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Further studies of the *engrailed* phenotype in *Drosophila*

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Although most mutations at the *engrailed* locus of *Drosophila* cause embryonic death when homozygous, they are viable in clones of cells. We describe the phenotype of such clones in the eye-antenna, proboscis, humerus, wing, legs, and terminalia. When in anterior compartments the clones are normal, but in most posterior compartments they are abnormal and fail to respect the anteroposterior compartment boundary. We find that the yield of *engrailed*-lethal clones in posterior compartments is often significantly lower than expected, indicating that these clones are lost during development. Mutant clones are abnormal in the analia and rare in the humerus, suggesting that both structures are of posterior provenance. These results support the hypothesis that the *engrailed*$^+$ gene is required exclusively in cells of posterior compartments to specify their characteristic cell affinities and pattern.

Key words: cell affinities/developmental compartments/*engrailed*/homoeotic genes

Introduction

Developmental compartments are precisely defined regions of the adult which are constructed by the complete set of cells descending from small groups of embryonic founder cells (Garcia-Bellido *et al.*, 1973; Crick and Lawrence, 1975). Garcia-Bellido (1975) has postulated that there are genes that act in one or a specific set of developmental compartments. These 'selector genes' are supposed to determine, at the cellular level, the developmental pathways followed by growing polyclones (Garcia-Bellido *et al.*, 1973; Crick and Lawrence, 1975). Segments are compartments (Lawrence, 1973, 1981a) and examples of selector genes whose operations are co-extensive with them are the elements of the *bithorax*-complex (Lewis, 1978; Struhl, 1981a). In addition most, if not all, segments are each subdivided into an anterior and a posterior compartment (Garcia-Bellido *et al.*, 1973; Steiner, 1976; Morata and Lawrence, 1979; Kornberg, 1981a; Struhl, 1981b) and the *engrailed*^{1} mutation (Eker, 1929) has effects only in posterior compartments (Morata and Lawrence, 1975, 1979; Lawrence and Morata, 1976; Lawrence *et al.*, 1979; Kornberg, 1981a, 1981b). This has led to the hypothesis that *engrailed*$^+$ is a selector gene which determines the development of posterior, but not anterior, compartments (Morata and Lawrence, 1975; Lawrence and Morata, 1976; Garcia-Bellido *et al.*, 1979).

Recently lethal alleles at the *engrailed* locus have been isolated (Kornberg, 1981b; Nüsslein-Volhard and Wieschaus, 1980) and the behaviour of cells homozygous for such alleles has been briefly described (Kornberg, 1981a, 1981b). Homozygous cells are viable and wild-type when in anterior compartments of legs, wings, and abdomen, even when the clones are very large. In posterior compartments the clones give abnormal patterns and, as with *en*^{1} cells (Morata and Lawrence, 1975), fail to respect the anteroposterior compartment border (Kornberg, 1981a, 1981b). Here we describe in more detail the phenotype of clones of cells that are homozygous for lethal *engrailed* alleles.

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Results

The eye-antenna

The developing eye-antenna segment is subdivided into an anterior and a posterior compartment much later than the labial and thoracic segments (Morata and Lawrence, 1979). The distribution of experimental and control clones is compared in Table I; they are similar. We have not detected any difference between the phenotype of control and experimental clones in either the anterior or the posterior compartment.

The proboscis

A large number of probosces were screened and the frequency of control and experimental clones compared; there was a shortfall of posterior experimental clones ($p = <0.001$, Table I), but the frequencies of anterior clones were similar. In addition, six experimental clones appeared to cross between the anterior and posterior compartments (Figure 1), whereas only two crossing clones were found in the controls. Between zero and two crossing clones are expected to result from two independent clones, one in each compartment, in the experimental series and between zero and three are expected in the control series; the number of experimental clones crossing the boundary is much greater than expected from two independent events ($p = <10^{-5}$). Finally, several experimental clones in the posterior compartment were associated with abnormal bristle patterns not found in control clones (Figure 1).

Table I. Clone frequency in various compartments

		Controls		*engrailed*-lethal	
Eye-antenna	A + P	23	n = 2870	17	n = 3206
	A	106		91	
	P	40		26	
Proboscis	A + P	2	n = 7290	6	n = 7694
	A	71		71	
	P	99		31	
Terminalia ♂	genitalia	21	n = 1145	28	n = 2329
	analia	12		28	
Terminalia ♀	genitalia	14	n = 848	20	n = 1623
	analia	15		11	

n = number of sides for all parts except the terminalia which, because clones frequently cross from left to right (Dubendorfer and Nöthiger, 1982), are treated as single units. Larvae were irradiated at 48 ± 4 h after egg laying with 1500 rads, and flies were mounted and screened under the compound microscope.

