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On the role of the *engrailed*$^+$ gene in the internal organs of *Drosophila*

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Our purpose is to assess the effects of lethal alleles of *engrailed* on cells of the internal organs of *Drosophila*. Using nuclear transplantation we make mosaic flies that contain regions made by *engrailed*-lethal cells that are genetically labelled. We find that *engrailed*-lethal cells cause defects in some parts of the epidermis and central nervous system. Most of the internal organs of the fly are assessed and of those, all organs and tissues derived from the endoderm or the splanchnic and somatic mesoderm are normal; flies carrying *engrailed*-lethal cells in large areas of these organs are viable. We postulate that segments of the mesoderm are single units of cell lineage and that, unlike the ectoderm, they are not subdivided into anterior and posterior compartments.

Key words: Drosophila/genetic mosaics/homoeotic genes/nuclear transplantation

Introduction

The main purpose of developmental genetics – to understand the wild-type function of genes – has always been bedevilled by the difficulty of distinguishing primary from secondary effects of mutations. It is also difficult to identify the cells that are first or most seriously affected by defective gene function and therefore mainly or entirely responsible for the mutant syndrome. The most successful approach to this problem has used *Drosophila* that are mosaic for mutant and wild-type cells, one cell type being labelled by a gratuitous cell marker (Demerec, 1936). Several mosaics can then be analysed to see which cells are responsible for the mutant phenotype. Mosaics can be made with unstable chromosomes (Sturtevant, 1929), with mitotic recombination (Stern, 1936) or by nuclear transplantation (Zalokar, 1971). The first two of these approaches have been applied to homoeotic genes (Lewis, 1963; Morata and Garcia-Bellido, 1976), and support a hypothesis that links cell lineage of the developing fly to the pattern of activity of a specific class of genes – the 'selector' genes (Garcia-Bellido, 1975). According to this hypothesis, developmental compartments form different patterns because in each of them there is a unique combination of active and inactive selector genes (reviewed in Morata and Lawrence, 1977; Struhl, 1982). Experimental evidence in support of this hypothesis has come largely from two genetic units, the bithorax complex (Lewis, 1963, 1978) and the *engrailed*$^+$ gene.

In the epidermis, the *engrailed*$^+$ gene appears to function exclusively in posterior compartments, so that cells carrying either viable or lethal mutant alleles of the gene are perfectly normal in anterior compartments but defective in posterior ones. In posterior compartments, *engrailed* mutant cells acquire anterior properties; they make anterior-like patterns and mix across the compartment boundary with anterior cells (Morata and Lawrence, 1975; Kornberg, 1981; Lawrence and Struhl, 1982). Since the segmented epidermis consists of alternating stripes of anterior and posterior cells which are set up at or close to the blastoderm stage (reviewed in Lawrence, 1981a) the *engrailed*$^+$ gene may be crucial for the maintenance of segmentation; indeed in larvae homozygous for lethal alleles of *engrailed*, the segments appear to fuse (Kornberg, 1981; Nüsslein-Volhard and Wieschaus, 1980).

Little is known about segmentation of the internal organs of *Drosophila* and less about the role of *engrailed*$^+$ gene there. Here we use a general cell label (*sdh*, Lawrence, 1981b) and nuclear transplantation to make marked mosaics in adults. We find that cells homozygous for lethal alleles of *engrailed* are defective in parts of the epidermis and central nervous system, but appear normal in all mesodermal and endodermal organs that we were able to score. This suggests that the *engrailed*$^+$ gene function is limited to the ectoderm and that the mesoderm is divided into segments that are not subdivided into anterior and posterior subsegments.

Results

Cuticle mosaics

In all, 54 mosaics were studied of which 28 were in *sdh*$^+$ flies; of these latter, only three carried patches of *straw pawn* bristles and were therefore made by cells homozygous for lethal alleles of the *engrailed* gene. Two were mosaic in the terminalia where the *engrailed* mutant cells showed the expected phenotype (Lawrence and Struhl, 1982). In the other mosaic mutant cells were confined to the dorsal part of the anterior compartment of the head which, as expected, was normal in structure. About one quarter of the mosaics should have involved *engrailed* cells; the shortfall is no doubt due to the death of any mosaic with extensive colonisation of a tissue where *engrailed*$^+$ function is essential (such as the epidermis).

