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Genetics

engrailed: A gene controlling compartment and segment formation in *Drosophila*

(embryogenesis/lethal mutation)

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ABSTRACT **A total of 58 mutations at the *engrailed* locus were isolated. Analysis suggests that this genetic locus is necessary for survival but required only in the cells of the posterior compartments. Inactivation of the *engrailed* locus renders the animal incapable of maintaining the separation between the groups of cells that constitute either the compartments that subdivide each segment or the individual segments themselves.**

During development, the prospective fate of cells in successive cell generations becomes more and more limited. Nowhere is this seen more vividly than in the development of the insect integument. After the formation of the cellular blastoderm during early embryogenesis, segmental borders confine the epidermal cells of *Oncopeltus* to grow only within their respective segments (1). In *Drosophila*, subdivision of the segments themselves (into areas termed compartments) similarly confines epidermal cells to grow within the restricted areas defined by the compartmental borders (2, 3). With the goal of a molecular understanding of these fascinating observations, this paper describes a genetic and developmental study of mutations of *Drosophila melanogaster* that impair the border-forming capabilities of epidermal cells. This study focused on a set of developmentally deficient mutants at the *engrailed* locus and found that the ability to form and respect these borders is essential to the viability of the organism.

engrailed is an autosomal locus first identified by a spontaneous lesion, en^1 (4). A recessive and homozygous viable allele, en^1 disrupts the normal development of the thoracic segments, causing in the adult fly a clefted scutellum, duplicated sex combs, additional bristles in the posterior regions of the legs of all three thoracic segments, and abnormal vein and bristle development in the posterior wing blade (5–7) (Fig. 1). In a detailed study of the effects of en^1, Lawrence and Morata found that the *engrailed* locus was required for the maintenance of the border that subdivides the wing blade into anterior and posterior compartments (8). This provided a genetic basis to the compartment hypothesis proposed by Garcia-Bellido (9).

In this study, the phenotype of en^1 was compared with that of other mutations at the same locus to determine whether this locus plays a role in the development of other body segments.

MATERIALS AND METHODS

All crosses were carried out in standard culture medium at 25°C. Descriptions of the chromosomes and mutants used can be found in Lindsley and Grell (10) or as indicated.

Isolation of *engrailed* Mutants. Mutagenesis to produce lesions at the *engrailed* locus was by the administration of ethyl methanesulfonate (EtMes) using the method of Lewis and Bacher (11), by x-ray or γ-ray irradiation of young adult males with 4000 rads (1 rad = 0.01 gray), or by the culture of second instar larvae in standard medium containing 0.1% formaldehyde (12). *engrailed* mutations were detected in the F1 generation by their failure to complement en^1 (all formaldehyde-induced alleles, $en^{LA14,15,16,17,18}$) and in the F2 generation by their failure to complement the phenotype of en^1 (en^{C2}, $en^{SFX30\text{-}37}$, $en^{SF\gamma\text{-}1}$), the lethality of en^{C2} ($en^{LA3,4,5,7,9,10,11,12,13}$), or the lethality of en^{LA4} ($en^{SFX1,12,22,24,26,29}$).

Clonal Analysis. Mitotic recombination was induced by x-irradiation (100 rads) at either 72 ± 2 hr, 96 ± 2 hr, or 120 ± 2 hr after egg laying. Adults of the genotype *stw pwn en/ M(2)c*^{33a}; *mwh*/+ were collected and preserved in isopropanol (13). The *Minute* mutation was present in trans to the *engrailed* mutation to increase the size of the *stw pwn en* M^+ clones (14).

Somatic clones in the wing blades were detected by examination with a compound microscope. The wings had been removed from carcasses and mounted in Euparol.

Analysis of Embryos. Embryos obtained from the mating of flies of the genotype *en*/+ were collected, dechorionated in 6% hypochlorite, and either mounted directly in Hoyer's aqueous mountant or fixed in glutaraldehyde-saturated heptane by the method of Zalokar (15) and mounted on microscope slides in Faure's aqueous mountant according to the method of Nusslein-Volhard (16). Specimens were examined by using phase-contrast or Nomarski optics.