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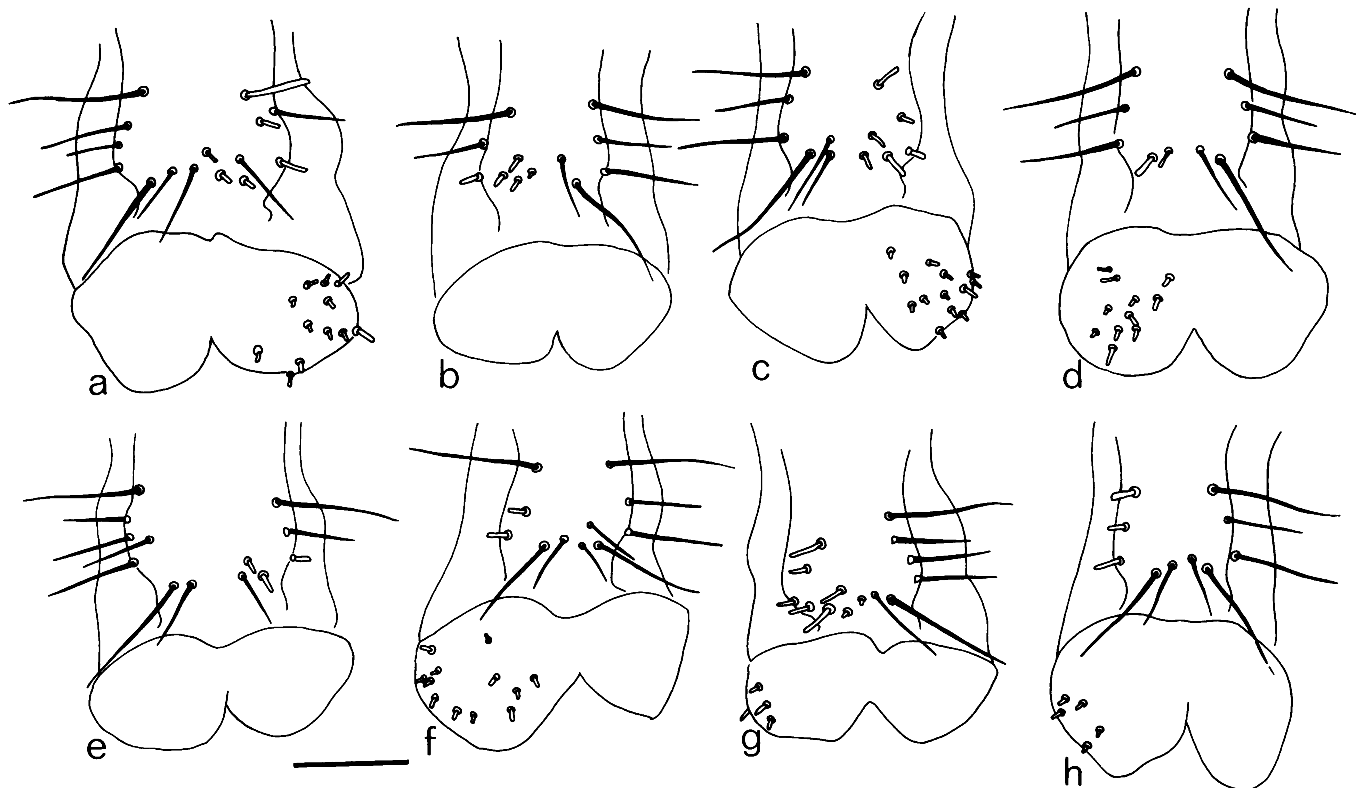

Fig. 1. Camera lucida drawings of clones in the proboscis. The marked bristles are stubby and pale as drawn; all bristles are shown on the prementum, while on the palps only the *stw pwn* bristles are indicated. **d** is an en^+ clone in the posterior compartment and **h** is an en^{IK} clone in the anterior compartment. Note that these clones mark adjacent, non-overlapping regions of both the prementum and the palps, and hence define the normal anterior and posterior compartments (Struhl, 1981b). The remaining examples are *engrailed*-lethal clones which mark bristles in both anterior and posterior territories; sometimes these clones are associated with odd arrangements of bristles (**a**, **b**, **g**). **a**, **f**, **g**, and **h**, are en^{IK}, **b**, **c**, and **e** are en^{IO} and **d** is en^+. Magnification x 150.

The humerus

The humeri are situated at the anterolateral margins of the thorax and their segmental origin is not clear. Even clones produced at blastoderm do not extend between the humerus and the notum or between the humerus and any part of the legs (Morata and Struhl, unpublished results). Experimental and control clones that marked either the humerus or the anterior notopleural bristle, which belongs to the anterodorsal compartment of the wing disc (Garcia-Bellido *et al.*, 1973, 1976), were induced by irradiation 96 h after egg laying. In both controls and experimentals the numbers of clones that marked the anterior notopleural bristle were similar; but there was a significant loss of those humerus clones that were *engrailed* ($p = <0.01$). The five clones that marked the humerus appeared normal in phenotype (Table II).

Table II. Clone frequencies in the humerus

n	Controls		*n*	*engrailed*-lethal	
	humerus	notopleural bristle		humerus	notopleural bristle
2000	23	33	2000	5	32

n = number of sides.
Larvae were irradiated at 96 ± 4 h after egg laying with 1500 rads, all putatives were dissected, mounted, and examined under the compound microscope to confirm the *stw pwn* phenotype.

