (M(1)osp/sp/y v f mal) carry the Minute gene. Male and female larvae can be distinguished from one another by gonad morphology. Males have testes which appear as bilateral translucent ovals located in the posterior of the larva. Females lack testes, therefore the translucent ovals cannot be seen. The chromosome carrying the Minute mutation also carries the dominant gene for wild-type (grey) body color. Larvae with the genotype M(1)osp/sp/y v f mal have wild-type body color which can be seen in the larvae as black mouth-parts.

Staining procedure

Late third-instar larvae were everted in ice cold Drosophila Ringer's solution, .02 M potassium phosphate buffer, pH 7.2
(Ephrussi and Beadle, 1936). All of the internal structures with the exception of the third leg, haltere and wing discs were removed from the carcass. The remaining tissue was then fixed in a Ringer's solution with a final concentration of 2% gluteraldehyde for 30 minutes. This was followed by three 10 minute washings in Ringer's solution. The carcasses with discs were transferred to glass depression slides containing AO staining solution and incubated for approximately 15 minutes in a dark chamber. The AO staining solution contained 10.5 ml of 0.2 M Tris-HCl, pH 8.0, 0.3 ml of 2 mg/ml phenazine methylsulfate (PMS), 0.05 ml of 0.1 M EDTA, 0.05 ml of benzaldehyde, and 12.5 mg of nitro blue tetrazolium (NBT). The benzaldehyde was used as enzyme substrate and the PMS was used to accelerate the transfer of electrons from the substrate (oxidation) to the NBT (reduction). In its oxidized form NBT is a yellow colored substance containing two nitrogenous rings. When reduced, these rings are broken producing a formazan compound which is blue in color (Pearse, 1972). The wing discs were then dissected from the carcasses and the staining reaction was terminated by placing them in ethanol-acetic acid 3:1 for 10 minutes. The discs were stored in glass depression slides containing lactophenol until they were analyzed.

**Clonal analysis**

The technique employed in this study is nearly identical to the Minute technique used by Morata and Ripoll (1975). In their
experiment, Minute larvae (M(1)0^SP and M(3)i^55) were X-irradiated to induce a mitotic exchange between the chromosome arm with the Minute locus and its homologue, the homologue having cell markers. This exchange is believed to occur at the heterochromatic regions of the chromosomes near the centromere (Kaplan, 1953). After the proper segregation, this exchange produced one daughter cell homozygous for the Minute mutation (M/M) which is cell lethal, and another daughter cell homozygous for the selected cell markers (M^+/M^+). The M^+/M^+ clone was shown to proliferate at a normal rate (8.5 hrs/division) while the surrounding M/M^+ background divided at a slower rate (12 hr/division) (Morata and Ripoll, 1975).

For this clonal analysis the recessive mal gene was used as a marker for wing disc cells. A mitotic exchange followed by the desired chromosome segregation, would result in one daughter cell being M/M and the other daughter being M^+/M^+ (homozygous mal). The homozygous M/M cell would die and the M^+/M^+ (mal/mal) clone would proliferate at a normal mitotic rate. Recombination events were observed by the presence of AO^- mal/mal clones in the AO^+ M/M^+ background. Clones were examined in the wing pouch of late third-instar larvae and interpretations were made concerning developmental compartment boundaries.

Wing discs displaying unambiguous AO^- clones within the AO pattern were chosen for closer examination. Because of the AO activity in the peripodial membrane (which covers the wing disc
epithelium) it was necessary to remove this membrane prior to
drawing and mounting the discs on slides. Discs with clones were
drawn with the aid of a camera lucida and then mounted on slides
in Faure's mounting medium and cover slipped. Photographs of
the discs were taken under a Zeiss compound microscope equipped
with Nomarski phase interference objectives. Clones were classified
according to their apparent point of origin and morphology within
the wing pouch. Boundaries were identified by the "significant"
linear obedience of clones within the Minute pattern.