RESULTS

Isolation of New *engrailed* Mutations. Fifty-eight independent mutations at the *engrailed* locus were identified among the progeny of flies that had been subjected to mutagenesis with chemical agents or radiation (Table 1). The evidence for mutation at the *engrailed* locus is as follows: (*i*) All recovered mutations failed to give complete complementation of en^1. (*ii*) en^1 and a representative new point mutant, en^{LA4}, were mapped relative to loci neighboring en^1—these two alleles, en^1 and en^{LA4}, mapped to an identical meiotic location, 64.0, on the second chromosome.* (*iii*) Of six mutations recovered with chromosomal rearrangements (these include one inversion, two reciprocal translocations, two insertional translocations, and one deficiency), all had chromosome breaks at 48A, and 48A was the only site of breakage common to these six strains. 48A is a cytological location consistent with the suggested meiotic location of the point mutants. Russell and Eberlein (18) have isolated two *engrailed* deficiencies that have chromosomal deletions consistent with this localization.) (*iv*) The frequency of

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Abbreviation: EtMes, ethyl methanesulfonate.

* The crossover point of meiotic recombinants between *cn* and *vg* was located relative to en^1 (334 recombinants) and en^{LA4} (124 recombinants). Meiotic locations for *cn* and *vg* were 57.5 and 67.0, respectively (10).

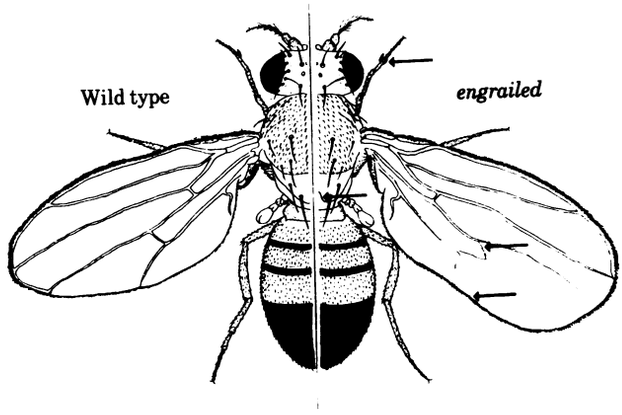

FIG. 1. Comparison of a wild-type and an en^1/en^1 adult male. Arrows indicate *engrailed* transformation of, from top to bottom, foreleg sex combs, scutellum, posterior wing veins, and posterior wing margin bristles.

appearance of *engrailed* lesions in the F2 generation was 0.0018 (5/2730) after EtMes mutagenesis and 0.0010 (18/17,160) after x-ray mutagenesis. This suggests that the extent of the *engrailed* locus measured in this study is comparable with that observed for an "average" locus and that only a single lesion is required to produce the engrailed phenotype.

All chromosomes bearing mutagen-induced *engrailed* mutations were lethal in the homozygous state. With only two exceptions [en^{LA3} (T(2;3) 48A;96C), and en^{LA9}], pairwise crosses between representative alleles of the EtMes-, x-ray-, γ-ray-, and formaldehyde-induced mutants, suggested that all transheterozygous combinations of lethal alleles were also lethal.† Complementation with en^1 divided the *engrailed*-lethal mutations into two groups, neither of which produced flies with engrailed transformations significantly more extreme in phenotype than en^1/en^1. One group, representing the majority of recovered mutants and consisting of all lesions with normal chromosome morphology, produced adult flies with a slight wing-blade transformation that overlapped wild type (see Fig. 2). Head, leg, thoracic, and abdominal morphology were normal. In contrast, the mutations with visible chromosome breakpoints produced adult flies with extreme *engrailed* transformations comparable to those seen in en^1/en^1 flies. One mutant, en^{C2}, had malformations not characteristic of en^1. The en^{C2}/en^1 individuals had wings and scutellum comparable to en^1/en^1 but in addition had duplicated antennal segments and fused leg segments. However, these abnormalities may arise from the effects of other lesions on the multiply broken en^{C2} chromosome, at least one of which is a cell-lethal mutation (data not given). The range in phenotype observed in the development of the wing blade by representatives of these mutant combinations is shown in Fig. 2.