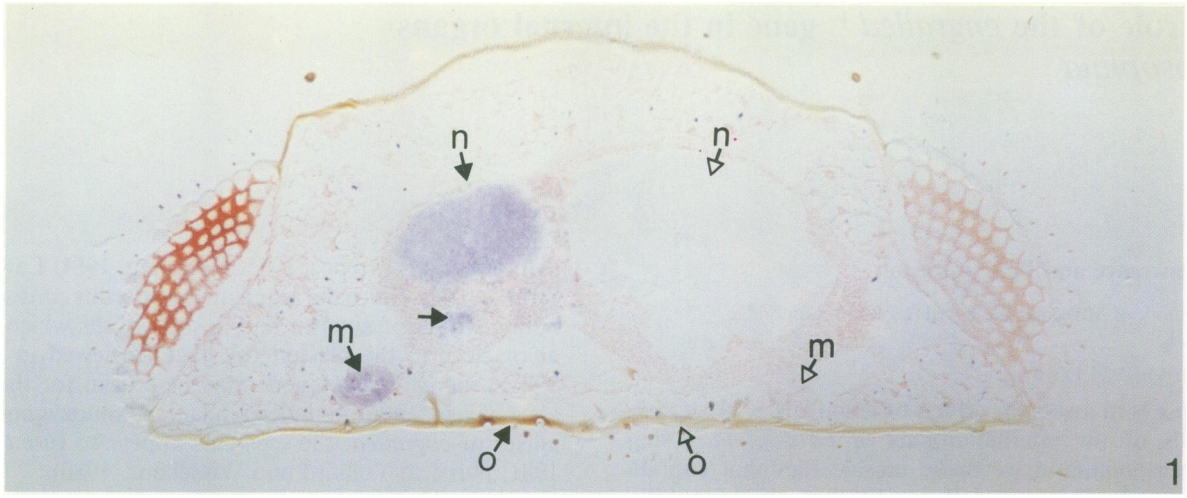
Internal mosaics

Of 26 mosaics in *sdh*^{8} hosts three were clearly mosaic for *engrailed*-lethal cells and we shall describe these in detail. Of the remainder, six were mosaic only in internal organs and therefore could not be allocated as *engrailed* or control mosaics. This leaves 17 control mosaics that contained patches of *engrailed*$^+$ *sdh*$^+$ tissue and from these one can deduce information about the normal cell lineage of the internal organs. These controls are being pooled with those from other experiments and we plan to publish a description of cell lineage later.

The three mosaics containing *engrailed*-lethal cells will be described in turn; in each case donor nuclei give rise to *engrailed*-lethal cells that are marked with *sdh*$^+$ while host nuclei are *engrailed*$^+$ and marked with *sdh*^{8}.

