Cell cycling and patterned cell proliferation in the Drosophila wing during metamorphosis

(Imaginal disc/morphogenesis/evagination/vein patterning)

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Contributed by Antonio García-Bellido, July 8, 1996

ABSTRACT In metamorphosing wing discs, progression through the cell cycle takes place, as in larval discs, in nonclonally derived clusters of cells synchronized in the same cell cycle stage. Contrary to early discs, there are temporal and spatial heterogeneities in cell proliferation associated with wing margin, vein, intervein, and middle intervein territories. Within these territories, there are no indications of a wave progression of the cell cycle. Mitotic orientations are, as in early discs, at random but there is a preferential allocation of postmitotic cells along the proximodistal axis, thus explaining the elongated shape of the resulting clones along this axis. Shapes of clones in mature discs and in evaginated wings are similar, thus excluding major morphogenetic movements during evagination. After the proliferative period, all the cells are arrested in G2 phase. The final number of cells of the wing is fixed independently of experimental perturbations that alter the cell division schedule. These results are discussed in the context of a model of wing morphogenesis.

The imaginal wing discs of Drosophila melanogaster are ectodermal monolayered sacs. They derive from the nonproliferating ectoderm of the embryo and proliferate extensively during the larval stages and the first 24 h of pupal development (1). During metamorphosis, the wing disc evaginates and, after apposition of both wing surfaces, differentiates the epidermic structures of the dorsal mesothoracic segment, with a characteristic pattern of veins and sensory organs.

Recent results have revealed the role of genes involved in the establishment and maintenance of compartment boundaries as organizers of growth during wing disc development (2, 3). However, the question of how proliferation is controlled within compartments remains poorly understood (for review, see refs. 4 and 5). Clonal analysis has shown that cell proliferation throughout larval development is intercalar (6). Moreover, regeneration experiments and analysis of genetic mosaics have shown that the control of wing size and shape is locally autonomous. Thus, cultured fragments of imaginal discs are capable of pattern regulation; the presence of cells from more than one compartment is unnecessary (7). Even single cells growing in a feeding layer can generate normal patterns (8).

On the other hand, haltere/wing territories in genetic mosaics grow until they reach their characteristic size (9). However, there are local nonautonomous interactions between mutant and wild-type cells in mosaics to accommodate for the disparity of size in mosaic borders, which reveals that cell proliferation is a collaborative event between neighboring cells (9, 10). Detailed analysis of proliferation patterns throughout early larval development in the wing disc has shown that its cells progress through the cell cycle in clusters of nonclonally derived cells, probably resulting from recruiting competent cells through a cell–cell communication process (11).

In this paper we examine the pattern of cell proliferation in the presumptive wing region in late third instar wing discs and in pupal wings to determine whether proliferation follows the same rules observed in early larval development (11). Proliferation patterns in late stages can be more accurately described than in early discs both topographically and temporally in relation to anatomical landmarks (veins and wing margin) and puparium formation, respectively. Our results show that the control of wing disc proliferation throughout pupal development is driven, as in larval discs, by interactions between neighboring cells until they reach their species characteristic size and shape. However, contrary to early discs, there are temporal and spatial heterogeneities in cell proliferation associated with wing margin, vein, intervein, and middle intervein territories.

MATERIALS AND METHODS Drosophila Strains. Vallecas, a wild-type strain; Star7056, an enhancer-trap line; hsp70-string (HS-stg); and hsp70-Dmcyclin E (HS-DmcyCE) are as described (12–14). Other stocks are as described (15).

Pupal Wing Staining. In situ hybridization, 5-bromo-2'-deoxyuridine (BrdU) labeling, Hoechst 33258 staining, and β-galactosidase detection were performed as described (11). Cell death was analyzed as described (16).

Clonal Analysis. Mitotic recombination was induced as described (10). The irradiated genotypes were (i) mwh/+, (ii) f50a/mwh P(f')/+, and (iii) y f50a/++; mwh/+. Adjacent mwh and f cells correspond to independent mitotic recombination events in neighboring cells. Its observed percentage (14%) in the pool of clones of at least two cells is high compared with the frequency of mwh or f clones per wing cell (which is 0.003 for the mwh clones, the lowest of both frequencies). Additional genotypes were (iv) pwn/+ and (v) pwn/++; HS-stg/+ . β-Galactosidase-expressing clones were initiated by the “flip-out” technique as described (2). From 50 to 320 clones were examined for each genotype and each developmental interval.

