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Regeneration and compartments in *Drosophila*

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SUMMARY

A clonal analysis was performed in order to study the process of regeneration in wounded wing discs of *Drosophila*. Regeneration was induced either by cutting the disc *in situ*, or by shifting gynandromorphic larvae whose male tissue was hemizygous for a temperature-sensitive cell lethal to the restrictive temperature. Fast growing M^+ clones, labelled with *y* and/or *mwh*, were produced by X-irradiation of the following genotypes: *y; sc*$^{J4(y+)}$ *M*(3)*i*^{55}/ *mwh jv* M^+ (series I), and *l*(1)*ts* 504 *sn*^{3} *l*(1)*ts* 5697/ *In*(1)*w*^{vC}; *M*(3)*i*^{55}/*mwh* M^+ (series II). The clones were induced either before or after the experimental lesion. Clones initiated one day prior to the lesion were able to cross compartment boundaries whereas clones initiated one day after the lesion did not do so. It is concluded that cells involved in the process of regeneration lose their compartmental commitment, but that later on the growing population of cells again becomes subdivided into the same compartments.

INTRODUCTION

The acquisition of a developmental programme and its stability or instability under experimental conditions are of central interest to developmental biologists. In many cases it is obvious that cells are reprogrammed in the course of regeneration. However, the cellular parameters underlying these ordered changes of developmental programmes are largely unknown, as is the relationship between regeneration and normal development. The imaginal discs of *Drosophila* offer ideal opportunities for an approach to these problems, and the tool of mitotic recombination can take the analysis down to the level of individual cells.

The present paper describes a clonal analysis of regeneration in the wing disc of *Drosophila*. The process of regeneration has gained a new interesting aspect since Garcia-Bellido and coworkers made the discovery of compartments (Garcia-Bellido, Ripoll & Morata, 1973; 1976; Garcia-Bellido, 1975). Briefly, the compartment hypothesis states that during development homogeneous populations of cells become progressively partitioned into subpopulations which remain clonally separated from one another. Even clones of cells whose

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genotype (M^+/M^+) allows for a much faster growth rate than that of their neighbouring cells (M/M^+) (Morata & Ripoll, 1975) respect and remain within well defined boundaries on the adult epidermis. These boundaries delimit compartments. It is postulated that each compartment contains a population of cells with a specific developmental programme common to all cells in that compartment. The maintenance of such programmes is thought to be under the control of so-called 'selector' genes whose state of activity specifies alternative compartmental qualities (Garcia-Bellido, 1975). Once a compartmental decision has occurred within a group of cells, this state of commitment remains clonally stable *in situ* (for reviews see Crick & Lawrence, 1975; Lawrence & Morata, 1976).

If the cells within a compartment have undergone a specific developmental commitment which is characterized by a specific pattern of gene activity, then the question arises as to how stable this developmental programme is. In particular, one would like to know whether the cells retain their commitment during additional experimentally induced divisions. Data from the existing literature already suggest that even the earliest compartment boundary which separates anterior from posterior within a disc (Garcia-Bellido *et al.* 1973, 1976; Garcia-Bellido, 1975; Steiner, 1976) can be crossed by the descendants of committed cells during regeneration (Schubiger, 1971; Bryant, 1975; Haynie & Bryant, 1976; Strub, 1977). This is especially clear for the leg disc: the upper medial quarter which is located entirely in the anterior compartment (Steiner, 1976) can regenerate posterior elements (Schubiger, 1971; Strub, 1977).

We decided to examine the phenomenon of regeneration by clonal analysis, with special emphasis on the *dynamics* of the process. In particular, we wanted to know whether compartment boundaries that are apparently abolished in a regenerating disc fragment are reformed in the course of regeneration. We present evidence that the offspring of committed cells can, in the course of regeneration, change their compartmental qualities. Our observations further suggest that previous compartment boundaries may become re-established in a growing population of regenerating cells.