Mosaic 54

In this mosaic, *engrailed* cells are confined to the head and parts of the gut. In the ectoderm most of the left side of the head and antenna is marked with *straw pawn* bristles and is normal in structure. The posterior head compartment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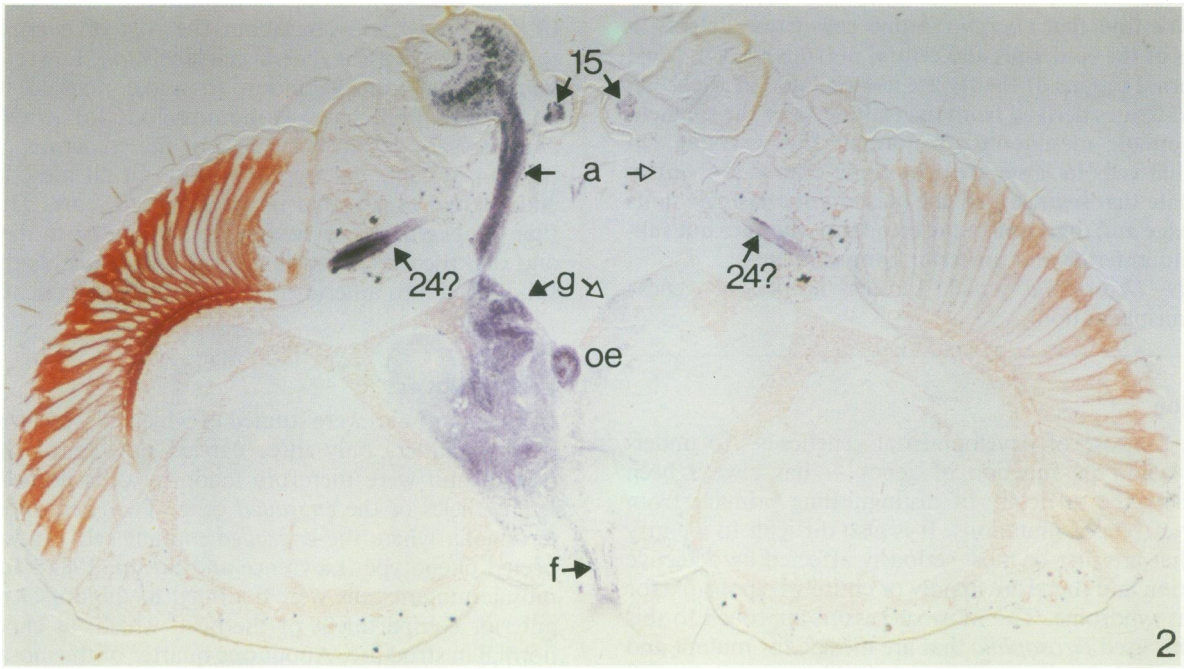
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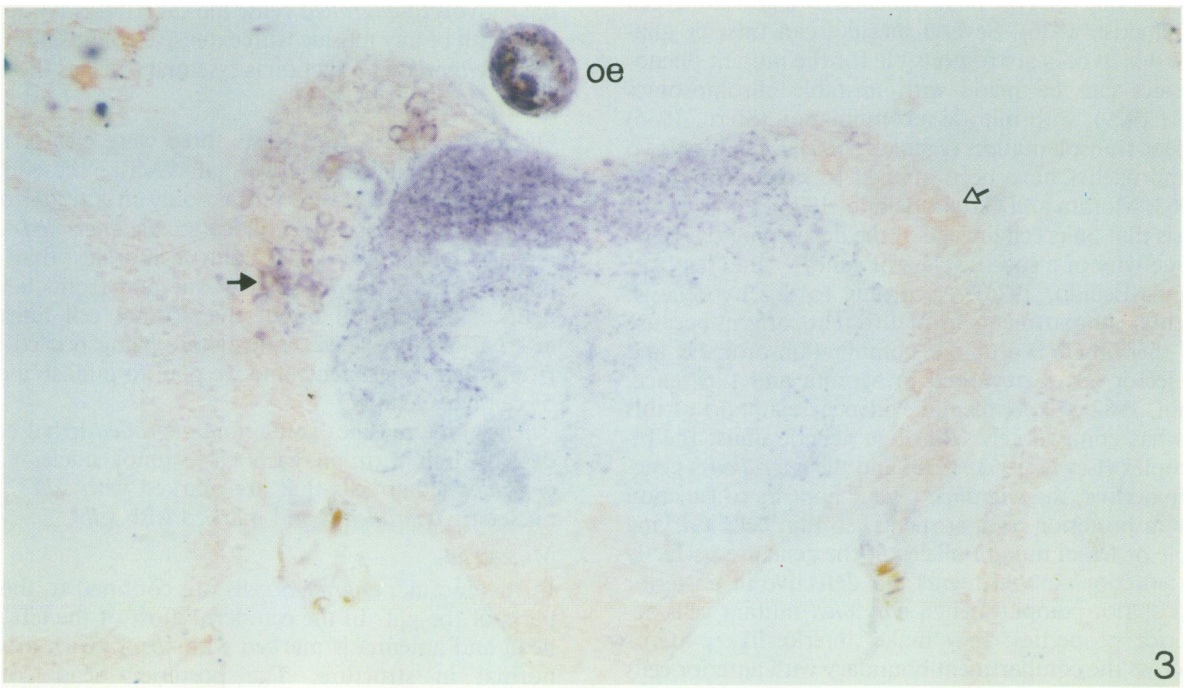
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(Morata and Lawrence, 1979) appears unmarked as is the maxillary palp and proboscis. Sections of the head show the brain is abnormal on the left side, especially dorsally where it is atrophied (Figure 1). More ventrally at the level of the antennal glomerulus, there are *engrailed* neurons but the structure is more symmetric, although the left antennal region is smaller than the right. There is extensive marking of cell bodies in the brain mainly on the left side, with *sdh*$^+$ fibres spreading contralaterally and posteriorly into the thorax (Figure 2). Control mosaics in the head are normal in structure even when much colonised by *sdh*$^+$ cells, so these effects are presumably due to the *engrailed* mutations. In the extreme ventral part of the brain the structure is more symmetric even though parts of the suboesophageal ganglia are extensively marked and appear normal in structure (Figure 3). These observations on the brain suggest that some parts are defective when they are *engrailed*-lethal while other parts are normal.

The ectodermal parts of the oesophagus and salivary gland are derived from the host: however the muscular wall of the oesophagus, which presumably derives from the splanchnic mesoderm (Robertson, 1936; Bodenstein, 1950) is *engrailed*-lethal, and is normal in structure. Muscle 16, which is just dorsal to the oesophagus and probably also derives from the splanchnic mesoderm (Miller, 1950; Lawrence and Johnston, unpublished results) is also *sdh*$^+$ and normal. The fat body, which is also presumably mesodermal (Poulson, 1950) is *sdh*^{8} in this mosaic.

Many of the somatic muscles of the head are *sdh*$^+$ and normal, and some are marked bilaterally (Table I).

The anterior midgut, which is endodermal and derives from a primordium in the head region (Poulson, 1950) is largely *sdh*$^+$ and therefore is made up of *engrailed*-lethal cells; it is normal in structure (Figure 4). The muscular lining of the anterior midgut is *sdh*^{8}.