RESULTS Proliferation Patterns During the Larval-Pupal Transition. Evolution of the Cell Cycle Stages. At the end of the third larval instar and in the wing region of the disc, two cell populations, one associated with the veins, the other with the interveins, can be distinguished by their proliferative dynamics. Thus, BrdU injected into late third instar larvae and revealed in 24-h pupal wings is detected only in intervein cells (11). Since there are not more than two cell divisions between the BrdU pulse and its visualization (ref. 1 and see below), dilution

Abbreviations: APF, after puparium formation; AEL, after egg laying.*To whom reprint requests should be addressed.

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of the label is not to be expected and the pattern of S-phase cells thus determined corresponds to the actual late third instar pattern. The nonlabeled regions include the zone of nonproliferating cells (17) and all vein territories. Moreover, in late third instar discs, string (stg) expressing cells (a marker of G2-M phase transition, ref. 13) are less frequent in vein than in intervein regions (11). Progression through the cell cycle takes place in small clusters of synchronized cells as in younger discs (11).

In contrast to the constant percentage of cells in the different cell cycle stages observed in early discs (11), during the last day of larval development, there is a steady increase in the fraction of disc cells in the G2 stage (18). To ascertain the extent to which pupal wing cells are in G2 phase, we have made use of HS-stg and HS-Dm CyclE flies. Overexpression of stg or Dm CyclE causes entry into M or S phases, respectively, of G2- or G1-arrested cells (13, 14). Overexpression of stg in pupae 4–8 h after puparium formation (APF), when the wing cells stay mitotically quiescent (see below), causes an almost generalized entry into mitosis of the wing cells, with the exception of the wing margin cells (Fig. 1A). Overexpression of Dm cyclE at this pupal stage provokes the entry into S phase of wing margin cells and only a few cells of the distal wing blade (Fig. 1B). These results indicate the arrest of most wing blade cells at the G2 stage and all the wing margin cells at the G1 stage at the beginning of pupal development.

Clonal Analysis. We have carried out a cell-lineage analysis with mwh mitotic recombination clones initiated during pupal development. The frequency of clones per wing (average value ± SEM, 76 ± 6 clones per wing) is constant during the first 12 h of pupal development; it raises during the next 8 h (up to 150 ± 16 clones per wing) and declines to zero at 24 h APF, suggesting that the pupal proliferative period extends from 12 to 24 h APF. The mean size of the clones is constant and larger than one (Fig. 2A). Surprisingly, even late initiated clones may have sizes of up to four cells. The sizes of these clones may reflect up to three pupal divisions per clone or correspond to independent mitotic recombination events in neighboring cells. To distinguish between these two alternatives, a cell lineage analysis was carried out inducing mitotic recombination in two different chromosomes (fneo/+; mwh+/+) in pupae 12–24 h APF. Fourteen percent of the resulting clones of at least two cells corresponds to adjacent mwh and f clones, a value higher than the one attributable to a random distribution. Since mitotic recombination occurs in G2 phase (6), the observed clustering of marked cells suggests clustering of G2 cells. Moreover, the use of the pwn cell marker, with a low mitotic recombination index (15), leads to clones containing only one cell after irradiating at 20–24 h APF (Fig. 2A).

To determine the fraction of dividing cells and the number of divisions per clone throughout pupal development, we have initiated clones of β-galactosidase-expressing cells (2) that can be induced at any cell cycle stage, thus reducing the contribution to clone size of recombination events in neighboring synchronized cells. These clones were initiated at 0–4 h APF and visualized in wings 24 h APF (Fig. 2B). The sizes of these clones range from 1 to 6 cells (16% of 1 cell, 59% of 2 cells, 8% of 3 cells, 14% of 4 cells, and 3% of 5 or 6 cells) indicating that 84% of the wing cells divide at least once during pupal development.

Mitotic recombination clones initiated during larval stages and visualized in adult wings show an elongated shape in the proximodistal axis (1). The final shape of these clones could be due to the expansion of the epithelium in the process of evagination of the wing disc leading to reallocation of neighboring cells. To that end we analyzed β-galactosidase expressing clones initiated during larval development (48 h or 72 h after egg laying (AEL) and visualized them both in late third instar (120 h AEL) and in wings 24 h APF. The clones visualized in larval discs are also elongated in the proximodistal axis: they are parallel to the wing margin in proximal wing regions and perpendicular to it in distal regions (Fig. 2C), as are clones seen in the pupal wings (data not shown). The shape of these clones relative to the wing axes (proximodistal length/anteroposterior width ratio, in number of nuclei) is 4.4 ± 1 nuclei when clones were visualized at 120 h AEL but 8.1 ± 1 nuclei when visualized at 24 h APF. This difference may be due to the preferential positioning of sister cells throughout the proliferative pupal period. Indeed, in 65% of twin clones generated in Fneo, mwh P(+)/+; pupae 0–4 h APF, sister cells are arranged along the proximodistal axis of the wing. Moreover, in 75% of β-galactosidase expressing clones initiated at 0–4 h APF and visualized at 24 h APF (Fig. 2B), sister cells are similarly arranged. Thus, it is unnecessary to invoke morphogenetic movements leading to repositioning of cells associated with the evagination of the wing disc, as postulated for the leg discs (19), to account for the elongated shape of clones.