MATERIAL AND METHODS

(*a*) *Assay system*

We have chosen to use the wing disc for our experiments because here the process of compartmentalization is well known and the compartment boundaries are well defined and identifiable. Fig. 1 (*a*) shows the imaginal wing disc and the approximate positions of the anterior-posterior (A/P), dorso-ventral (D/V) and notum-wing (N/W) compartment boundaries. Fig. 1 (*b*) shows the position of these boundaries in the imaginal mesothorax. The A/P boundary is thought to be established at or very soon after blastoderm formation in the egg since clones have never been observed to cross this line (Garcia-Bellido *et al.* 1976;

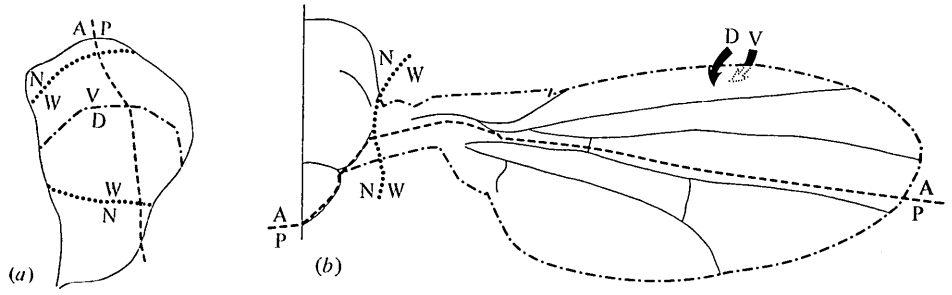

Fig. 1. The wing disc and its adult derivatives. (*a*) Wing disc with the three compartment boundaries separating the anterior/posterior (A/P, ----), dorsal/ventral (D/V, –·–) and notum/wing (N/W, ····) compartments. Shaded area represents the region through which the cuts of series I were made. (After Garcia-Bellido & Nöthiger, 1976.) (*b*) Adult half mesothorax with the three compartment boundaries.

Steiner, 1976; Lawrence & Morata, 1977). The D/V and N/W boundaries appear to arise at about the end of the first larval instar (Garcia-Bellido *et al.* 1976). In order to identify clones at the A/P boundary as being anterior or posterior, we used the following criteria: sensilla campaniformia are restricted to the anterior compartment on vein III, the anterior part of the proximal crossvein, and on the thickened radial vein. They were therefore considered to represent anterior elements. A vein IV which had no sensilla and was connected to both a proximal and distal crossvein was considered to be a posterior element.

(*b*) *Techniques*

Two different techniques were used in order to produce lesions and regeneration *in situ*.

Series I

Regeneration was induced by cutting wing discs *in situ* (Bryant, 1971). Larvae were etherized and pressed between two glass slides so that the wing disc could be seen clearly. The ventral part of the larval cuticle was brought against the dorsal part by means of a curved tungsten needle. The wing disc was trapped between the two cuticles and squeezed into two parts without puncturing the larva. The cut was made approximately at level 3–4 of Bryant's (1975) scheme which means that the A/P boundary and the D/V boundary were cut in every operation (Fig. 1 *a*). Only those larvae were used in which the disc was observed to have been separated into two pieces. In 10 cases the larvae were dissected after the operation and the wing disc was actually found to be cut into two pieces. Only the wing disc on the right side of the larva was operated; the left disc served as a control. The survival rate was some 80%. The cuts were made in young 3rd instar larvae 4, 5 or 6 days after oviposition

(pupariation of the $M(3)i^{55}/+$ larvae occurred at about 7 days at 25 °C, standard *Drosophila* food).

Larvae were of the genotype y; $sc^{J4}{}^{(y+)}_{z}$ $M(3)i^{55}/mwh\ jv\ M^+$ (for a description of mutants see Lindsley & Grell, 1968; Garcia-Bellido *et al.* 1976). X-irradiation resulted in clones of the genetic constitution y; *mwh jv* M^+. The induction of *mwh Minute*$^+$ clones in a heterozygous M/M^+ background permits the recovery of larger clones due to the difference in cell division rate between the homozygous M^+ and heterozygous M/M^+ cells (Morata & Ripoll, 1975). A dose of 1000 rad was delivered with the following parameters: 50 kV, 25 mA, 11·6 cm focal distance, 309 rad/min, 1 mm Al filter; Dermopan-2 Siemens. Wings were mounted in Fauré's solution and analysed under a compound microscope. Only those clones were considered that touched the compartment boundaries and delineated them clearly over a considerable distance (Table 1).