Mosaic 43

In this fly the *sdh*$^+$ cells are largely confined to the thorax and anterior abdomen. In the cuticle the *engrailed*-lethal cells are found in the left anterior wing and in the left leg of the second thoracic segment (T2). This leg was torn off and was rescued from the pupal case, it is marked in both anterior and posterior compartments, and is similar to the grossly deformed legs that are made when large regions of posterior compartment are colonised by *engrailed*-lethal cells (Kornberg, 1981; Lawrence and Struhl, 1982). Although the left haltere is present the left leg of T3 is completely missing and the thorax is correspondingly misshapen. The central nervous system (CNS) is extensively marked on the left side, *sdh*$^+$ fibres extend contralaterally and to the brain. The CNS is asymmetric and malformed although how much of this is due to indirect effects because of the loss of the T3 leg is unclear.

Mesodermal organs are extensively marked. The fat body stains in part of the head, all the thorax and abdomen. Many somatic muscles are *sdh*$^+$ on the left side, where all muscles belonging to the dorsal and ventral parts of T2, T3, A1 (first abdominal segment) and A2 are *sdh*$^+$ and are normal in pattern – except where their attachment sites are missing or deformed, as in ventral parts of T2 and T3 (Figure 5). The most anterior cells of the heart are *engrailed*-lethal, as is the scutellar pulsatile organ on the left side. The splanchnic mesoderm that forms the muscles around part of the midgut in the abdomen is *sdh*$^+$ and normal in structure. The endodermal midgut is derived from the host.

In summary, this mosaic confirms the results of mosaic 54; when *sdh*$^+$ and *engrailed*-lethal all ectodermal organs are defective in parts, while all mesodermal organs are normal.

Mosaic 17

This mosaic has considerable *engrailed* territory which is largely internal and extends from T3 in the thorax to the terminalia. In the cuticle it is only marked in the male genitalia and analia and we could find no *sdh*$^+$ cell bodies in the CNS. This lack of coincident marking in the epidermis and CNS is unusual; in every case of control mosaics examined so far when the adult cuticle is *sdh*$^+$ so are corresponding parts of the CNS (n = 40). This might suggest a loss of the corresponding cells in the CNS of Mosaic 17, presumably because they were *engrailed*-lethal. In Mosaic 17 the internal organs of the male genitalia are extensively marked; both the proximal and distal parts of the ejaculatory ducts as well as the sperm pump are partly *sdh*$^+$. The accessory glands are unstained, but they do not always stain reliably in controls. The hindgut ectoderm and the malpighian tubules are derived from the host.

The mesoderm is extensively *sdh*$^+$. In the thorax the somatic muscles of T3 on the right side are *engrailed*-lethal. Although the staining is not ideal in the middle segments of the abdomen, there is no doubt that all the somatic muscles of the male abdomen from A1 – A6 as well as the genital and anal muscles are *sdh*$^+$. The muscles which attach to the sperm pump and enwrap the ejaculatory ducts are *engrailed*-lethal as is the splanchnic lining of the hindgut and parts of the midgut. The heart is almost entirely *sdh*$^+$ and the fat body is heavily stained throughout the thorax and abdomen (Figure 6).

The *sdh*$^+$ territories in these three mosaics add up to the majority of all the internal organs. The only important exceptions are the posterior midgut, hindgut and malpighian tubules.

Fig. 1. Section of dorsal part of head of Mosaic 54. *engrailed*-lethal *sdh*$^+$ territories (filled arrows) are pale blue, *engrailed*$^+$ *sdh*^{8} territories (open arrows) pale rose. Although the section is well oriented the brain is asymmetric with the left *sdh*$^+$ side being atrophied especially in the neuropil (*n*). The left eye is darker than the right because it is *cinnabar*$^+$, while the right is *cinnabar*^{1}. The left occipital cuticle (*o*) is darker because it is *yellow*$^+$ while the right is *yellow*. Muscle 1 (*m*) is *sdh*$^+$ on the left and not on the right. Araldite section, head stained en bloc for succinate dehydrogenase, counterstained with eye pigment. The host genotype was *M(3)w*$^+$ */M(3)w*^{124}.

Fig. 2. Section of ventral part of head of Mosaic 54. The cuticle, eye and antenna of the left side are *engrailed*-lethal and marked with *yellow*$^+$, *pawn*, *cinnabar*$^+$ and *sdh*$^+$. Muscles 15 and 24? contain *engrailed*-lethal nuclei on both sides, although they are paler and therefore of mixed origin on the right and the muscular coat of the oesophagus (*oe*) is also *sdh*$^+$. Note the brain is somewhat undeveloped on the left side (where the antennal nerve and glomerulus (*g*) are *sdh*$^+$) although the optic lobes are symmetric. *sdh*$^+$ fibres (*f*) extend posteriorly to the thorax. Muscle 24? does not correspond well with any of the muscles described in Miller (1950); the muscle illustrated may be connected to muscle 24 of the anterior thorax. The outer muscular layer of the oesophagus (*o*) is *sdh*$^+$.