Clonal Analysis. To determine whether the organismal lethal *engrailed* alleles en^{LA4}, en^{LA7}, and en^{LA10} are required for the viability of individual cells and investigate the effects of these mutations on the developmental fate of cells, mitotic recombination clones were induced. The cross performed in these experiments produces predominantly two types of clones. After somatic crossing-over in the proximal region of the right arm of the second chromosome, *Minute*$^+$ cells homozygous for *straw* (10), *pawn* (12), and *engrailed* are produced. *straw* and *pawn* are recessive alleles that transform the bristles and hairs of the adult cuticle to abnormal color and shape. Located proximally to *engrailed*, they gratuitously mark the clones of homozygous *engrailed* cells. After somatic crossing-over in the left arm of the third chromosome, cells homozygous for the mutation *multiple wing hair* are produced. These two sets of clones permit direct comparison with mitotic clones that are composed of en^+ or en^- cells. (As the results obtained with all three *engrailed*-lethal mutations were similar, only the behavior of wing-blade cells homozygous for en^{LA4} will be summarized here.)

Table 1. *engrailed* mutants

Mutant	Mutagen	Chromosome morphology
en^1	Spontaneous	Normal
en^{C2}	EtMes	In(2R) 47A; 48A
en^{LA3}	EtMes	T(2; 3) 48A; 96C
$en^{LA4,5,7,9,10,11,12,13,14,15,16,17,18}$	EtMes	Normal
$en^{SFX1,12,22,24,26,29,30,33,34,35,36,38,39,40}$	x-Ray	Normal
en^{SFX31}	x-Ray	Df(2) 48A; 48B5
en^{SFX37}	x-Ray	T(2; 3) 46C; 48A; 81F
en^{SFX32}	x-Ray	T(2; Y) 48A
en^{SFX24}	x-Ray	T(2; 3) 48A; 79F
$en^{SF\gamma1}$	γ-Ray	Normal
$en^{SFH1,2,3,4,5,6,7,8,9,10,11,12,13,14}$*	HCOH	Normal
H144-B107†	x-Ray; synthetic	Df(2) 47E; 48A

* Because of the probable recovery of mutant clusters that do not represent separate mutagenic events, only single representatives from 14 separate mutageneses are included here. In total, 80 *engrailed* mutants were examined and all had normal chromosome morphology.

† This deficiency was generated from the indicated strains obtained from the collection of Lindsley et al. (17).

Larvae were irradiated to induce mitotic recombination; as expected, clone frequency increased and clone size decreased with increasing developmental age for both *stw pwn* $en^{LA4}/$*stw pwn* en^{LA4} clones and for *mwh/mwh* clones. Clone appearance as a function of genotype, location, and time of induction is summarized in Table 2. The mitotic recombination clones induced during mid to late third instar (120 hr after egg laying) were found with equal frequency in the anterior and posterior wing compartments. This suggests that, at this time of development, the numbers of cells in the anterior and posterior compartments are equal and that the viability of cells homozygous for the lethal

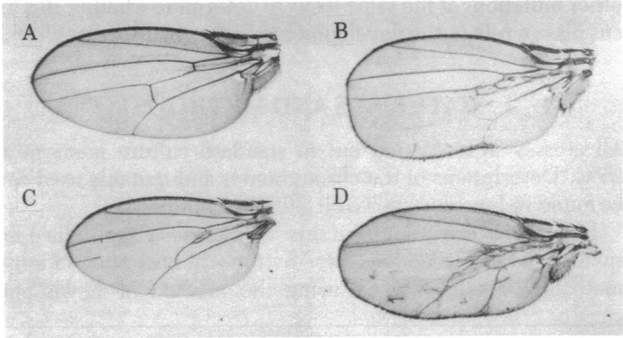

FIG. 2. Comparison of wing blades. (*A*) Wild-type, (*B*) en^1/en^1, (*C*) en^1/en^{LA4}, (*D*) en^1/en^{SFX32}. (Bright-field optics.)