Series II

Extensive cell death was induced in the developing imaginal discs by exposing larvae of a temperature-sensitive (*ts*) cell lethal stock to the restrictive temperature (30 °C). The stock used contained the mutations *l(1)ts 504* (Simpson & Schneiderman, 1975) and *l(1)ts 5697* (Arking, 1975). Larvae were of the following genotype: *l(1)ts 504 sn*^{3} *l(1)ts 5697*/$In(1)w^{vC}$; $M(3)i^{55}/mwh\ M^+$. Irradiation and mitotic recombination in the 3. chromosome will produce large *mwh* M^+ clones. Furthermore, gynandromorphs with male tissue hemizygous for the *ts* lethals will arise due to the loss of the unstable ring *X* chromosome, $In(1)w^{vC}$ (Hinton, 1955). A dose of 1000 rad was delivered with the following parameters: 40 kV, 20 mA, 12 cm focal distance, 500 rad/min, 1 mm Al, Theta X-ray machine, machlett tube. Mesothoraces were mounted in Euparal and inspected under a compound microscope. As in series I, only those clones that touched a compartment boundary were considered.

Statistical analysis

The expected number of apparent overlapping clones, simulated by two independent clonal inductions in the same wing, was calculated as follows: For the wing in series I we are dealing with four compartments (DA, DP, VA, VP; see Fig. 1) whose boundaries (A/P and D/V) had been established long before our earliest irradiation (Garcia-Bellido *et al.* 1976). We assumed that the four compartments contain equal numbers of cells, which is not quite true, but has no significant effect on the expected frequencies. The frequency of clone induction per wing (f) is $f = n/N$, where n is the observed number of clones touching compartment boundaries and N the number of wings analysed. The estimated probability of a clone being induced in one of the four compartments is $f/4$. Considering one of the two boundaries the probability of obtaining two clones in adjacent compartments becomes $2\ (f/4)^2$, since for each boundary there are two combinations which can simulate an overlap. For the A/P boundary, these are one clone in DA, the other in DP; or one clone in VA, the other in VP. The expected number of apparent overlaps $F_{ex} = 2(f/4)^2N$. This is the formula that we used to calculate the expected numbers in Table 1. In those experiments (series II) in which the wing-notum boundary was also analysed (Table 2) the number of compartments scored was 6 Fig. 1), and the formula was adjusted accordingly.

A standard χ^2 test was applied to compare the number of observed overlaps with the number of expected double inductions among all clones analysed. For this purpose, the numbers obtained for each of the compartment boundaries were added. In those cases in which a number was smaller than 4 and a χ^2 test should not be applied, we have used the number 4 as the expected value for calculating χ^2. The corresponding p values therefore give an upper limit. Nevertheless, in the two critical cases (top row in Table 1 and Table 2) the difference between observation and expectation is significant.

RESULTS

Series I

In spite of the fact that the disc could clearly be seen to have been cut, most of the operated wings were normal in shape. However, signs of the operation were apparent on almost all the wings as scars and duplications of certain wing elements, such as wing margin, veins, sensilla. Since in most of the flies with 'normal' wings we did not find an isolated fragment, we conclude that the two pieces healed together after the operation. Thus, we are probably dealing with regeneration of an intercalary type reported by Haynie & Bryant (1976). Apart from some wings that were cut several times (operated on day 6, Table 1, second row) the morphology of most of the wings was normal enough to allow the identification of all veins and specific sensilla and thus the position of the compartment boundaries. This was possible in all experiments for the D/V boundary which follows the wing margin.

The data of series I are presented in Table 1. Three sets of experiments were performed: in order to study the dynamics of regeneration, larvae were irradiated either 24 h prior to the operation (first two rows in Table 1), or at the same time as the operation (third row), or 24 h after the operation (fourth row). In all cases both the irradiation and the operation were performed long after the time when the A/P and D/V compartment boundaries had been established. The unoperated left wings (controls) of all those animals irradiated on day 5 have been pooled together with a large number of wings from unoperated animals.