Fig. 3. Section through suboesophageal ganglion of Mosaic 54. The left side of the brain is largely *engrailed*-lethal and *sdh*$^+$ and yet the brain is more symmetric.

Table I. Head musculature in Mosaic 54

Muscle	Left		Right	Comments
1	+		−	
2	−		(+)	Weak but definite staining
3	+		+	
4	+?		+?	Not identified with certainty
5	−		−	A clear group of muscles in the distal
6	−		−	proboscis which probably form
7	−		−	a separate compartment
8	−		−	(Vijayraghavan and Pinto, 1984)
9	+		+	
10		+		Medial
11	+		+	
12	+		+	
13	?		?	We did not distinguish this muscle
14	+		−	
15	+		−	
16		+		Medial and probably splanchnic
24?	+		+	We were uncertain about the
27?	+		+	naming of these muscles

The muscles are named after Miller, 1950. Some are entirely or partially *sdh*$^+$ and *engrailed*-lethal (+), others are entirely *sdh*^{8}, (−) being derived from the host.

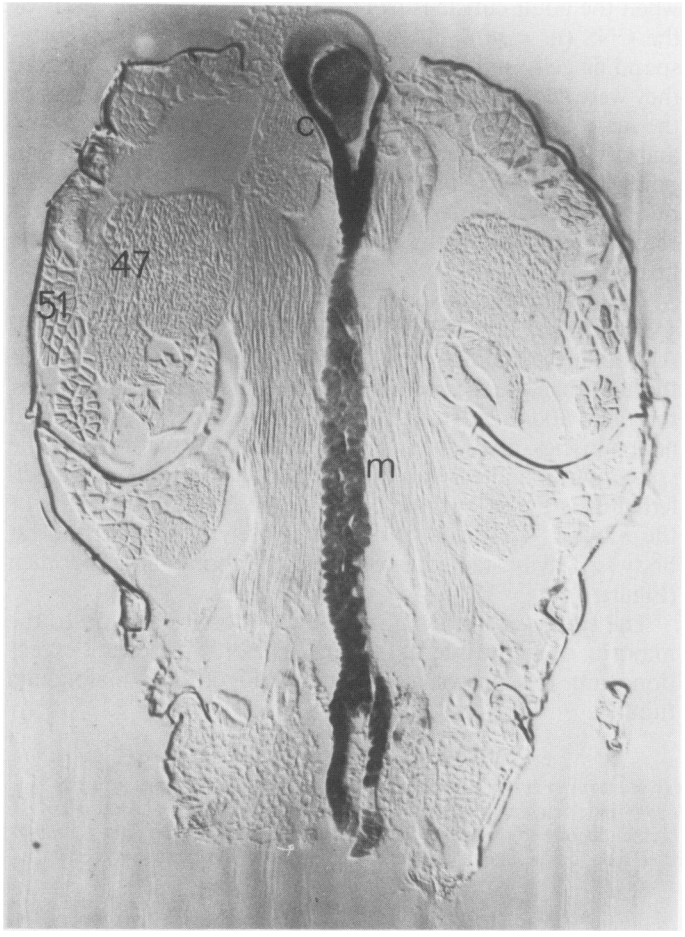

Fig. 4. Horizontal section of thorax of Mosaic 54 which passes through part of the cardium (*c*) and anterior midgut (*m*). Note that the endodermal parts are stained while the muscular lining of the gut (which is derived from the visceral mesoderm) and the somatic muscles are not (for numbered muscles, see Miller, 1950; Lawrence, 1982). The host genotype was *M(3)w*$^+$ */M(3)w*^{124} Nomarski interference optics. x110.