Wings

Experimental clones in the anterior wing and notum were normal (number examined (n) = 50) and identical to the controls (Figure 2). Posterior clones in the wing were abnormal ($n = 43$). They caused some enlargement of the part of the wing they marked, and also areas outside that territory (Figure 3). The posterior margin bore large socketed bristles (Figure 3), and a well-developed triple row (normally found on most of the anterior margin) was found more distally. Nearer the tip of the wing there was a socketed double row of bristles (as is found on the anterior tip of the wing). The vein pattern was grossly disturbed and all veins bore scattered campaniform sensilla of the type normally found on vein III in the anterior compartment. Posterior clones failed to respect the anteroposterior compartment boundary, frequently extending up to, and beyond, vein III (Figure 3). These clones formed abnormal patterns in both the anterior and posterior parts of the wing (enlarged regions, irregular veins, Figure 3). All these features are similar to the phenotype of $engrailed^1$ clones (Garcia-Bellido and Santamaria, 1972; Morata and Lawrence, 1975; Lawrence and Morata, 1976).

Clones of the lethal alleles of *engrailed* appeared to cross the anteroposterior border more readily than $engrailed^1$ clones. This was confirmed by several unusual clones that were undoubtedly of posterior provenance (they were posterior in their proximal part, and marked enlarged and slightly abnormal wing regions) but were located almost entirely in territory that is normally part of the anterior compartment. These clones frequently respected the anteroposterior compartment boundary from the anterior side

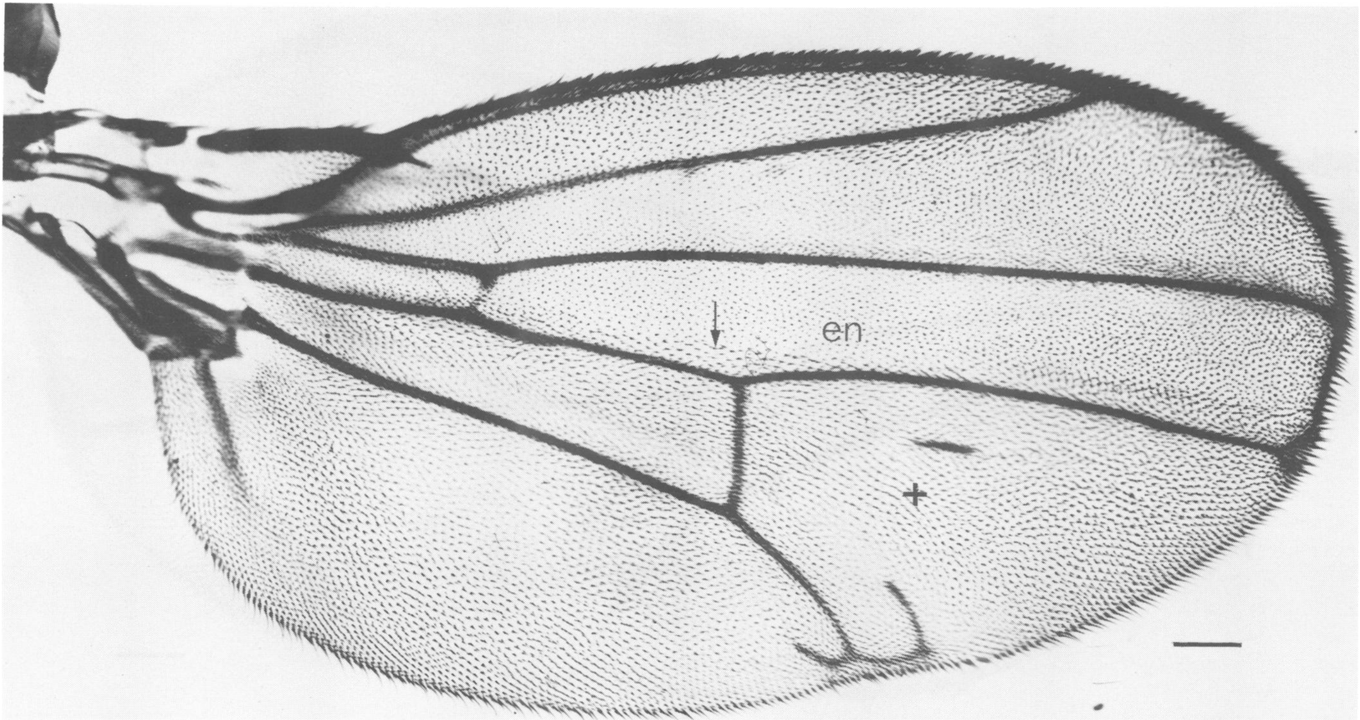

Fig. 2. A *stw pwn* en^{IO} clone on dorsal and ventral surfaces of the anterior compartment of the wing. Note that the marked trichomes (*en*), which are finer than the wild-type ones (+), respect the anteroposterior boundary (arrow). Magnification x 90. Scale bar = 100 μm.

