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The *engrailed* Locus of Drosophila: In Situ Localization of Transcripts Reveals Compartment-Specific Expression

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Summary

The *engrailed* locus plays a unique and critical role in organizing the segmented body plan of Drosophila. Embryos lacking *engrailed* function die with fused, abnormal segments. Adult mosaics with patches of *engrailed* cells similarly suffer defects in all of their segments, but only with mutant cells that are in the posterior developmental compartment of each segment. The non-uniform requirement for *engrailed* function reflects the position-dependent expression of the *engrailed* locus and we demonstrate it here unambiguously by directly visualizing *engrailed* transcripts in frozen sections of embryos and larvae and in whole imaginal discs. These results demonstrate that developmental compartments subdivide the embryonic insect segments. In these and in the compartments of the later developmental stages the *engrailed* locus is expressed in the posterior but not the anterior compartments. With its role in controlling the developmental pathway of the posterior compartment cells, the *engrailed* locus may be an example of a binary developmental switch.

Introduction

Repetitive patterns are a common motif in the organization of the body plan in many animals. Striking instances are the serial arrangement of annelid and arthropod segments and of chordate vertebrae. Possibly, such repetitive architecture reflects the reutilization of a single genetic program that specifies a basic developmental unit. In studies of the fruit fly, Drosophila melanogaster, cell-lineage analysis has demonstrated further developmental subdivision in the insect segments (Garcia-Bellido et al., 1973). Each adult segment is composed of two areas, anterior and posterior compartments. Unlike the segments that are anatomically delineated, no morphological evidence of the intrasegmental borders can ordinarily be seen. The existence of the two compartments has been deduced solely from studies of the growth histories of their constituent cells; they are populated by developmentally distinct groups of cells that never mix. Their importance as units of development has been inferred from the demonstration that a variety of mutations disrupting development affect only one of the two compartments in a segment (e.g., *bithorax*, *postbithorax*, and *engrailed*, reviewed by Morata and Lawrence, 1977), suggesting that developmental compartments may provide the spatial domains for the regulation of these genes.

The initial generation of the segmented body pattern of Drosophila has been analyzed genetically in considerable detail. Mutations in any of 15 identified loci disrupt segment pattern or reduce segment number (Nusslein-Volhard and Weischaus, 1980). These genes function in concert to ensure the proper shape, size, and position of each of the metameric subdivisions.

One of the first of these segmentation loci to have been identified is *engrailed*, now known to be a functional genetic unit of at least 70 kb of DNA as defined by chromosomal breakpoint mutations (J. Kuner, M. Nakanishi, Z. Ali, B. Drees, E. Gustavson, J. Theis, L. Kauvar, T. Kornberg, and P. O'Farrell, unpublished). Most of the *engrailed* mutant alleles are fully recessive and cause disrupted and fused segments with ensuing embryonic death (Kornberg, 1981a). All of the body segments are affected. Because genetically mosaic flies, even those with large areas of homozygous *engrailed* mutant tissue, can survive to the adult stage, a patchwork reconstruction of the *engrailed* mutant phenotype can be obtained for the adult. Such studies reveal that in the absence of *engrailed* function, normal development was not perturbed in the anterior compartments of the proboscis, antennae, thoracic legs, wings, first abdominal segment, or male genitalia (Lawrence and Morata, 1976; Kornberg, 1981a, 1981b; Lawrence and Struhl, 1982). In contrast, all of the posterior compartment cells required *engrailed* function to develop normal patterns. Indeed, posterior *engrailed* cells even fail to respect the anterior–posterior compartment border, escaping their growth restrictions within the posterior compartment (Lawrence and Morata, 1976). One of the functions of the *engrailed* locus, therefore, is to maintain the subdivisions of the insect segment, the compartments.