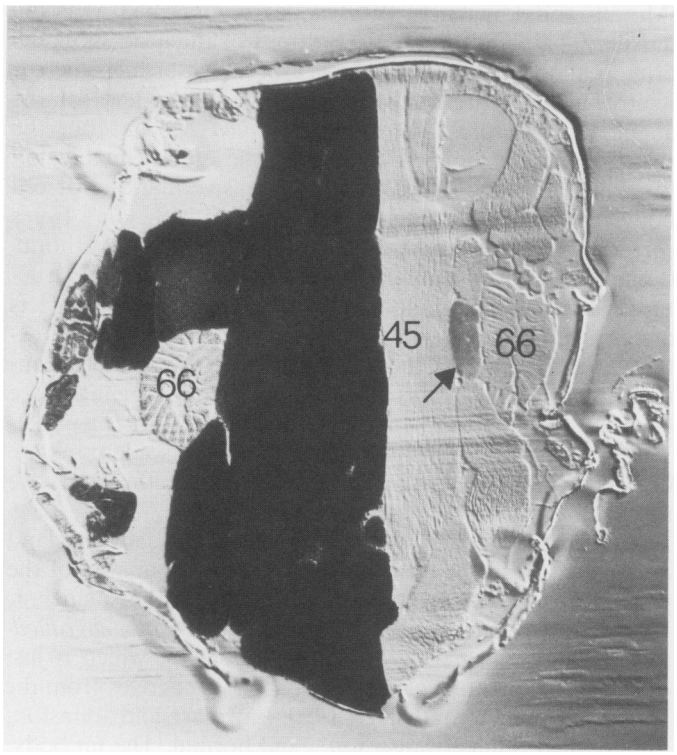

Fig. 5. Horizontal section of thorax of Mosaic 43. Stained muscles are on the left and belong to T2 (numbered). T1 muscles are *sdh*^{8}. On the right one fibre of muscle 48 is slightly stained (arrow). We believe that this is due to the damage connected with the loss of the T3 leg and the release of some myoblasts that have fused indiscriminately (see Lawrence and Brower, 1982). This has not happened in control mosaics. The host genotype was *M(3)w*$^+$. x110.

Discussion

The purpose of these experiments is to evaluate the requirement for the *engrailed*$^+$ gene in the internal organs of *Drosophila*. The method is to make mosaic flies, parts of which are made by cells homozygous for *engrailed*-lethal alleles. The *engrailed* territories in the three mosaics found were large and only partially overlapping and most of the internal organs were assessed. We find that *engrailed*-lethal cells do not develop normally in posterior parts of the epidermis and in some regions of the CNS, but in all the mesodermal and endodermal organs examined *engrailed*-lethal cells form normal patterns.

Although this conclusion is based upon adults, it almost certainly applies to larvae as well. Such extensive colonisation by *engrailed*-lethal cells of the mesoderm of the adults could not have occurred without corresponding colonisation of the larval mesoderm. In Mosaic 17 for example all the somatic and much of the splanchnic mesoderm in the entire abdomen was *engrailed*-lethal; unless the larval mesoderm comes from a different part of the blastoderm − which seems most unlikely − the larval mesoderm of much of the abdomen must have been *engrailed*-lethal as well. Therefore the survival of this individual to adulthood is good evidence for lack of significant effect of *engrailed*-lethal in the mesoderm (confirming an earlier study that was restricted to the adult thoracic muscles, Lawrence, 1982). In the case of Mosaic 54 the anterior midgut of the adult, which derives from the midgut of the larva (Poulson, 1950) was almost entirely *engrailed*, so a general lack of effect of *engrailed*-lethal in the endoderm can also be presumed.

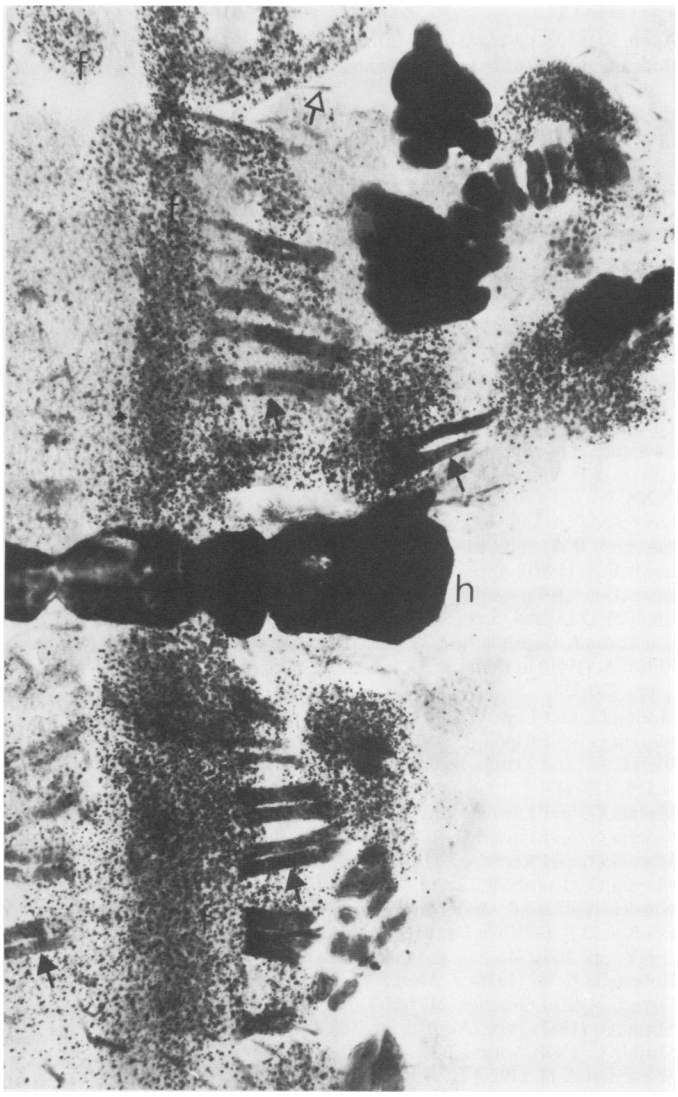

Fig. 6. Whole mount of part of abdomen of Mosaic 17. The second and third abdominal tergites (A2 and A3) are displayed. The heart (*h*) and dorsal somatic muscles (filled arrows) are *sdh*$^+$ as is the fat body (f). Note the small *Ki Sb*^{63b} *M(3)w*^{124}/+ bristles of the host (open arrow) which are *engrailed*$^+$.