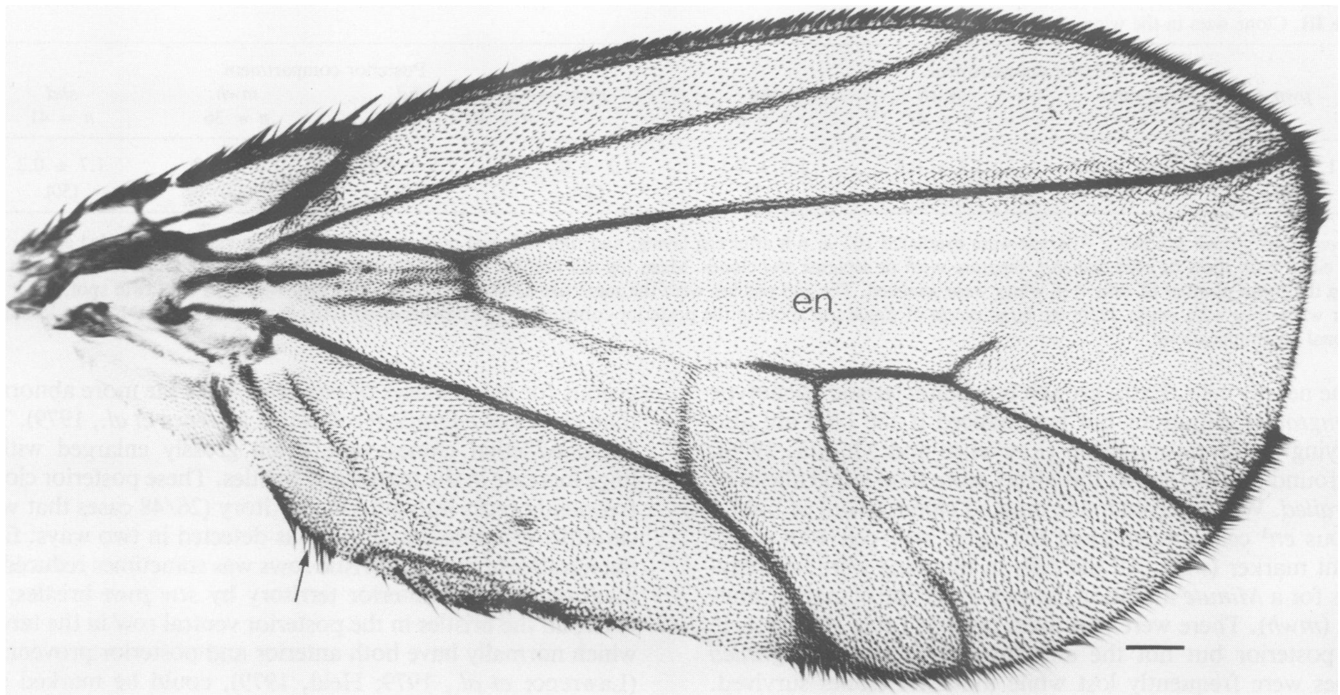

Fig. 3 A *stw pwn* en^{IO} clone which extends into both anterior and posterior territory. The clone is both dorsal and ventral in the middle of the wing (*en*) but is only dorsal more proximally where, at the posterior margin, it marks socketed bristles (arrows). Magnification x 80. Scale bar = 100 μm.

(Figure 4). Our impression was that their margin along this line was not quite as straight as the margin of normal and *engrailed*-lethal anterior clones.

One perplexing aspect of the *engrailed* phenotype is the enlargement of posterior territory. A possible explanation is that the transformation of posterior cells into anterior-like cells might be analogous to transplanting anterior cells into the posterior polyclone and, if so, intercalary growth would be elicited at the border between the clone and the surround. [In both *Drosophila* (Haynie and Bryant, 1976) and other insects (Bohn, 1967; Nübler-Jung, 1979; Wright and Lawrence, 1981) translocations of cells in the anteroposterior axis stimulate intercalary growth.] We consequently tested whether extra growth occurs in both the *engrailed* clone and