† The en^{LA3} chromosome was homozygous lethal but heterozygous viable with all other *engrailed* mutations; it is classified as an *engrailed* mutation because of its cytological breakpoint at 48A and its failure to fully complement the morphological phenotype of other *engrailed* mutations. The mutation en^{LA9} complemented the lethality of en^{C2} but failed to complement all other *engrailed* mutations. Although the genotype en^{C2}/en^{LA9} had severely reduced fertility and reduced viability, the phenotypic transformation was less severe than en^1/en^1.

Table 2. Mitotic recombination clones in wing blade

Time of induction, hr	Flies, no.	Clones, no. *stw pwn* en^{LA4} Anterior	*stw pwn* en^{LA4} Posterior	*mwh* Anterior	*mwh* Posterior
120 ± 2	160	53	56	91	106
96 ± 2	680	206	128	281	207
72 ± 2	1680	88	38	61	45

mutation en^{LA4} is unimpaired. (The greater relative recovery of *mwh/mwh* recombinant clones can be attributed to the greater relative distance of this locus from the centromere.) Earlier irradiations produced larger clones, again without evidence of decreased viability of cells homozygous for en^{LA4} in the anterior or posterior compartment. As observed (3), frequency of clone induction was greater in the anterior compartment, suggesting the larger relative target size of the anterior compartment at early times.

The phenotype of the en^{LA4}/en^{LA4} clones differed dramatically according to the compartmental origin. Anterior clones were normal in phenotype, producing veins and wing margin bristles and hairs in normal patterns. Twenty-four large clones covering more than 20% of the wing surface were found to meet the anterior-posterior compartment border without crossing and so define the position of this border. The border defined by these clones was indistinguishable from that defined in en^+ flies (2, 3). In contrast, clones of en^{LA4}/en^{LA4} cells in the posterior compartment failed to produce normal patterns; those that formed the posterior margin of the wing blade produced socketed bristles (normally found only on the anterior wing margin), even if induced late in development and generating but a single bristle. Vein and bristle patterns were in every case abnormal. Seven large clones were found that failed to define the anterior-posterior compartment border and crossed into anterior territory. These properties of en^{LA4} clones are similar to the behavior of clones homozygous for the viable allele, en^1 (7, 8).

Lethality of *engrailed* Mutations. The inability of homozygous *engrailed* mutants to produce viable adult flies is due to abnormal embryonic development. Development proceeded until the late embryonic period and was arrested after secretion of the larval cuticle. Wild-type embryos at this stage have nearly completed larval differentiation; low-power microscopy shows segmentally distributed tracheal branches, chitinized mouth parts, posterior spiracles, opalescent malpighian tubules, and numerous cuticular processes that include, on the ventral surface, regular rows of small chitinized hooks called denticle belts. These denticle belts clearly mark each of the thoracic and abdominal segments (Fig. 3) and so indicate the extent of segmental differentiation.

Several hundred preparations of embryos carrying various *engrailed* mutations, homozygous or trans-heterozygous, were examined. Characteristic defects were evident in the *engrailed* embryos: The mouth parts were poorly formed, the tracheal trunks were disrupted and fragmented, the posterior spiracles were nonextruded, the malpighian tubules were misshapen, and the denticle belts were disoriented and abnormally placed. However, the severity of these transformations varied considerably both among individuals of a given genotype and between different genotypes. Among the *engrailed* point mutants, the en^{LA4} embryos showed the most extreme transformations. With respect to the pattern of denticle belts that mark each segment, normally regular rows were reduced in number and disoriented (see Fig. 3). The denticle belts frequently appeared fused, and the extent of fusion varied between individuals: In some, a single pair of adjacent bands was partially fused, but in others, as many as eight bands fused in a pairwise fashion. Heterozygotes of the synthetic deficiency H144-B107 and en^{C2} produced the most extreme disruptions of denticle-belt pattern, some having as many as four belts of denticle teeth fusing into a single array.