In the controls the majority of the clones followed compartment boundaries without crossing them. The few cases where marked cells were found on either side of a compartment boundary can be accounted for by two simultaneous inductions (see Table 1 in which the expectations for two simultaneous inductions – double inductions – are calculated).

In both experimental sets in which the larvae were irradiated before the operation, most of the clones also respected the compartment boundaries. But in this case the number of clones crossing the boundaries was significantly greater than that expected from independent double inductions ($P < 0{\cdot}001$) and also exceeded that observed in the controls (Table 1). This was also the case though less pronounced for wings of animals that were irradiated and operated on the same day. In those experiments in which larvae were irradiated one day

Table 1. *Number of clones overlapping A/P and D/V compartment boundaries on the wings of animals of series I* (y; sc$^{J4(y+)}$ M(3)i^{55}/mwh jv M^{+}) *which were operated (oper.) and irradiated (irrad.) at the times shown*

		A/P compartment boundary				D/V compartment boundary				Total overlapping clones (A/P+D/V)		
			Clones				Clones					
Time irrad. (days)	Time oper. (days)	Number of wings analysed	Touching and overlapping*	Observed overlapping†	Expected double inductions‡	Number of wings analysed	Touching and overlapping*	Observed overlapping	Expected double inductions‡	Observed overlapping§	Expected double inductions§	P‖ (χ^2 test)
4	5	456	63	7 (+3)	1·1	456	65	6	1·2			
5	6	123	52	8 (+1)	2·7	179	70	10	3·4		13 > 2·3	< 0·001
5	5	164	41	7 (+1)	1·3	170	43	0	1·4	a	18 > 6·1	< 0·001
5	4	167	56	3 (+1)	2·3	173	69	2	3·4	a	7 ⩾ 2·7	< 0·15
4	—¶	460	73	2 (+2)	1·4	460	77	2	1·6	a	5 = 5·7	~ 0·75
5	—¶	879	227	11 (+5)	7·3	879	274	3	10·7	b	4 = 3·0	—
										b	14 = 18·0	~ 0·4

* Overlapping clones were treated as two clones resulting from two independent inductions, and are included as two events in this column.

† Numbers in parentheses refer to those clones that covered the A/P boundary, but did not include veins III and IV and were therefore excluded from the analysis (see pp. 231, 239).

‡ For calculation of expected double inductions see p. 232.

‖ P gives probability with which observed and expected numbers are not different (see also p. 233).

§ The samples connected by brackets were compared in a 2 × 2 contingency table. The χ^2 test yielded the following P values: < 0·02 for a, ~ 0·65 for b, < 0·01 for c, < 0·001 for d (see also p. 233).

¶ Non-operated controls.