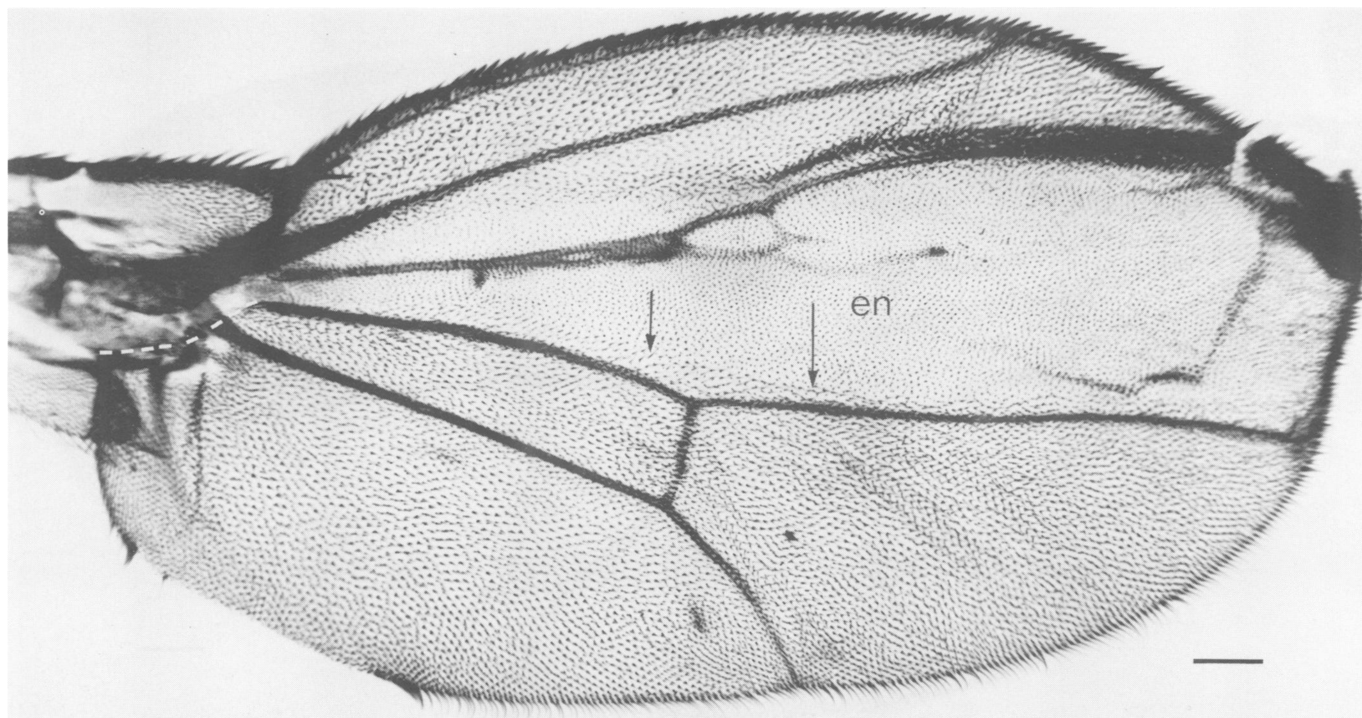

Fig. 4. A *stw pwn* en^{IK} clone of posterior provenance which, although it fills some posterior territory in the proximal part of the wing (indicated by the dotted line), is largely anterior in location. Note that the clone (*en*) respects the anteroposterior boundary from the anterior side (arrows), although the clone border seems more uneven than usual (compare Figure 2). Magnification x 90. Scale bar = 100 μm.

Table III. Clone sizes in the wing

Anterior compartment				Posterior compartment			
pwn ———	*shd*	*mwh*	*shd*	*pwn* ———	*shd*	*mwh*	*shd*
n = 25		*n* = 47	*n* = 5	*n* = 10		*n* = 36	*n* = 41
1.2 ± 0.2 (16)	1.3 ± 0.3 (20)	1.3 ± 0.3 (20)	1.5 ± 0.1 (32)	1.3 ± 0.3 (20)	1.5 ± 0.3 (32)	1.5 ± 0.2 (32)	1.7 ± 0.2 (50)

pwn en^1/shd^{IN}; *mwh* $M(3)w^{124}$/+ larvae were irradiated 102 ± 6 h after egg laying, and flies collected over the first 24 h of emergence. Recorded clones were either twin spots of *pwn* and *shd,* separate *mwh* or separate *shd* clones. Mean log cell number per clone is given with the s.d., the figures in brackets giving the mean number of cells. 138 wings were screened. The *shd* patches were the same size as *mwh* controls when they were part of a twin spot, but larger when they were single. Note the large excess of single *shd* spots in the posterior compartment. The *shavenoid (shd)* locus is at 2–64 (Nüsslein-Volhard, personal communication).

in the nearby cells. Extra growth is a characteristic phenotype of *engrailed* mutations but is enhanced if the cells are also carrying a *Minute* mutation (Lawrence and Morata, 1976). We found that en^1 shows this effect more than lethal alleles of *engrailed*. We therefore made marked clones (*pwn*) of homozygous en^1 cells and marked the sister en^+ cell with a different marker (*shd*). All the cells in the wing were heterozygous for a *Minute* mutation, as well as a control marker mutation (*mwh*). There were two main results (Table III): first, in the posterior but not the anterior compartment, *engrailed* clones were frequently lost while the sister clones survived. Second, the *pwn* en^1 and *shd* en^+ sister spots were a similar size to each other and the *mwh* controls, which argues against intercalation. We found, as noted before, that *engrailed* clones which reach the posterior wing margin often cause large outgrowths (Lawrence and Morata, 1976). Probably, therefore, the mutation causes the extra growth not by inducing intercalation but by its effects on the wing margin.

Legs

Anterior clones were normal in every respect (*n* = 19, 12, and 11 for legs I, II, and III). Posterior clones of cells homozygous for lethal alleles of *engrailed* were far more abnormal than clones of *engrailed*^{1} cells (cf. Lawrence *et al.*, 1979). The *engrailed*-lethal clones were often grossly enlarged with a huge increase in the number of bristles. These posterior clones frequently cross into anterior territory (26/48 cases that were carefully examined) and this was detected in two ways: first, the number of anterior bristle rows was sometimes reduced by invasion into the anterior territory by *stw pwn* bristles; second, all the bristles in the posterior ventral row in the tarsus, which normally have both anterior and posterior provenance (Lawrence *et al.*, 1979; Held, 1979), could be marked and therefore be part of the posterior clone. The pattern produced by the clones differed both from the wild-type pattern and from the *engrailed*^{1} pattern. For example, the clones did not form the large bristles characteristic of the posterior femur of the wild-type prothoracic leg (Steiner, 1976; Morata and Kerridge, 1981). They did not make an ectopic sex comb as do clones of *engrailed*^{1} (Tokunaga, 1961). Most of the bristles were of intermediate size; also they were crowded and were often abnormally oriented (Figure 5). There were sometimes extra elements: for example, ectopic bristles resembling the anterior apical and pre-apical bristles were found in the mid-