Cell Proliferation During the Pupal Period. Mitotic Patterns. At the beginning of the pupal period (0–12 h APF), mitotic figures are found only at the anterior wing margin. They correspond to the precursors of the wing margin bristles (20) (data not shown). Mitotic activity in the wing blade is resumed at 12 h APF lasting until 24 h APF (Fig. 3 B and C) consistent with mitotic recombination data (see above). Mitotic figures appear in the wing blade, in proximal (vein trunks and hinge region, data not shown) as well as in distal regions. During the proliferative period, the number of mitotic figures per wing increases from 140 ± 20 (at 12–16 h APF) to 450 ± 29 (at
16–20 h APF) falling to zero after 24 h APF. Mitotic cells are found either as single cells or forming clusters of neighboring cells (Fig. 3B and C), with an average cluster size of 2.7 ± 0.12 cells per cluster (n = 95 clusters of 2–10 cells), similar to that found during larval development (11). Notice that pupal wing cells enter into mitosis in small groups of neighboring cells despite the fact that wing cells are arrested in G2 phase at the onset of metamorphosis. This suggests a synchronization step between neighboring cells at the G2–M phase transition.

The topographic distribution of the mitotic cells with respect to veins has been monitored using the Star7056 line, which shows β-galactosidase activity only in veins, wing margin, and sensory organ precursors (12). During the period of 12–16 h APF, mitotic figures appear in the precursors of anterior wing margin bristles, as in previous pupal stages, in posterior wing margin cells, and at the distal tips of L3 and L4 veins, and spread later to the whole length of the veins (data not shown). During the next 8 h, mitotic cells and mitotic clusters are found...
stg-expressing cells are more sparse and found mostly at the wing margin (data not shown). stg is within any ventral or compartment proximodistal axis. Thus, the observed random mitotic orientation must be followed by preferential allocation of postmitotic cells along the proximodistal axis.

G-M Phase Transition. The evolution of the pattern of expression of the stg gene (13) during pupal development correlates with the mitotic patterns described above. At 12−16 h APF, stg transcripts accumulate mostly at the wing margin and at the distal part of the wing blade (Fig. 3A). At 16−20 h APF, there is a large increase in the number of stg-expressing cells distributed throughout the wing blade, including hinge and vein trunk regions (data not shown). At 20−24 h APF, stg-expressing cells are more sparse and found mostly at the wing margin (data not shown). stg is expressed in clusters of neighboring cells (Fig. 3A and C), whose size (4.98 ± 0.22, n = 113 clusters of 2−10 cells) is similar to that of the mitotic and S-phase clusters as in larval development (11). Clusters appear within any intervein region at any distance to veins. Progression of the stg expressing clusters into the next mitosis, visualized by double staining with Hoechst 33258, does not show any noticeable polarity in relation to the veins, dorsoventral or anteroposterior axes, or compartment borders (Fig. 3C).

S Phase. We have confirmed previous observations (20) regarding the start of DNA synthesis at 8−12 h APF in the wing margin cells and the arrangement of S-phase cells in longitudinal stripes in vein regions at 12−16 h APF (Fig. 3D) and, later, at 16−20 h APF (Fig. 3E), in the intervein territories adjacent to the veins. The distribution of these stripes is symmetric in both wing surfaces. Cells in middle intervein regions, with a width similar to that of veins, never enter S phase during pupal development. There is little BrdU incorporation in the hinge region, vein trunks (20), and L2 vein (Fig. 3D and E). BrdU incorporation ceases altogether in pupae older than 20 h APF.