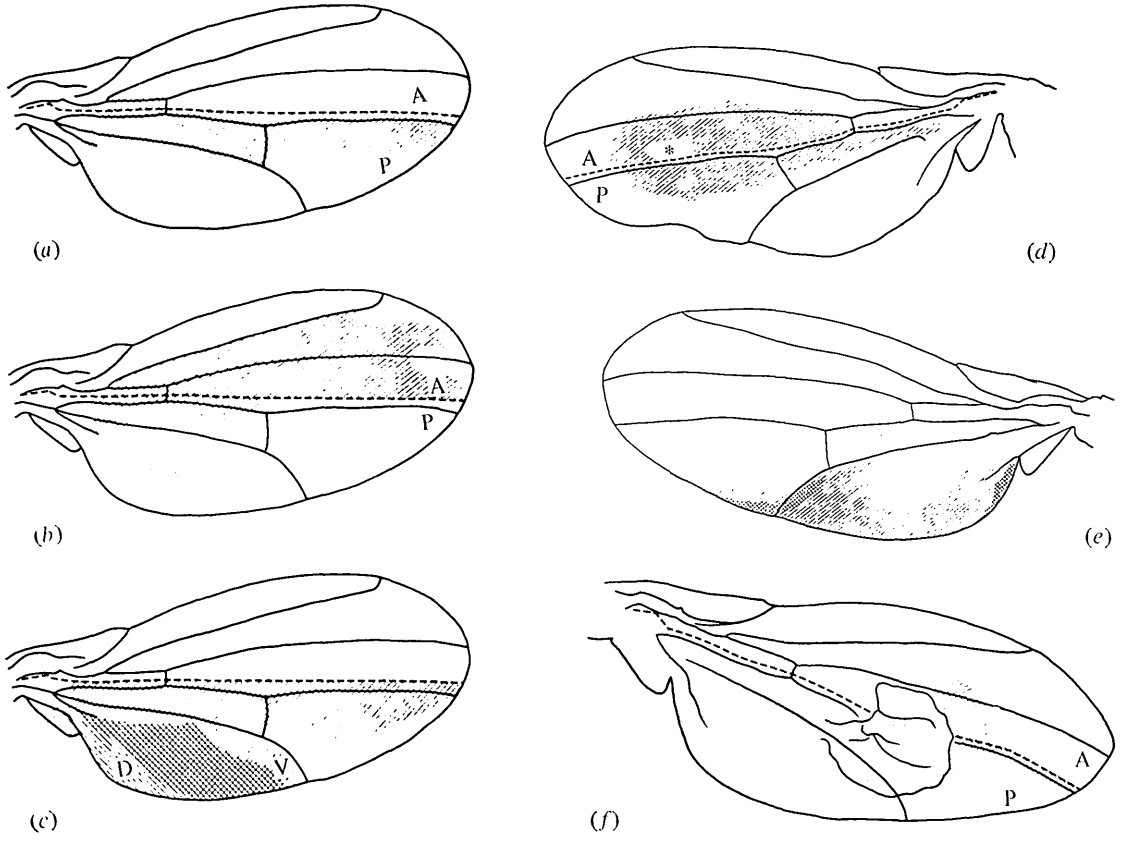

Fig. 2. Some examples of clones crossing boundaries; (*a–c*) from series I; (*d–f*) from series II. (*a*, *b*) Two clones crossing A/P boundary; (*c*) clone crossing D/V boundary; (*d*) clone crossing A/P boundary. Note islands of mwh$^+$ trichomes (*). Such islands are quite frequently observed in fast growing M$^+$ clones even in the absence of cell death (Morata & Simpson, unpubl.); (*e*) clone crossing D/V boundary; (*f*) slightly abnormal wing with blister, with distal clone crossing A/P boundary.

after the operation, however, the number of overlapping clones was no higher than either that expected from double inductions ($P \sim 0{\cdot}75$) or from that observed in the controls ($P \sim 0{\cdot}65$).

Fig. 2 (*a–c*) show camera lucida drawings of clones and wings from series I. In the experiments in which animals were irradiated on day 5 and operated on day 6, instead of one single cut, several cuts were made. In these animals the wings were not always normal, and of 179 wings only 123 permitted a reliable analysis of the A/P boundary whereas the D/V boundary could be analysed in all cases (Table 1).

Table 2. *Genotype of mesothoraces and number of clones overlapping A/P, D/V and N/W compartment boundaries in gynandromorphs* (In(1) w^{vC}/l(1)ts 504 sn^3 l(1)ts 5697; M(3)i^{55}/mwh M^+, *series II*) *grown at* 23 °*C, irradiated and shifted to 30* °*C at the times shown*

Time irrad. (days)	Time shift-up (days)	Number of half-thoraces analysed	% thoraces that were ♀	% thoraces that were ♂	% thoraces that were Mosaic	Clones: Touching and overlapping*	Clones: Overlapping A/P†	Clones: Overlapping D/V	Clones: Overlapping N/W	Total overlapping clones (A/P+D/V+N/W): Oberserved overlapping	Total overlapping clones (A/P+D/V+N/W): Expected‡ double inductions	(χ^2 test) *P*§
4	5	168	84·6	8·3	7·1	38	2 (+1)	3	3	8 > 0·5¶		< 0·03
4	—‖	203	46·3	36·4	17·3	29	0 (+1)	0	0	0 = 0·2		—
5	4	131	85·5	5·3	9·2	26	1 (+1)	0	1	2 = 0·4		—
5	—‖	144	45·1	27·1	27·8	21	0 (+0)	0	0	0 = 0·2		—

* Overlapping clones were treated as two clones resulting from two independent inductions, and are included as two events in this column.