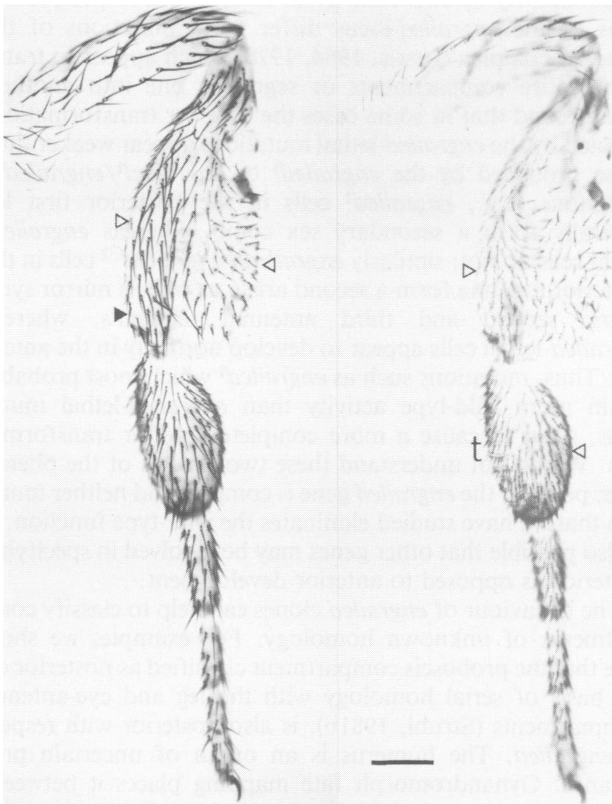

Fig. 5. A *stw pwn* en^{IO} clone in the posterior part of a metathoracic leg which is extremely enlarged. The **left** picture shows the anterior face of the leg, and the **right** (at a lower plane of focus) the posterior face. Note the marked bristles (open arrows) are small, poorly oriented, and cover the entire posterior face and about half of the anterior face. Depauperated transverse rows of the posterior compartment of the basitarsus are formed by the clone (t). Magnification x 84. Scale bar = 100 μm.

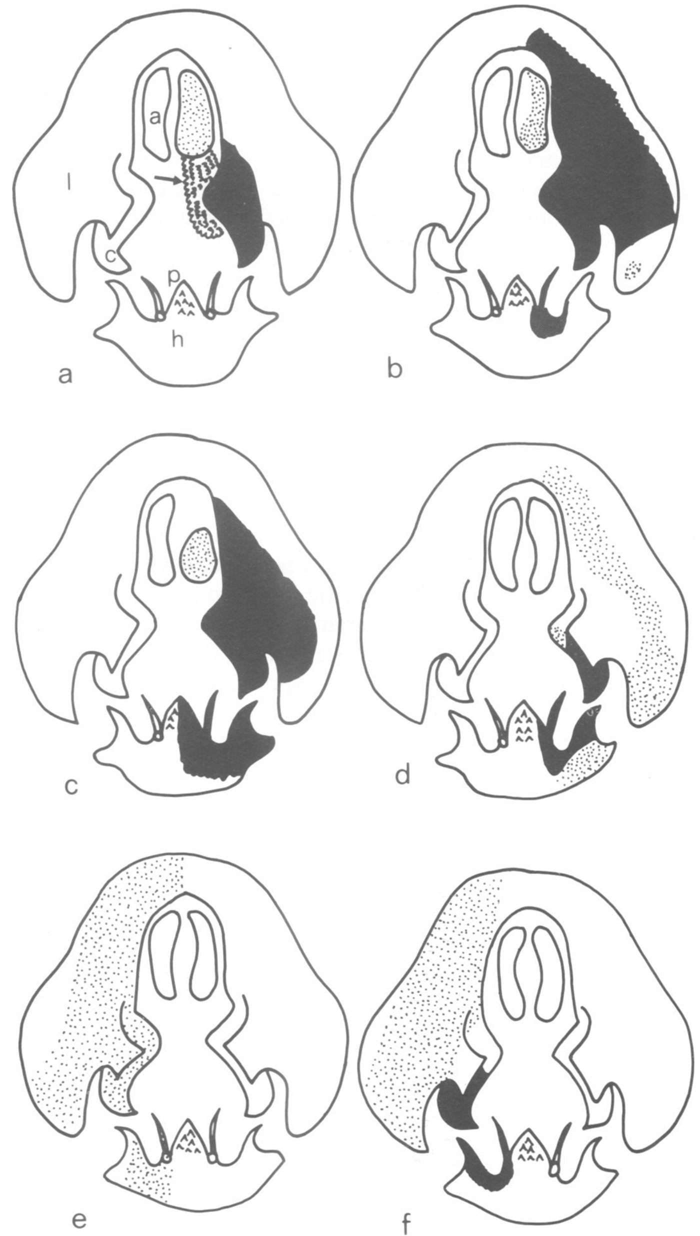

Fig. 6. Clones in the terminalia. **a**, **b**, and **f** are *stw pwn* en^{IO}, **c**, **d** are *stw pwn* en^{IK} and **e** is a *stw pwn* en^{+} clone. Note that clones in the analia cause abnormally shaped plates and can be associated with deletion of genitalia (the approximate areas missing are shown in black, and the shading indicates territory marked with *stw pwn*). In **a** there is some unidentified cuticle (arrow) associated with a marked anal plate. **d** and **f** are mutant clones in the genitalia which are also associated with missing parts. a = anal plate, c = clasper, h = hypandrium, l = lateral plate, p = penis. (Diagram after Dubendorfer and Nöthiger, 1982.)