Wing pouch morphology

Scanning electron photomicrographs of the wing disc revealed
folds bordering the presumptive wing margin (Eskens et al., 1981).
Another fold, signified by a notch at the periphery of the wing
pouch, was also found in mitotic recombination studies involving
Minute imaginal wing discs (Kuhn et al., 1981). Because of the
similar locations of these folds to the proposed compartmental
borders in the wing pouch, the folds were examined further.

Imaginal wing discs were dissected from Oregon-R-C late third-
instar larvae. In order to fix the disc epithelium and maintain
wing pouch morphology, the discs were immersed in a Ringer's solu-
tion with a 4.7% gluteraldehyde concentration for 30 seconds.
Fixation was followed by washing the discs in 0.05 M Tris-HCl,
Ringer's solution, .02 M potassium phosphate buffer, pH 6.9, and
then staining the epithelium for AO activity. Termination of the
staining reaction was accomplished by placing the discs in a glass depression slide with ethanol-acetic acid 3:1 for 10 minutes. The wing discs were stored in lactophenol until they could be examined. The peripodial membranes were later removed and the discs were mounted on slides in Faure's mounting medium. The discs were manipulated so that they could be photographed on their sides and on end before the Faure's solution solidified. Folds in the areas of the proposed anterior-posterior border and presumptive wing margin were identified in the wing pouch and compared to the AO pattern, clonal restrictions and scanning electron photomicrographs of imaginal wing discs. The above changes in gluteraldehyde concentration and duration of fixation as well as the washing phase of the AO staining procedure apparently did not affect the AO pattern of the intensity of tissue staining. The only significant difference between the two procedures was the amount of time required.
Results

More than 3700 wing discs were stained in this analysis. Eighty-seven discs having unambiguous clones were selected for further examination. This yields a recombination frequency of 2.34%. This percentage as well as the following results were reinforced by a joint study performed in Sprey's laboratory at the University of Lieden in the Netherlands (Kuhn et al., 1981).

Anterior-posterior boundary

Due to the AO staining pattern in the imaginal wing disc only AO− clones residing in the AO+ anterior surfaces of the wing pouch were beneficial concerning a possible anterior-posterior (A/P) boundary. Fifty-seven discs had AO− clones induced in the anterior (AO+) portion of the wing pouch. Twenty-one clones, originating in the dorsal or ventral wing surfaces approached the AO+ - AO− interface. Sixty-nine percent of these clones stopped slightly anterior to this interface producing a narrow band of AO+ cells perpendicular to the presumptive wing margin. Examples of such clones can be seen in Figs. 2A, B and C. The AO+ band in Fig. 2A can be seen between the AO− clone confined to the anterior dorsal surface and the AO− posterior dorsal wing pouch. Figure 2B similarly shows the narrow band between an anterior ventral clone and the AO− posterior ventral wing pouch. The clone in Fig. 2C occurs in both anterior dorsal and ventral surfaces
showing the band of activity there. Therefore, the narrow band of AO activity is a part of the posterior compartment.

Apparent AO clones, in the posterior wing pouch were observed in 30 of the 87 discs examined. This can only be seen as in interruption in the AO+ presumptive wing margin. Eight clones approached the A/P border from the posterior wing margin. Approximately 62% of these clones stopped at a common position that protruded slightly into the posterior wing pouch. This posterior protrusion can be seen in Figs. 2D, E and F.

Dorsal-ventral boundary

It was previously mentioned that only the anterior dorsal and ventral surfaces of the wing pouch were AO+. For this reason only the 57 discs with anterior clones were used in considering the dorsal-ventral (D/V) boundary. Referring back to Fig. 1A, the dorsal (D) and ventral (V) wing surfaces are labeled according to their orientation in the adult wing. Twenty-three AO clones approached the ventral edge of the presumptive wing margin. Approximately 48% of these clones did not transgress this edge of the margin. This restriction can be seen in Figs. 3A, B, and C. The clone in Fig. 3A extends along the ventral edge of the wing margin for a considerable distance. Figure 3B also shows an AO clone running along most of the ventral edge of the presumptive wing margin. The clone in Fig. 3C, though it occurs more anteriorly, also shows the restriction along the margin's ventral edge.
Regarding the dorsal edge of the wing margin, 27 clones approached it from either the dorsal surface or the wing margin. Nine clones (33%) obeyed the dorsal edge of the wing margin in linear fashion as illustrated in Figs. 3D, E, and F. The clone in Fig. 3D can be seen following the dorsal edge of the margin eliminating the AO activity in most of the anterior dorsal disc surface. Figure 3E also shows a clone stopping at the dorsal edge of the presumptive margin. The disc in Fig. 3F has two clones, one in the anterior ventral, the other in the anterior dorsal surface. Notice that the clone in the dorsal surface extends for a considerable distance down the margin's dorsal edge.