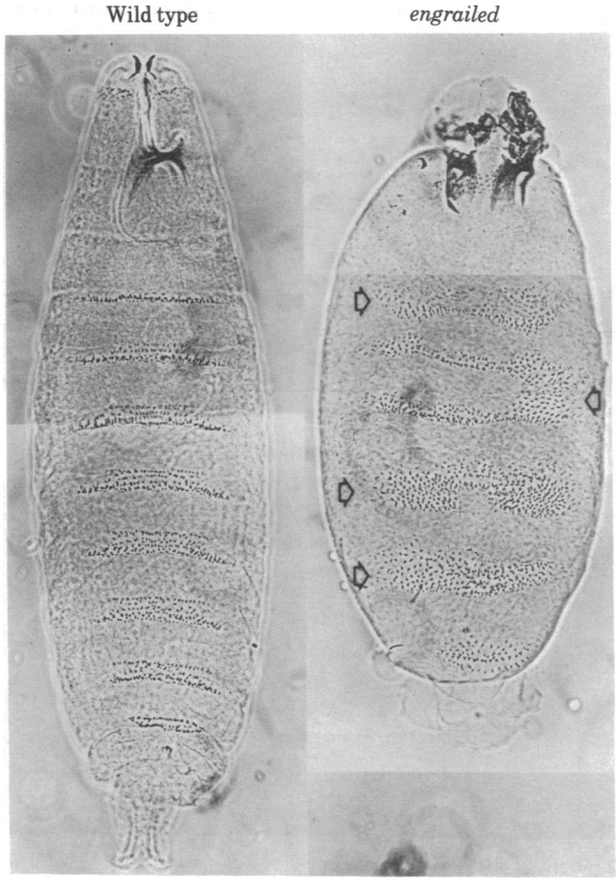

FIG. 3. Comparison between wild-type and en^{LA4}/en^{LA4} 18-hr embryos. Dechorionated embryos were removed from their embryonic case with fine tungsten needles, mounted in Hoyer's aqueous mountant, and incubated at 45°C for ≈12 hr. Arrows indicate regions of denticle belt fusion. Phase-contrast optics.

Several interesting features of the denticle-belt transformation are noteworthy. First, the transformation can be attributed directly to the mutations in the *engrailed* locus. These abnormalities were evident in embryos homozygous for all of the *engrailed* point mutants and most of the trans-heterozygous combinations examined. Given that different *engrailed* mutations were induced in several different genetic backgrounds, isolated after a variety of screening procedures at a relatively high frequency, and cleansed of linked mutations by several recombinations, it is unlikely that a closely linked mutation associated with all the alleles described in this study is responsible for the embryonic transformation. Second, the denticle-belt fusion reflects a disruption and fusion of the basic segmental organization of the insect. At the late gastrula stage before the larval cuticle has been secreted (dorsal closure, ≈9 hr after fertilization), fused abdominal segments are evident (Fig. 4), a phenotype that closely parallels the denticle-belt-fusion pattern seen in older embryos of the same genotype. Third, the denticle belts, in addition to their disoriented pattern, were abnormal in two other respects. The denticle rows of the mutant embryos were laterally shortened relative to the longer rows of wild-type embryos, and, frequently, an entire belt or the half belt from one