dle of the posterior compartment of the mesothoracic femur, and sometimes there were several extra claws, although the posterior claw could also be defective or wanting. The excessive growth of the clone could be associated with extra growth of unmarked cells, just as happens in the wing when *engrailed*^{1} *Minute* clones are made near the posterior margin (Lawrence and Morata, 1976). Clones were associated with fusion of the leg segments and occasionally produced leg duplications. Sometimes parts of the clone had sorted out into separate vesicles, as happens with en^1/en^{C2} clones in the posterior wing and antenna (Morata *et al.*, in preparation).

Terminalia

The terminalia are probably subdivided into at least three compartments whose homology with segments and subsegments is unknown (Nöthiger *et al.*, 1977; Dubendorfer and Nöthiger, 1982). The female genital compartment is most anteriorly located, next comes the male genitalia, and most posteriorly there is a compartment which, in the female, consists of a pair of anal plates and, in the male, both an anal plate and parts of the penis (Dubendorfer and Nöthiger, 1982). In the two sexes, the genital compartments appear to be constructed by polyclones which arise in different places; however, the analia develop from homologous primordia (Belote and Baker, 1982; Wieschaus and Nöthiger, 1982). As reported by Dubendorfer and Nöthiger (1982), we found that control clones marked either analia or genitalia but not both (Figure 6). Clones of male *engrailed*-lethal cells were defective in several respects: in the genitalia they were smaller than controls and were often associated with missing parts of the claspers, lateral plate, and hypandrium (6/28, e.g., Figure 6d,f); clones in the analia formed unusually shaped anal plates and were often associated with missing genital parts (10/28, Figure 6a,b,c). Male analia clones also occasionally formed some unidentified cuticle that could extend from the anal plate towards the penis. In females, mutant analia clones resembled en^{+} control clones and the genitalia were normal, as far as we could tell. (Unfortunately, the *stw* and *pwn* mutations are not ideal markers of the thorn bristles and T8

sensillae – the only parts of the female genitalia that can be marked.)

Discussion

The primary aim of these experiments is to learn more about the role of the *engrailed*$^+$ gene. To this end, we have generated clones of cells homozygous for lethal alleles of this locus and have described their phenotype in the head, thorax, and terminalia. Unlike the original *engrailed*^{1} mutation which is homozygous viable, such lethal alleles cause apparent fusion of the embryonic segments (Kornberg, 1981b; Nüsslein-Volhard and Wieschaus, 1980). It is therefore likely that the phenotype of *engrailed*-lethal mutations approaches the null phenotype more closely than that of the *engrailed*^{1} mutation, and hence that it may provide new insights into the normal role of the *engrailed*$^+$ gene. Our results confirm and extend Kornberg's finding (1981b) that *engrailed*-lethal clones are normal in anterior, but abnormal in posterior, compartments, and support the hypothesis that the *engrailed*$^+$ gene is a selector gene which has a specific function in all the cells of one set of developmental compartments, but no function in the remaining set (Morata and Lawrence, 1975).

The *engrailed*$^+$ gene may be required to 'label' cells of posterior polyclones such that their affinities become distinct from anterior cells (Morata and Lawrence, 1975; Lawrence and Morata, 1976). Our present results strongly support this view since they indicate that the affinities of posterior cells are further transformed by *engrailed*-lethal mutations than by the *engrailed*^{1} mutation. First, *engrailed*-lethal cells generated in the posterior wing compartment mix preferentially with anterior cells, and can even respect the anteroposterior boundary from the anterior side (Figure 4). Second, we show that in both the legs and the proboscis, posterior cells that are *engrailed*-lethal cross into anterior territory whereas *engrailed*^{1} clones do not appear to cross (Lawrence *et al.*, 1979; Struhl, 1979). Third, we find a significant loss of *engrailed*-lethal clones in the posterior, but not the anterior, compartment of the proboscis; shortfall of posterior *engrailed*^{1}/*engrailed*^{C2} clones is also observed in the eye-antenna (Morata *et al.*, in preparation). This loss of posterior *engrailed*-lethal clones might result from a change in the affinities of mutant cells so that they sort out from their posterior neighbours. Indeed, Morata *et al.* describe a posterior *engrailed* clone that appears to be caught in the process of sorting out into a separate vesicle. All these results suggest that one main function of the *engrailed*$^+$ gene is to give posterior cells particular surface properties which distinguish them from anterior cells (Morata and Lawrence, 1975).