The apparent dual restriction along the ventral and dorsal edges of the presumptive margin make it appear as a subcompartment as can be seen in Figs. 3G, H, and I. The clone in Fig. 3G obliterates nearly all of the AO staining in the most anterior wing pouch. The AO\(^+\) presumptive wing margin does, however, appear to be flanked more posteriorly by the clone occupying both the dorsal and ventral wing surfaces. In Fig. 3H the clone can be seen eliminating the AO activity in the posterior and most of the anterior margin. Measurements taken with an ocular micrometer indicate that the clone might have also eliminated some of the AO activity in the dorsal and ventral disc surfaces adjacent to the proposed A/P border. Finding discs such as Fig. 3H were rare events, however, the fact that they were observed in two independent studies tends to rule out the possibility of them being mere
Figure 3. AO⁻ clones following the D/V restriction, located at the ventral edge of the presumptive wing margin, are pictured in A, B, and C (X80). A second restriction is depicted along the dorsal edge of the presumptive wing margin in D, E, and F (X80). The AO⁻ clones in D and E are shown following the dorsal wing margin edge. In F, two clones can be seen in the anterior surfaces. One is confined to the ventral surface near the A/P border. The other originates in the dorsal surface and follows the dorsal edge of the wing margin. Clones depicting the presumptive wing margin are pictured in G, H, and I (X80). The clone in G can be seen in the most anterior portion of the wing pouch. The AO⁺ wing margin protrudes into the anterior wing pouch flanked by the clone in the dorsal and ventral surfaces. In H and I, clones are shown within the wing margin. The clone in H obliterates the AO staining in the posterior and most of the anterior wing margin. In I, the clone can be observed in the anterior wing margin, flaring out at the very tip of the anterior compartment. cl, clone; dwm, dorsal edge of the presumptive wing margin; vwm, ventral edge of the presumptive wing margin.
artifacts. The clone in Fig. 3I can be seen within the anterior wing margin, flaring out into the dorsal and ventral surfaces at the tip of the pouch.

Central spot

During this study, an intensively stained oval, located in the presumptive wing margin, proved to be resistant to AO- clonal extension. Residing in the center of the wing pouch this oval was found to be part of the anterior compartment. We have termed this persistent feature the "Central Spot." Of the 14 clones that approached this spot from either anterior disc surface, 92% respected it. Figures 4A through F depict AO- clones respecting the "Central Spot." Figure 4A shows a disc with dual clones that approach and stop at the anterior "Central Spot." The spot can best be seen in Fig. 4B. The clone in this disc eliminated the posterior wing margin except for a posterior protrusion. This marks the posterior edge of the "Central Spot." Figures 4C, D, and F show clones originating in the anterior dorsal wing surfaces obeying the "Central Spot."