These findings do not rule out all function of the *engrailed*$^+$ gene in the internal organs since slight effects of the mutation might not be noticed. More significantly, there might be other functions in the *engrailed*$^+$ gene that are not affected by the lethal mutations. There is already evidence that *engrailed*-lethal mutations may not be null alleles: the pattern in the cuticle made by cells homozygous for these alleles appear normal in the posterior compartment of the antenna (Lawrence and Struhl, 1982) yet some viable *engrailed* combinations (*en*^{1}/*en*^{1}, *en*^{Cl}/*en*^{1}, Morata *et al.*, 1983; *en*^{1}/*en*^{CX1}, Holmgren, unpublished) do show an *engrailed* phenotype in the posterior antenna.

These caveats aside, it is worth discussing the implications of our main conclusion for segmentation. Segments are fundamentally units of cell lineage (Lawrence, 1973, 1981a) and in the epidermis each segment is subdivided into an anterior and a posterior compartment (Garcia-Bellido *et al.*, 1973), the *engrailed*$^+$ gene being required only in the cells of posterior compartments (Morata and Lawrence, 1975). The effects of *engrailed*-lethal on some − but not all − parts of the CNS is consistent with the reasonable expectation that the CNS is structured like the epidermis with alternating stripes of anterior and posterior cells.

Table II.

Eggs injected	Eggs hatched	No. of adults			
		sdh$^+$*M*$^+$	*sdh*$^+$*M*	*sdh*^{8}*M*$^+$	*sdh*^{8}*M*
6288	1734	258 **(13)**	297 **(16)**	241 **(12)**	168 **(13)**

The numbers of mosaics are indicated in bold figures. Only 28% of injected eggs hatched and of these only 56% reached adulthood; the overall mosaic frequency was 54/964 or 6%. In the second chromosome hosts can be either *CyO/sdh*^{8} (*sdh*$^+$) or *sdh*^{8} while in the third chromosome they can be *Ki Sb*^{63b}*M(3)w*/+ (*M*) or *TM2*/+ (*M*$^+$).

There is evidence that compartition of the mesoderm is different from that in the ectoderm: cell lineage studies on the somatic mesoderm of the adult thorax (Lawrence, 1982) and of the abdomen (Lawrence and Johnston, 1982) did not reveal any antero-posterior subsegments, although the thorax is divided into segmental lineage compartments. This, with our present results on *engrailed*, can be interpreted as follows: we postulate that the somatic mesoderm is divided into segmental units by cell lineage in a simpler way than the ectoderm. We believe that the segments of the mesoderm are not subdivided into anterior and posterior subsegments and therefore there cannot be any function for the *engrailed*$^+$ gene in the mesoderm. There may be other selector genes which are active only in posterior compartments and, with *engrailed*$^+$, make up the genetic address 'posterior' (Garcia-Bellido, 1975; Garcia-Bellido *et al.*, 1979; Lawrence and Struhl, 1982; Brower, 1984). These genes would also not be expected to function in the mesoderm.

This hypothesis may help explain why the patterns of expression of the *Ubx*$^+$ gene in the mesoderm and ectoderm are out of register (Akam, 1983). The *Ubx*$^+$ gene is a selector gene that is partly responsible for segment diversification (Lewis, 1978) and in the epidermis *Ubx*$^+$ functions in a defined area that is limited, not by a segmental border, but by an antero-posterior boundary (e.g., Morata and Kerridge, 1981; Struhl, 1984). Yet, in the mesoderm, if (as we suspect) antero-posterior boundaries do not exist, the expression of *Ubx*$^+$ cannot be limited by them and the gene is therefore more likely to be expressed in whole segmental units in the muscles and other mesodermal organs.

Our hypothesis also has implications for segmentation in the early embryo. It predicts either that *engrailed*$^+$ transcription [which we imagine would be in the form of evenly spaced stripes of one half one segment in width (Kornberg, 1981; Lawrence, 1981a)] be confined to the ectoderm or that it be only transiently expressed in presumptive or patent mesoderm.