† Number in parentheses refer to those clones that covered the A/P boundary, but did not include veins III and IV and were therefore excluded from the analysis (see pp. 231, 239).

‡ For calculation of expected double inductions see p. 232.

§ *P* gives probability with which observed and expected numbers are not different (see also p. 233).

‖ Non-shifted controls.

¶ For the calculation of the χ^2 an expected value of 4 was assumed (see p. 233).

Series II

Gynandromorphs of the genotype *l(1)ts 504 sn*^{3} *l(1)ts 5697/In(1)2w*^{vC}*; M(3)i*^{55}*/mwh M*$^+$ were constructed. The male XO tissue will show the cell lethal phenotype and the female XX tissue will not. X-irradiation will result in *mwh M*$^+$ clones. If the clones are induced in the female tissue of a mosaic wing disc and the animals are placed at 30 °C, cell death in the male cells will take place concurrently with the growth of a *mwh M*$^+$ clone in the female cells. In a way analogous to series I, two sets of experiments were performed: animals grown at 23 °C were either irradiated at 96 h and shifted up to 30 °C at 120 h, or shifted up to 30 °C at 96 h and irradiated at 120 h. Control animals were left at 23 °C. Wings and thoraces of all gynandromorphs, that is all animals which showed male tissue on any part of the body, were analysed.

The data are presented in Table 2 which also shows the percentage of the wing discs that were female, male or mosaic in these gynandromorphs. It can be seen that compared to the non-shifted controls, most of the gynandromorphs surviving at the higher temperature bore purely female thoraces. This is probably partly a reflection of the fact that at 30 °C those animals that were mainly composed of female tissue may have survived better. However, in mosaic wing discs, death of the male mutant tissue would also be followed by regeneration of the female wild type tissue. Duplications composed of entirely female tissue have been observed in gynandromorphs of cell lethal mutants grown at restrictive temperature (Russell, 1974; Simpson & Schneiderman, 1975). Duplications are believed to arise through the same process as that leading to regeneration and are often observed after surgical removal of part of an imaginal disc (Bryant, 1975). The fact that regeneration may have occurred in some of our wing discs is consistent with the observation that of all the overlapping clones, six were in entirely female wings and four were in mosaic wings.

In series II the notum-wing (N/W) compartment boundary was also analysed. Clones were found crossing the A/P, D/V and N/W boundaries. As in series I, clones overlapped two compartments when the animals were irradiated prior to the temperature shift, but did not do so when irradiated after the shift. Fig. 2(*d–f*) show camera lucida drawings of clones and wings from series II.

Mention should be made here of an observation which, however, has not been analysed further. In some wings of both series I and II, groups of bristles with sockets, sometimes clearly identifiable as anterior triple row bristles, were found situated on the posterior wing margin.

DISCUSSION

The few clonal analyses that had been coupled with regeneration experiments (Ulrich, 1971; Postlethwait, Poodry & Schneiderman, 1971; Nöthiger, 1976) were done at a time when neither the technique to produce fast growing clones nor the idea of compartments were known. They, therefore, cannot provide

information with respect to the relationship between normal development and regeneration. But as we stated in the Introduction, some recent experiments, notably those of Haynie & Bryant (1976), had nevertheless demonstrated that in a population of dividing cells changes in the compartmental commitments can occur. It was therefore not surprising that we obtained *clones* that crossed compartment boundaries after experimentally induced lesions of the wing disc. If compartments represent populations of cells that have acquired specific developmental programmes, then the descendants of a committed cell can, under experimental conditions, acquire new developmental programmes. However, the frequency of overlapping clones was low. This may mean that in our experiments compartmental restrictions were rapidly re-established during regeneration. This conclusion is strongly supported by the fact that a significant number of overlapping clones was only found in those series in which irradiation, i.e. clone induction, preceded the operation (series I) or temperature shift (series II). When the clones were induced after the lesion of the disc, all apparent overlaps could be accounted for by two independent inductions (Tables 1 and 2). Unless the clone is already present and close to or at the compartment boundary when regeneration starts, its descendants will reach the critical region too late and find the compartment boundary already re-established.