Those stripes are actually formed by discontinuous clusters of cells synchronized at S phase. Clusters are easily distinguished when only cells with labeled euchromatin or heterochromatin are considered (Fig. 3F). The size distribution of these clusters is similar to that found during larval development [average sizes of heterochromatin and euchromatin clusters of 2−10 cells are 4.07 ± 0.55 cells per cluster (n = 61) and 3.44 ± 0.45 (n = 100), respectively]. To calculate these figures, we assume the sizes of initial clusters to be at least a half of those visualized at 24 h APF since during the chase period cells divide at least once (Figs. 2 and 5). Clusters with heterochromatin or euchromatin labeled cells are intermingled, coexisting at any distance with respect to veins and compartment borders (Fig. 3F), thus excluding any wave progression of the cell cycle in relation to veins.

To see whether the clustered pattern of DNA synthesis follows automatically that of the previous mitosis, we have altered the timing of mitoses and examined the subsequent S phase. After stg overexpression at 4−8 h APF, most of the cells enter prematurely into mitosis (Fig. 1A) but afterward only a small fraction of the cells enters into S phase (Fig. 4A). Interestingly, these S-phase cells appear in clusters distributed along the longitudinal veins. By contrast, the cells in the intervein do not immediately enter into S phase, indicating again the absence of a vein to intervein wave. Four to 16 h after the treatment, although the pattern of S-phase cells is similar to that seen in wild type, the fraction of these cells is reduced (compare Figs. 4B with 3E). These effects do not appear in control heat-shocked wild-type wings. Therefore, we assume that the fraction of cells in S phase at 12−20 h APF is reduced because the overexpression of stg has caused a desynchronization of vein cells, some of them entering into S phase immediately after the heat shock and some of them entering much later. These results indicate that S phase does not immediately follow the G2−M phase transition and suggests the existence of a synchronization step between neighboring cells in the G1−S phase transition.

The analysis of the resulting adult wings after overexpression of stg in 4−8 h APF reveals no increase in the size (in number of cells) of the wing (albeit there are manifest effects on cell differentiation in the wing margin chaetae, data not shown). That may reflect lack of extra cell divisions or that the extra cells are removed later by cell death. Mitotic activity (total number of mitoses per wing) during the proliferative period subsequent to the heat shock (12−32 h APF) is reduced to roughly half of that found in untreated pupal wings. Moreover, mitotic clones induced after such treatment in pwn/+; HS-stg/+ pupae appear smaller (Fig. 2A). On the other hand, neither the heat shock by itself nor the associated stg overexpression increase the normally occurring apoptotic cells any time after the treatment (data not shown). These results indicate that the only consequence of stg overexpression is to advance the timing of mitosis without changing their total number. The depletion in the number of cells that enter further division indicates that the final number of wing cells is determined by the dynamics of proliferation and not by external physiological conditions such as hormones.

The Post-Mitotic Period. During the last 4 h of the pupal proliferative period (20−24 h APF), there is mitotic activity but no DNA synthesis. Overexpression of stg at this stage induces extra mitoses not followed by an extra S phase and it has no effect at 24−28 h APF (data not shown). This indicates that differentiating pupal wing cells are arrested in G1/G0 phase. Indeed, overexpression of Dmcyclin E at 20−24 h APF causes entry into S phase of the wing cells with the exception of wing margin and middle intervein cells (Fig. 4C). Induced S-phase cells are not clustered. Overexpression of Dmcyclin E at 24−28 h APF triggers replication only of intervein cells adjacent to the veins (Fig. 4D) and it has no effect at later stages. These
results suggest a heterogeneous progression of the wing blade cells, associated to vein, intervein and middle intervein territ-
ories, from G1 to a final Dmcyclin E-nonsensitive G0 stage.
After the extra S-phase induced by Dmcyclin E overexpres-
sion, adult wings show no increase in their number of cells; in
fact, such treatment does not induce extra mitoses or extra cell
death (data not shown). Surprisingly, the size of these cells is
also normal despite entering differentiation in the G2 stage.

DISCUSSION

Cell proliferation dynamic of pupal wings is very similar to that
of larval discs (11). Cell proliferation is in both periods of
intercalar type throughout the pupal wing and it takes place in
clusters of cells synchronized in the same cell cycle stage.
Clusters retain the larval average size. Cluster synchronization,
as in larval discs, occurs at least in two transition steps: (i) The
clustered entry of pupal wing cells into mitosis after the G2
synchronization at the onset of the metamorphosis confirms
the G2–M phase transition as a synchronization step. (ii) The
clustered entry into the S phase after mitotic synchronization
induced by stg overexpression confirms that there is a second
synchronization step at the G1–S phase transition.