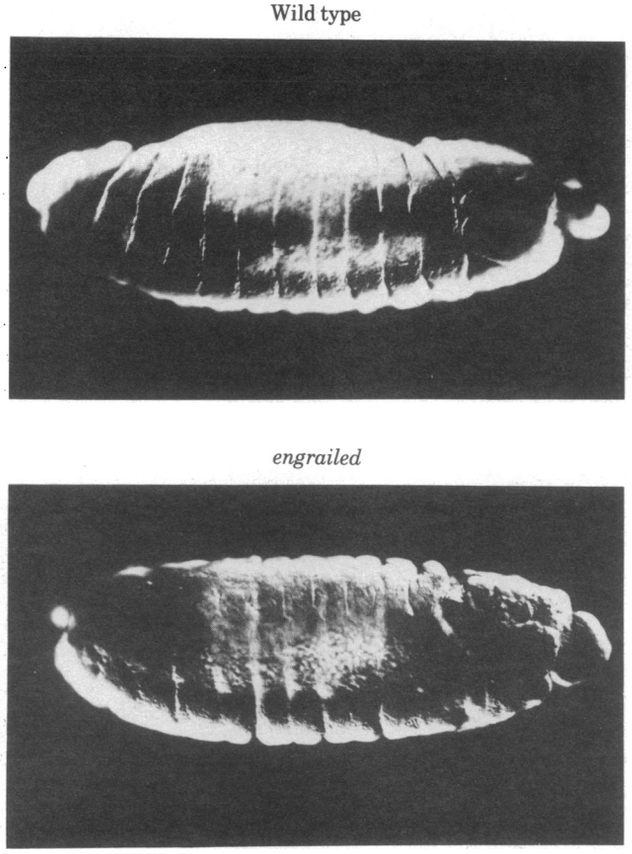

FIG. 4. Comparison between wild-type and en^{LA7}/en^{LA7} 8- to 10-hr embryos. Embryos were fixed by the method of Zalokar (13), examined by using Nomarski optics. In the *engrailed* embryo, fusion of abdominal segments 7 and 6, 5 and 4, 3 and 2, and 1 and the third thoracic segment has occurred. The proportion of embryos from the mating of *en*/+ parents with fused segments (12/83; 15%) was less than the Mendelian expectation (25%). This may have resulted from the recognition of only the more severely fused embryos or variability in the time of fusion. Fused embryonic segments were not observed among the progeny of wild-type parents.

side was absent but without indication of any segment fusion. Fourth, the pattern of denticle-belt or segmental fusion was regular and dependent on genotype. The pairwise fusion of adjacent abdominal segments followed either of two patterns: (*i*) abdominal segments 7 with 6, 5 with 4, 3 with 2, and 1 with the metathorax (characteristic of en^{LA4} embryos; see Fig. 3) or (*ii*) abdominal segments 8 with 7, 6 with 5, 4 with 3, and 2 with 1 [characteristic of en^{C2}/Df(2) 47E;48A embryos, data not shown].

To determine the earliest stage of embryogenesis at which *engrailed*-associated abnormalities become visible, gastrulating embryos were examined. Abnormalities of segmentation are recognizable at germ-band extension (≈6 hr after fertilization), when segment boundaries are morphologically discernible. However, all progeny of crosses between pairs of *en*/+ parents appeared normal at this stage, although, by the time of dorsal closure, fused abdominal segments are evident (see Fig. 4). This delayed effect on segmentation may indicate a role for the *engrailed* locus in the maintenance but not in the initial definition of segmental borders or it may indicate a leaky phenotype of the mutations studied here. Clarification will require the further analysis of cell-viable deficiencies.

The cause for the death of *engrailed* embryos is not known, but one observation suggests that the cuticular defects are not the primary cause. en^{LA9} embryos, homozygous or trans-heterozygous with other *engrailed*-lethal alleles, die late in embryogenesis and, although some exhibit abnormalities characteristic of the engrailed transformation, others differ little from the wild type.

DISCUSSION

The original spontaneous *engrailed* allele en^1 is unusual in several respects. The partial complementation of en^1/en^{lethal} combinations may represent the complementation of pseudoalleles within a complex locus, but the failure to recover additional en^1 alleles suggests that either the en^1 site is small and insensitive to the mutagens used in this study or it is insensitive to single-site lesions. Moreover, the difference in behavior of the allele, en^1, in heterozygous combinations with *engrailed* lesions that do or do not include chromosomal breaks suggests that en^1 may be sensitive to the position effects of some chromosomal rearrangements.

Despite the lethality of the *engrailed* mutations, clones of cells deficient for the locus can survive if surrounded by wild-type cells. Mitotic recombination generates clonal homozygosity in a heterozygous animal and so can be used to mark cells for the analysis of lethal mutations that are not cell lethal; the clonal patches produced provide a piecemeal description of mutant cell behavior in a wild-type background. The behavior of somatic clones of en^{LA4}/en^{LA4} in the wing-blade suggests that this mutation, although lethal to homozygous animals, is viable in cells and that at least in the posterior compartment, expression of the mutant phenotype is cell autonomous. Consistent with observations made with the mutation en^1 (7, 8), en^{LA4}/en^{LA4} cells develop normally in the anterior compartment but fail to produce normal posterior structures or to remain confined to the limits of the normal posterior compartment. Given the similarity of the phenotype to en^1, the conclusion seems warranted that the wild-type development of anterior en^{LA4}/en^{LA4} cells is due not to a lack of autonomous expression but to the lack of requirement for the *engrailed* locus in these cells. The abnormal posterior development is indicative of a requirement for the *engrailed* locus in posterior cells.