In those cases in which the clones were induced after the lesion and did not cross compartment boundaries any more, the size of the clones becomes crucial. Is the lack of overlapping clones simply a function of their smaller size due to later irradiation, or does it reflect a biologically significant phenomenon? When judging the size of the clones, we reasoned that their total size is less relevant in this context than their length touching a boundary. In the critical experiments in which the larvae were operated on day 4 and irradiated on day 5, no overlapping clones were found (Table 1). In this experiment, we considered only those clones that touched a boundary with at least seven cells. In our sample, two thirds of the clones followed a boundary for more than 18 cells. The average clone along the D/V boundary measured 33 cells for the controls and 28 cells for the experimental wings, and for the A/P boundary these values were 29 and 34 cells, respectively. The length of the clones was about the same in experimental and control wings which also supports the conclusion that the compartment boundaries had already been re-established at or shortly after the time of clone induction.

A comparison of the different experimental sets listed in Table 1 provides further arguments for the discussion and practically eliminates the problem of critical clone size. The experiment in which clones were induced on day 5 and the wing discs were cut on day 6 shows that these clones are still big enough to cross compartment boundaries. When irradiation and operation were done on the same day (day 5), a clone may occasionally have been able to cross a boundary, but the difference between observation and expectation is hardly significant. Finally, when the discs were operated (day 4) before irradiation, the

clones did not cross any more. This gradual increase in P values with discs that were all irradiated on day 5 speaks in favour of true developmental restrictions as the cause for absence of overlapping clones in those experiments where clone induction followed the lesion.

The low frequency of overlapping clones probably also reflects the way in which regeneration occurred. Regeneration in imaginal discs is epimorphic, i.e. it requires cell division (Ulrich, 1971; Schubiger, 1973; Bryant, 1975; Dewes, 1975). In a recent model for epimorphic regulation, French, Bryant & Bryant (1976) postulated that regeneration is essentially intercalary and that growth results from the juxtaposition, by wound healing, of cells of unlike positional values which then divide to fill in the missing values. Both our series, cutting the disc and exposing a *ts* cell lethal to restrictive temperatures, should lead to intercalary regeneration the extent of which is basically dependent on the amount of damage. Particularly in series I, in which the disc was simply cut into two pieces, which then healed together again (see p. 233), only a limited regenerative growth is expected. Consequently, the probability for a clone to cross the narrow 'bridge' where this growth takes place should be rather low, even when the clone was induced one day prior to the lesion. In fact, assuming a cell cycle length of about 8·5 h (Garcia-Bellido & Merriam, 1971), the clone should not comprise more than four cells at the time of the operation.

In those cases where regeneration did not lead to entirely normal wings, it is possible that the A/P boundary might have been simply displaced. As stated on p. 231, we only recorded as A/P overlaps those clones that covered vein IV as a posterior and vein III with sensilla as an anterior structure. A small number of clones that covered the presumed A/P boundary, but did not extend to both vein IV and vein III were excluded. These cases were so rare that they remain without influence on the general picture. For complete information, they are listed in Tables 1 and 2 in parentheses behind those numbers that were used for the statistical analysis. A more severe displacement of the A/P boundary would probably lead to abnormal wing morphology. As documented by Fig. 2, most of the regenerated wings looked normal. Fig. 2(f), however, shows a case of an abnormal wing with a blister on the same surface as the clone that apparently overlaps A and P compartments. This clone, in contrast to most others, has borders which are contiguous on either side of the presumed A/P boundary, i.e. it is almost symmetrical about this line. One would not expect overlapping clones to have contiguous borders on either side of the compartment boundary. If compartment boundaries are re-established during regeneration, and a clone becomes subdivided by a newly forming boundary such that part of it is in one compartment and part in another, then different growth patterns in the two compartments may lead to a spatial separation of the two parts of the clone.

The results reported in this paper rely on statistical analysis and depend at least in part on arbitrary criteria. We have tried to keep these in bias against our hypothesis whenever we felt insecure in our judgement. Nevertheless, our data

support the conclusion that compartmental commitments can be changed during regeneration, and – more important – that compartment boundaries are re-established in the course of the process. If selector genes are involved in the formation of compartments, the hypothesis can be put forward that regeneration makes use of the same genetic signals as normal development.

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