Mitotic orientation during larval and pupal development is
random with respect to the planar axes of the epithelium.
However, cell lineage clones analyzed in this work show a
preferential elongated shape in the proximodistal axis. More-
over, during evagination the spatial relationships between
neighboring cells do not change indicating that the final
elongated shape of the clones, and by extension that of the
adult wing, results from the relative allocation of postmitotic
cells during the larval and pupal proliferating stages, indepen-
dently of their randomly oriented cell divisions.

In early discs, clusters appear distributed throughout the
entire disc, without any visible pattern in relation to compart-
ment boundaries (11). However, in late discs and pupal wings,
there are discernible growth heterogeneities, including the
timing of entry into the differentiating stage, of wing margin,
vein, intervein, and middle-intervein regions (summarized in
Fig. 5A). Such heterogeneities can be associated to gene
expression patterns [i.e., veinlet (rhomboid), blistered, and
extramacrochaetae] known to be operative in wing venation
and morphogenesis (4). Cell cycle progression of these clus-
ters, as occurs in larvae, moves along any direction of the wing
blade. Our observations do not support the proposition of
proliferating waves starting at veins and progressing to the
intervein regions (20). By contrast, cell proliferation in the
wing disc during late third instar and pupal development is
locally controlled, i.e., by neighbor cell interactions. In fact,
alterations of the schedule of cell proliferation (by inducing
premature entry into mitosis) does not cause any extra divi-
sions (Fig. 5B) indicating again the autonomous local control
of wing disc proliferation that leads to the achievement of a
constant adult final size and shape. Postmitotic cells become
progressively arrested at G0 phase where they enter differenti-
tation. Surprisingly, the Dmcyclin E-induced extra S period,
which brings many wing cells to a G2 stage (4C), does not cause
larger cell size (see ref. 22).

How cell proliferation is locally controlled can be envisaged
in a generative model (Entelechia) of wing morphogenesis (4).
Thus, intercalary cell proliferation is driven by differences in
positional values along the x and y axes. These values are
heterogeneously distributed throughout the disc with, for
example, maximal values associated with clonal restriction
borders (compartment boundaries and veins) (6) and increase
with cell proliferation. Neighboring cells communicate their
values, via ligands/activated receptors, eliciting cell division
when differences between one cell's values and those of its
neighbors surpass a given threshold. Cell division then
progresses along local waves between maxima and minima in
both wing axes. Differences along the two axes generate in
their intersection major discontinuities in groups of cells not
related by lineage. Mitotic orientation in these clusters is at
random but the daughter cells are allocated depending on their
positional values, as shown in cell reaggregation experiments
(23). Eventual mismatching values between neighboring cells
lead by signal deprivation to cell death (24). Final wing shape
and size in number of cells will then result from the correct
solution of intercalar positional values along both x and y axes
when cell proliferation has reached the Entelechia condition.
Gene expression, leading to territorial specification (e.g.,
veins) and cell differentiation (e.g., sensory elements) may
appear at precise positions in the wing landscape, correspond-
ing to the so-defined positional values.

We are grateful to Peter Bryant, Bruce Edgar, Juan Modolell, J. L.
Gómez-Skarmeta, C. Estavour, and P. Fernández-Fuñez for construc-
tive suggestions on the manuscript; to P. H. O’Farrell and C. F. Lehner
for providing fly stocks; and to R. Hernández, A. López, and P. Martín
for technical assistance. M.M. is a postdoctoral fellow of the Funda-
ción Rich. This work was supported by Grants PB92-0036 (to A.G.-B.)
and PB93-0181 (to J. Modolell) from The Dirección General de
Investigación Científica y Técnica and an institutional grant from The

![Fig. 5](https://example.com/fig5.png)

Fig. 5. Pattern of proliferating cells in the pupal wing blade. (A) Cells enter into puparium formation (PF) in G2 (grey background) and differentiate 24 h later in G0 (black background). Progression through the cell cycle occurs in clusters of neighboring cells synchronized at S (○), G2/M (●), and M (●). Vein, intervein, and middle intervein territories show heterogeneous proliferation dynamics. Middle intervein cells divide only once. Vein and intervein cells undergo two mitotic rounds but only a full cell cycle. Cell cycling starts in veins earlier than in intervein regions. Progression from G1 (white background) to G0 phase starts at middle intervein, followed by vein and later by intervein regions. (B) Overexpression of stg at 4–8 h APF causes a premature entry into mitosis of all wing blade cells, followed by a clustered entry into S phase of only the vein cells. The remaining cells are arrested in G1 phase. Afterwards, the normal clustered pattern of proliferation is resumed (albeit expanded because of the heat shock treatment) but the number of cycling cells is reduced.