Considerable evidence has been presented for the anterior-posterior compartmental subdivision of segments other than the mesothorax. Included to date are the labial (19), eye-antennal (20), prothoracic (21, 22), and metathoracic (21, 22) segments, the genitalia (23), and the first abdominal segment (unpublished results). Do the engrailed-lethal mutations perturb the development of segments other than the mesothorax? Mitotic recombination experiments with the alleles en^{LA4} and en^{LA7} suggest that, at least in the prothoracic, metathoracic, and first abdominal segments, anterior *en/en* cells develop normally, whereas posterior cells do not (unpublished results). These analyses of the *engrailed* mutants, then, are consistent with the conclusion that the *engrailed* locus is required in and only in posterior cells and that it is involved in the anterior-posterior compartmental subdivision of these segments.

Previously, there was no evidence for or against the existence of compartmental clonal restrictions in the embryonic or larval cuticle [these epidermal cells undergo very few divisions (24), probably precluding such a demonstration by clonal analysis]. However, the embryonic lethality of the *engrailed* mutants described here suggests that the compartmental rules that govern the behavior of the imaginal cells apply to larval and embryonic cells as well. First, the large number of independently isolated alleles of *engrailed* exhibited similar embryonic and adult defects and all defined a single complementation group. It is unlikely that two separate sites exist within the locus, one that functions in adults and another that has very different functions

in embryos. It seems more likely that the embryonic defects of *engrailed* mutants are due to defects specifically in previously unrecognized early posterior compartments.

Second, the phenotype of the *engrailed* embryo suggests a failure in segmentation. Segments were observed to fuse after their initial formation. It therefore appears that the lack of posterior *engrailed* cells has rendered the embryo incapable of maintaining segmental borders, just as this deficiency in posterior adult cells renders the adult incapable of maintaining the compartment borders within each segment. This would predict that *en/en* posterior cells should be capable of crossing segmental as well as compartmental borders. Clones of *stw pwn* cells in the first and second abdominal segments respect a border that separates these segments. Clones grow along this border, but do not cross it. In contrast, clones in the posterior region of the first abdominal segment that are homozygous for either en^{LA4} or en^{LA7} cross this segmental border (unpublished results). This suggests that, in both the adult and the embryo, the *engrailed* locus is required for maintaining segmental, as well as compartmental borders.

The hypothesis that the *engrailed* locus is involved in the compartmental and segmental subdivision of the embryo does not suggest a simple explanation for the patterns of segment fusion that were observed. The different fusion patterns may reflect the different steps in the process of segmentation that are affected by different *engrailed* alleles. Consistent with this suggestion is the genotype-dependent variability and the phenotypes of other mutants affecting segmentation that have been isolated. The isolation (25) of mutations in 15 loci that are required for the normal segmentation of the *Drosophila* embryo (included in this collection are five *engrailed* mutants that are allelic with the mutants described here) has led to identification of several other genes whose deficiency results in phenotypes similar to that described here for the *engrailed* mutants. Thus, it is likely that the abnormal segmental phenotype of *engrailed* embryos results not from pattern duplication or homeotic transformation specific to the *engrailed* mutants but rather from the interruption of normal morphogenesis.

Lawrence and Morata have suggested that the cellular differences between the anterior and posterior cell populations reduce intermingling of cells at the compartment boundary and that these cellular differences depend on the *engrailed* gene function in posterior cells (8). The data presented here support this hypothesis and argue further that, in constructing the body cuticle, this border-forming mechanism has been reiterated many times in the embryo, in the larva, and in the adult.

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