Although the functional role of the "Central Spot" is presently unknown, preliminary data using the wing mutant Xasta suggests that it may be involved in the development of the distal wing blade. Figure 4G shows the surface of the distal wing blade from a fly with the Xasta mutation. A large indentation is noticeable near the third wing vein. Figures 4H and I show a fold
Figure 4.  Pictured in A through F are AO^- clones outlining the "Central Spot" (X80). Two clones, one in the dorsal, the other in the anterior compartment are shown stopping at the edges of the "Central Spot" in A. The clone in B is shown approaching the "Central Spot" from the posterior wing margin. In C and D the clones are shown approaching the anterior "Central Spot" from the anterior dorsal compartment. The lateral edge of the "Central Spot" can be seen in E and F due to the presence of clones in the dorsal anterior compartment. The distal wing blade from a fly with the Xasta mutation is pictured in G (X32). An indentation can be seen very near the distal third wing vein. H and I show mature wing discs dissected from Xasta mutants that were stained for AO activity (X80). Both photographs show a fold running perpendicular to the wing margin in the anterior compartment. acs, anterior "Central Spot"; cs, "Central Spot"; wm, presumptive wing margin; I, II, III, VI, V; the wing veins found in the adult wing margin and blade surface.
in the larval wing disc from Xasta mutants stained for AO activity. This fold is located in the anterior wing pouch and presumably corresponds to the indentation near the third vein in the adult wing. It can also be seen that the anterior portion of the "Central Spot" lacks the intense staining characteristic of this area (see Fig. 3B). The reduced staining of the "Central Spot" as well as the interruption of the anterior wing margin, shown in Fig. 3I, suggests that a relationship may exist between the spot and the tissue around the distal third and fourth wing veins.

**Imaginal wing pouch folds**

A relationship between wing pouch morphology and compartmental boundaries was suggested by results obtained in Sprey's laboratory (Kuhn et al, 1981). Figure 5A is a scanning electron photomicrograph of an imaginal wing disc. Folds can be seen flanking the dorsal and ventral edges of the presumptive wing margin. When wing discs were mounted on their sides, these folds could be seen under the compound microscope as illustrated by Figs. 5B and C. Their location closely corresponds to the two clonal restrictions found along the dorsal and ventral edges of the presumptive wing margins (see Figs. 3A through F). Another interesting feature demonstrated by Sprey's work is a notch located in the periphery of the inner fold of the wing pouch. Figure 5D shows the notch positioned very near the proposed A/P boundary. The discs in Figs. 5C and D show the notch in the dorsal side of the pouch.
Figure 5. A scanning electron photomicrograph showing two folds flanking the presumptive wing margin can be seen in A (X120). The folds flanking the margin are shown in discs mounted on their sides in B and C (X80). The anterior compartment in these wing discs faces the viewer. In E, F, and G, the wing discs show significant staining in the posterior disc surfaces (X80). The notch marking the A/P border can also be seen in these wing discs. Lighter staining in the posterior ventral compartment allows the localization of this notch relative to the A/P. Pictured in G, H, and I are wing discs mounted on end (X80). The notch marking the A/P can be seen in the wing margin. The ventral compartment faces the viewer. The notch in H can be seen slightly anterior to the posterior edge of the "Central Spot", in the anterior compartment. AC, anterior compartment; D, dorsal wing surface; f, fold; n, notch; PC, posterior compartment; pcs, posterior "Central Spot"; wm, presumptive wing margin.
Again, when we used the unorthodox method of positioning stained discs on end, this notch was easily seen in the presumptive wing margin as shown in Figs. 5G, H, and I. In Fig. 5H, the position of this notch in relation to the "Central Spot" indicated that it is very near the proposed A/P boundary.

Other potential clonal restrictions in the anterior wing pouch

In the presumptive anterior compartment two darkly stained bands appear in the dorsal and ventral wing surfaces of the AO pattern. Between these bands is a region (interband) that frequently does not stain as intensely (see Fig. 1A). A clonal analysis by Kuhn et al. (1981) suggested that limited restrictions may exist between these bands. The proposed border for the most anterior dark band is the line between it and the interband. Twenty percent of the 20 clones that approached this presumptive border obeyed it. A second border between the posterior dark band and the interband has also been suggested. Of the 48 clones that approached this line, 5 (11.6%) followed it for variable lengths. These percentages suggest that if these restrictions exist they are not particularly effective at limiting clonal growth.
Discussion

The development of the imaginal wing disc has been shown to be accompanied by progressive commitments by groups of cells to narrower and narrower developmental potentials (Bryant, 1970). X-ray induced mitotic recombination has been used to demonstrate the confinement of cells with the same compartmental affiliation to a particular area in the wing blade (Garcia-Bellido et al., 1973; Bryant, 1970). Although the establishment of compartments occurs at various developmental times during embryonic and larval development, most analyses have been limited to extrapolations back from adult structures to the embryonic and larval precursors. This approach has been informative with regard to the timing and extent of boundary formation, but it provides no information on the developmental compartments within the imaginal discs. To narrow this gap between the time of clonal induction and the observation of the clones we have looked at compartmentalization in the late third-instar imaginal wing disc.