One intriguing aspect of the *engrailed*^{1} mutant phenotype is that mutant cells in posterior compartments form structures normally found in the corresponding anterior compartment (Tokunaga, 1961; Garcia-Bellido and Santamaria, 1972; Lawrence and Morata, 1976; Morata and Lawrence, 1979). As we describe, some of these transformations are characteristic of *engrailed*-lethal clones and, as with the *engrailed*^{1}, the anterior elements appear in approximate mirror symmetry with respect to their anterior counterparts. However, note first that the phenotype cannot be described simply as the transformation of posterior compartments into anterior compartments, because normal patterns are not formed even when the posterior compartments are constructed predominately by *engrailed*-lethal cells. In this respect, mutations of the *engrailed*-locus differ from mutations of the *bithorax*-complex (Lewis, 1964, 1978) which appear to transform entire compartments or segments one into another. Note second that in some cases the anterior transformations effected by the *engrailed*-lethal mutations appear weaker than those produced by the *engrailed*^{1} or *engrailed*^{1}/*engrailed*^{C2} mutations (e.g., *engrailed*^{1} cells in the posterior first leg generally form a secondary sex comb, whereas *engrailed*-lethal cells do not; similarly *engrailed*^{1}/*engrailed*^{C2} cells in the posterior antenna form a second arista as well as mirror symmetric second and third antennal segments, whereas *engrailed*-lethal cells appear to develop normally in the antenna). Thus, mutations such as *engrailed*^{1} which most probably retain more wild-type activity than *engrailed*-lethal mutations, seem to cause a more complete anterior transformation. We do not understand these two aspects of the phenotype; possibly the *engrailed* gene is complex and neither mutation that we have studied eliminates the wild-type function. It is also possible that other genes may be involved in specifying posterior as opposed to anterior development.

The behaviour of *engrailed* clones can help to classify compartments of unknown homology. For example, we show here that the proboscis compartment classified as posterior on the basis of serial homology with the leg and eye-antenna compartments (Struhl, 1981b), is also posterior with respect to *engrailed*. The humerus is an organ of uncertain provenance. Gynandromorph fate mapping places it between, but separate from, the labial and mesothoracic segments; this allocates it to the prothorax (Struhl, 1981c). Our results, which show a shortfall of *engrailed*-lethal clones compared with an internal control, suggest that the humerus is of posterior homology. There is other evidence that this is so. The argument depends on the generalisation that mutations of the *bithorax*-complex change segmental determination without altering the number of segments present, or the anterior or posterior status of subsegments. It is found that, in the legs, cells lacking the *Ubx*$^+$ gene in the posterior mesothorax and posterior metathorax can be transformed into posterior prothorax (Morata and Kerridge, 1981; Kerridge and Morata, 1982). Likewise, in the postnotum (posterior dorsal mesothorax) *Ubx* tissue sometimes forms small patches of bristles that look like humeri (Lewis, 1964; Kerridge and Morata, 1982). If this dorsal transformation is in the same direction as the transformation in the legs, these bristles are indeed humeral and the humerus is therefore posterior prothorax.

The behaviour of *engrailed*-lethal clones in the terminalia is not so easily explained. Certainly, the abnormal structure of the analia clones in the male suggest that the analia-penis compartment (Dubendorfer and Nöthiger, 1982; Wieschaus and Nöthiger, 1982) is homologous to other posterior compartments. Additional and stronger evidence for this comes from the behaviour of *Polycomb* clones. These clones transform all segments of the body towards the terminalia. In several cases in the legs and antenna both analia and penis were made by these clones; in all of these cases the clones originated in posterior compartments (Struhl, unpublished data). *engrailed*-lethal clones in the male genitalia might also be of posterior provenance. The normal behaviour of mutant clones in the female genitalia is consistent with their being of anterior provenance. In summary, we believe the evidence that the male analia-penis compartment is of posterior pro-

venance is good, but the status of the male and female genital compartments is obscure.

Materials and methods

Clones of lethal alleles of engrailed

Two lethal alleles of *engrailed* (en^{IK} and en^{IO}, generously provided by C.Nüsslein-Volhard and E.Wieschaus) have been studied. The homozygous mutant cells were marked with *straw* and *pawn* (Garcia-Bellido and Dapena, 1974); in addition the *Minute* technique was used so that the clones grew excessively (Morata and Ripoll, 1975). Males *cn bw* $M(2)c^{33a}/bw^{V32g}$ were crossed to females *stw pwn cn* en^{IO} (or en^{IK}) sdh^{8} *bw/CyO* (as experimentals) and *stw pwn* sdh^{8} *bw/CyO* (as controls) and the progeny irradiated with 1500 rads at 48 h after egg laying unless stated otherwise. All experiments were performed at 25°C. Normally the controls and experimental crosses were performed separately but, in studies of the humerus, both control and experimental females were crossed to males in the same bottles. The progeny could be distinguished by eye colour (experimentals *cn bw*; controls *bw*), and equal numbers of each class were collected from a bottle on any day. This was done to ensure true equivalence of the flies (when bottles are aged for 96 h before irradiating, crowding and other factors can have large effects on the rate of development). sdh^{8} is a gratuitous cell marker (Lawrence, 1981b) which was not used in this study. No differences were noted between en^{IO} and en^{IK}, so the results with both were pooled. Irradiated flies were dissected and the relevant pieces mounted in Struhl's mountant (1981b).

Acknowledgements

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