Materials and methods

Rationale

When compared with other methods of making genetic mosacis, nuclear transplantation has both advantages and disadvantages. On the credit side there is the complex flexibility it gives: almost any genetic combination can be made; for example, nuclei carrying *yellow*$^+$ (on the first chromosome) lethal mutations in the bithorax complex (on the third chromosome) and marked with *sdh*$^+$ (on the second) could be transplanted into *yellow sdh* hosts which are also heterozygous for any convenient *Minute*. In this case, as in the experiments reported in this paper, the donor-derived cells are blue (when stained for succinate dehydrogenase) and are easily seen against the white *sdh* cells which are derived from the host. This allows identification of mosaics in all

tissues including the CNS, something which we failed to do after inducing *sdh* clones (white) in the *sdh*$^+$ (blue) CNS by mitotic recombination (Lawrence and Johnston, unpublished results). The mosaics are also induced early in development, before blastoderm. This can be an advantage for some experiments.

On the debit side, the method is much more laborious than mitotic recombination or ring loss. Also, because the size of the patches varies considerably we imagine that donor nuclei are incorporated into the cleaving population of host nuclei at diverse times. This makes analysis of cell lineage tricky as patches will often derive from groups rather than single blastoderm cells. Further, since donor nuclei must be produced as the result of a cross between two different parent genotypes, it can be difficult to distinguish the mosaics of interest. This method has been developed independently by Fischbach and Technau (1984).

Genetics

For nuclear transplantation the following cross was used to make host eggs:

$$\sigma y;\ cn\ sdh^8/CyO;\ Ki\ Sb^{63b}\ M(3)w^{124}/TM2\ x\ \varphi\ y;\ cn\ sdh^8$$

Of the F1, half are useful for internal mosaics (they are homozygous for the temperature-sensitive and cell autonomous label *sdh*^{8}, Lawrence, 1981b) and, of these, half are *Minute*, giving slow growing hosts and an advantage to donor cells (Morata and Ripoll, 1975). The original *sdh*^{8} stock was male sterile (Lawrence, 1981b), but this turned out to be due to another mutation which we removed from the chromosome. The current *sdh*^{8} stock is fully viable and fertile at 20°C, but lethal at 25°C.

For donors the cross was *stw pwn en*^{lO}/*CyO* x *stw pwn en*^{lK}/*CyO*. Of the F1 only one quarter of the eggs are *engrailed*-lethal (Nüsslein-Volhard and Wieschaus, 1980), half are wild-type in phenotype and one quarter are *CyO/CyO* (two mosaics presumed to be partly of this genotype were found, the donor-derived tissues appeared normal except for the bristles which were stubby). All the donor nuclei are *sdh*$^+$ and *Minute*$^+$. Unfortunately, *engrailed* mosaics can be objectively recognised only when part of the cuticle is derived from donor nuclei; in those cases the bristles are not wild-type as in controls, but are marked with *straw* and *pawn* (Garcia-Bellido and Dapena, 1974). Homozygous *pawn* flies are poorly viable but the adults that do emerge appear normal, apart from bristle phenotype. We therefore assume that *pawn* is a gratuitous marker. For genetic nomenclature see Lindsley and Grell (1968).

Transplantation

Methods were largely as described elsewhere (Zalokar, 1971). Here we used needles of ~15 μm in diameter that were sharpened on a microforge. Sufficient donor nuclei for several hosts were sucked into the needle and injected into chosen locations in the host egg. Mosaic frequencies varied considerably and we now suspect that nuclei were sometimes sucked in too rapidly and killed. Host eggs were 40 ± 15 min at 20°C and donors were chosen from eggs aged 4 h ± 30 min. Donors were used when they were in late blastoderm, just before cellularisation.

For numbers, see Table II. The survival rate was low and we believe this was partly due to the weakness of the host eggs, which were derived from *sdh*^{8} mothers.

Mosaics

The *yellow sdh*$^+$ flies were examined under the dissecting microscope for *yellow*$^+$ patches in the cuticle. Mosaics were mounted on slides.

The *sdh*^{8} flies were screened under the microscope for *yellow*$^+$ patches of cuticle and then dissected; the heads were opened by cutting off the tip of the proboscis, the thoraces by removing a small portion of the dorsal notum, the legs by cutting at the proximal femur and the abdomens by making the lateral cut with fine scissors. These parts were heated in *Drosophila* Ringers solution at 52°C for 15 min and stained for succinate dehydrogenase for 2 days (Lawrence, 1981b). They were then fixed in 3 parts ethanol:1 part acetic acid for 1 h, cleared in isopropanol and examined. Mosaics were kept; the abdomen was dissected and mounted on a slide in Euparal, and the thorax and head were impregnated with agar and then taken through acetone to araldite and sectioned at 5–10 μm. There was sometimes slight staining in *sdh*^{8} controls but, with experience, this could be distinguished from *sdh*$^+$ patches in mosaics which were very dark and had sharp boundaries. The fat body was the only exception; it was difficult to score individual cells and we had to be content with allocating large areas of this tissue. There may be some non-autonomy of *sdh* staining in the fat body.

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