When considering the location of the anterior-posterior (A/P) boundary in the pouch of the wing disc, it was determined that it closely corresponded with the junction between the AO⁺ anterior and AO⁻ posterior wing surfaces as previously suggested (Kuhn and Cunningham, 1977a, 1977b). The A/P border appeared to lie slightly anterior to this junction which was denoted by a narrow band of AO
activity perpendicular to the presumptive wing margin. Although this band's intensity and frequency of occurrence were greater in gynanders (Kuhn et al., 1981), it was observed in 9 of 13 instances in this study. The differences can be accounted for by two possible factors. First, the discs from the gynandromorph analysis were stained for a prolonged period to enhance this characteristic. Second, M+/M+ recombinant cells residing in the Minute wing disc have a proliferative advantage. This could conceivably cause cell overgrowth into the proposed A/P boundary by M+/M+ cells, eliminating the narrow band of activity. Minute cell elimination through cell competition with M+/M+ recombinants has been observed by Morata and Ripoll (1975) and it appears that this competition becomes less significant near compartment boundaries (Simpson, 1979). Brower et al. (1981) reported instances where M+/M+ anterior clones pushed rather than grew into the proposed A/P border. In their study they stained epithelial tissue. This did not allow the use of known staining pattern landmarks in interpreting their data. Instead they relied on approximate boundary locations provided by fate map studies of imaginal wing discs (Bryant, 1975). If border pushing is possible, then one would expect to observe that faint band in a more posterior position in the wing pouch. This was not observed.

Using the discs stained in Sprey's laboratory, which had significant staining in the posterior half of the wing pouch, the proposed A/P boundary could be seen in clones originating in
presumptive posterior wing surface cells. This pronounced effect was believed to be due to the presence of the Minute mutation. Linear clonal restrictions perpendicular to the presumptive wing margin were observed and localized by the presence of a peripheral notch believed to be very near the A/P at the edge of the wing pouch (Fig. 5D). The only deviation of the straight line believed to mark the A/P border was observed in the area of the presumptive wing margin. All three studies (Kuhn et al., 1981), revealed a posterior protrusion interrupting the A/P line within the margin. We believe that this protrusion is the posterior edge of the "Central Spot", an area that is clonally restrictive early in development. The posterior edge of the "Central Spot" acts as the A/P boundary within the wing margin.

When looking for the dorsal-ventral (D/V) boundary, past studies revealed that it is imposed along the adult wing margin parallel to the triple row of bristles separating the two wing surfaces (Bryant, 1970; Garcia-Bellido and Merriam, 1971). When looking for the D/V border in the imaginal wing pouch we assumed that a clonal restriction running parallel to the presumptive wing margin was signified by a greater percentage of clonal obedience near the margin area. Forty-eight percent of those clones approaching the ventral edge of the presumptive wing margin appeared to obey it. This location was similarly found to be at least partially restrictive in the gynandromorph analysis. Thirty-three percent of the clones approaching the dorsal edge of the wing margin respected
it. These percentages, though not particularly dramatic, do suggest two boundaries, running along the ventral and dorsal edges of the $A0^+$ wing margin, separating the margin from the wing surfaces. The percentages also tell us something about the temporal establishment of these restrictions. If both restrictions were imposed simultaneously then the expectation for the percentages of clonal obedience would be nearly equal, which was not the case. Rather, the boundary on the ventral edge of the $A0^+$ wing margin was either more restrictive or was formed earlier than the one at the dorsal edge. Therefore the data on the D/V border suggests that the ventral edge of the presumptive wing margin is the initial restriction followed later by a second restriction along the dorsal edge.

In *Drosophila hydei*, van Breugel and Grond (1980) found evidence of two compartmental restrictions along the adult wing margin. *Drosophila hydei* has five bristle rows, two ventral, two dorsal, and a single medial row along its wing margin. Using X-ray induced clones with the cuticular marker yellow, they showed that the ventral edge of the margin was clonally restrictive 72 hours after oviposition followed later by another restriction between the two dorsal rows. The apparent similarity in the larval but not in the adult state of *D. melanogaster* to this more primitive species could be a reflection of evolutionary modification. This idea is supported by the biogenetic law which simply states that "ontogeny recapitulates phylogeny" (Balinsky, 1975). So that as *D.*
melanogaster evolved away from a five bristle row wing margin to a triple row, the dual restriction in the adult wing margin may have been altered as well.

If the D/V boundary in the wing disc of D. melanogaster results from two restrictions, then the margin should be subcompartmentalized. In both Minute studies (Kuhn et al., 1981) AO- clones were observed restricted to the presumptive margin. They extended from the posterior to anterior wing pouch. These clones, though rare, passed through the A/P boundary which has been shown to be established between 3 and 7 hours into development (Morata and Lawrence, 1977) long before the D/V restriction(s) are formed (Garcia-Bellido, 1973; 1976). How could a clone originating after D/V boundary formation pass through the A/P restriction? One remote possibility is that two clones, one in the anterior, the other in the posterior wing margin, were produced simultaneously. They could then grow within the margin meeting at the A/P border. Double clones were observed in only 9 of the total 3700 discs stained. Another explanation may be that a single clone originated within the "Central Spot" (at the junction of the anterior and posterior compartments). The M+/M+ cells proliferated in both directions, thereby eliminating the AO+ cells within the margin.

To account for the discrepancies between the D/V boundary found in the adult wing and the imaginal disc we considered directed mitotic spindle axes and folds near the presumptive wing margin as the most probable. Recombination studies that create
twin spots early in fly development show that cell divisions within an imaginal disc are organized in a common direction (Bryant and Schneiderman, 1969; Postlethwaite and Schneiderman, 1971). Bryant (1970) in his clonal analysis of the adult wing and notum found that cell proliferation in the wing surfaces occurs in the longitudinal direction with respect to the fly's body. In order for the wing margin to maintain its position at the perimeter of the wing blade, the margin would have to grow laterally in relation to the wing surfaces. This would mean that as cells migrate to the margin from the presumptive surfaces they would be influenced by the mitotic direction and also grow laterally. Inspection of the imaginal wing pouch, using a scanning electron microscope, revealed two invaginations flanking the presumptive wing margin. The proximity of these folds seems to correspond to the D/V restriction observed along the edges of the presumptive margin. These folds could slightly detour clones trying to transgress the margin, or at least obscure their detection. Detection of such clones could only be accomplished after they moved down one side of the fold and resurfaced within the margin. The above suggest that the imaginal wing pouch need not have clonally restrictive D/V borders. Work with other imaginal discs supports this possibility. To date there has been no indication that a D/V border exists in the eye-antennal disc (Morata and Lawrence, 1977), or in the proboscis (Struhl, 1981).

It is possible to account for the appearance of a D/V border
in the adult wing cuticle by studying the morphogenetic events accompanying the eversion of the wing disc during pupation. Waddington (1940) revealed that the wing undergoes dynamic changes. Due to folding and movement of epithelial cells, the imaginal wing margin cells could become internalized. Therefore, clones within the wing margin would have no observable phenotype. Fristrom et al. (1977) showed that cell movement during pupation was responsible for the eversion of the leg disc in Drosophila. Major changes occur in the wing margin with respect to the formation of the adult wing veins as well. Apparently prepupal veins arise in the perimeter of the wing pouch approximately five and a half hours after pupation. Cross sections of the wing bud show that the marginal veins exist along the entire margin. These are referred to as the prepupal blood lacunae. Sometime later in development they are replaced by a second set of blood lacunae known as the pupal wing veins. When the pupal wing veins replace the prepupal wing veins the posterior wing margin is not replaced. Waddington does not account for this loss and it is apparent that the cellular movements in the development of the wing deserve a re-evaluation using present day techniques.

An unexpected result of this research was the discovery of the "Central Spot." This deeply stained oval located in the anterior wing margin was clonally restrictive 92% of the time. The functional significance of the "Central Spot" is not presently understood. Preliminary data using AO and wing mutant Xasta
showed an interruption of the anterior half of the "Central Spot" and part of the presumptive wing margin (Figs. 4G, H and I). The Xasta phenotype in the adult is denoted by a deep notch in the distal part of the wing blade near the third wing vein (Fig. 4G). This suggests that the "Central Spot" may be important for distal wing formation between the third and fourth wing veins.

Another interesting phenomenon associated with the Minute study was the discovery of a notch located in the periphery of the wing pouch (Kuhn et al., 1981). By taking the wing discs through a dehydration process, this notch was noticeable in many, but not all, of the wing discs. Its position with regard to the normal A0 pattern appears to be very near the proposed A/P boundary. When we rotated the stained discs on their ends, the notch was visible along the wing margin anterior to the posterior protrusion of the "Central Spot." The proximity and subtleness of this notch leads us to believe that it may be a morphological feature of the A/P boundary. Its presence could be due to differences between the surfaces of the anterior and posterior cells that exist next to one another. Dissociation and reaggregation experiments with disc cells from similar imaginal discs have shown that differential cell surface affinities exist between different regions of the disc (Garcia-Bellido, 1967).
Summary

Clonal analyses have shown that the adult wing of D. melanogaster is divided into developmental compartments. Clonal restrictions were found to separate the anterior and posterior (A/P) wing blade as well and the dorsal and ventral (D/V) wing surfaces. A clonal analysis was used to locate the compartmental borders in the late third-instar imaginal wing disc of D. melanogaster.

The distribution of aldehyde oxidase (AO) in the mature wing disc has been used in studies concerning compartmentalization. It has been suggested that the AO pattern can be useful in localizing the A/P and D/V boundaries in the imaginal wing pouch. To identify the boundary locations AO<sup>-</sup> (mal) clones were induced within the AO pattern using the Minute technique. The AO pattern in the wing pouch consists of AO<sup>+</sup> anterior and AO<sup>-</sup> posterior halves. An AO<sup>+</sup> presumptive wing margin (wm) bisects both halves separating the dorsal and ventral surfaces of the wing pouch. The A/P boundary was located slightly anterior to the interface between the AO<sup>+</sup> anterior and AO<sup>-</sup> posterior halves of the wing pouch. This result was based on the appearance of a narrow band of AO<sup>+</sup> cells between AO<sup>-</sup> clones in the anterior compartment and the AO<sup>-</sup> posterior half of the wing pouch. This narrow band was observed 69% of the time. Clones approaching the A/P from the posterior presumptive wm stopped at a common position in the posterior compartment. This restriction marks the posterior edge of the "Central Spot" (cs).
The cs was an intensely stained oval within the anterior wm that was restrictive 92% of the time to clones approaching from the dorsal or ventral anterior compartments. Two D/V restrictions were observed along the dorsal and ventral edges of the presumptive wm. The ventral edge of the margin was clonally restrictive 48% of the time. Thirty-three percent of the clones approaching the dorsal edge of the wm respected it in linear fashion. The dual D/V restrictions made the wm appear as a subcompartment.

Subcompartments denoted by dark bands of AO activity in the anterior compartment have been suggested. An area of lighter staining produces an interband between the dark bands in the anterior compartment AO pattern. The subcompartmental restrictions are believed to coincide with the straight edges between the dark bands and the interband. The restriction for the anterior dark band was clonally obeyed 20% of the time. The restriction for the posterior dark band showed linear obedience 11.6% of the time.

Folds were found in the imaginal wing disc epithelium that closely corresponded to the areas where compartment boundaries were found. A notch in the periphery of the wing pouch is believed to mark the A/P boundary. Two folds were found flanking the presumptive wm in the areas where the D/V restrictions were observed. Wing disc morphogenesis as well as directed mitotic spindle axes relative to compartmental boundaries are discussed.
**LITERATURE CITED**