This genetic evidence, that all cells of the posterior compartments have an absolute requirement for *engrailed* function, has suggested that the *engrailed* function itself selects a posterior compartment developmental pathway by its presence, or an anterior compartment pathway by its absence (Garcia-Bellido, 1975). In the work reported here, direct examination of the expression of the *engrailed* locus is used to define precisely where the gene functions. With a cDNA probe derived from the most abundant transcript of the *engrailed* region, the presence of homologous RNA was monitored in tissue sections made from Drosophila embryos and larvae, and in whole mass-isolated larval imaginal discs (organs that give rise to the adult cuticular structures after metamorphosis). These results demonstrate that expression of the *engrailed* locus occurs only in the posterior compartment cells of each segment.

Results

The *engrailed* locus is the source of several developmentally regulated transcripts ranging in size from 1.4 to 3.6 kb (Drees and Kornberg, unpublished data). The most abun-

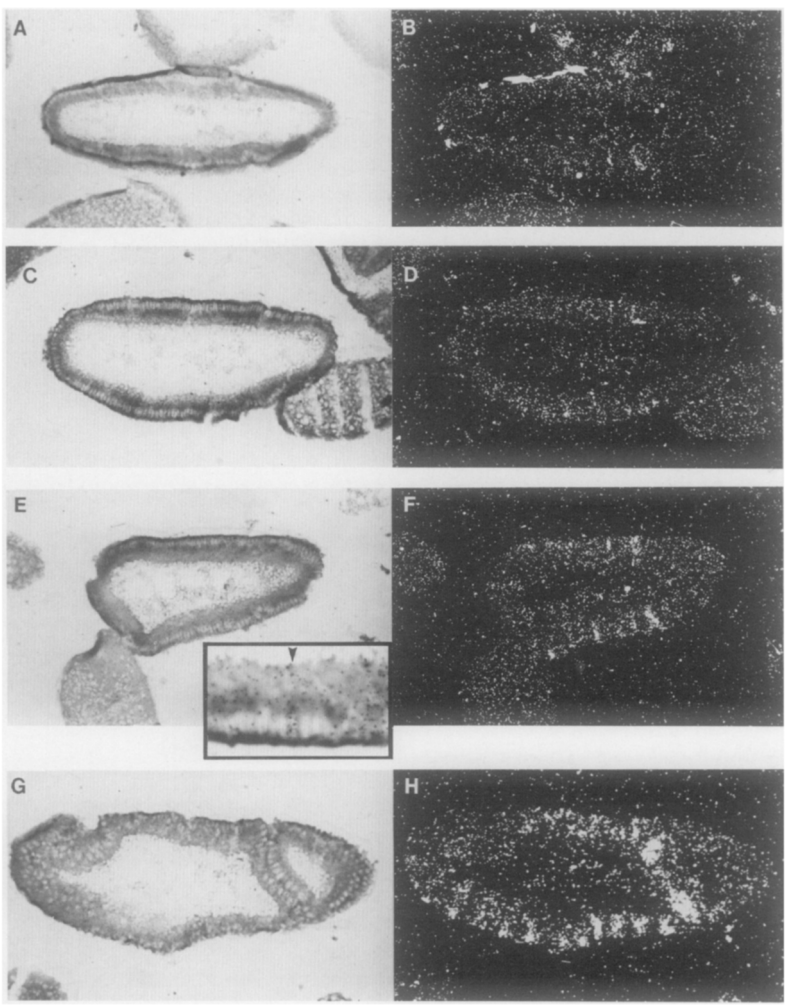

Figure 1. Localization of *engrailed* Transcripts in Sections of 3–4 hr Embryos

Bright-field (left) and corresponding dark-field (right) micrographs were taken after autoradiography. Orientation of embryos is anterior right. The cephalic furrow is visible as an indentation at one-third embryo length from the anterior pole. The embryos in (A), (C), and (E) have just initiated gastrulation whereas in (G), germ-band extension has commenced. The inset in (E) is an enlargement of a portion of the cellularized region showing hybridization in only a single cell along the longitudinal axis. The posterior midgut invagination is visible in (G) as an indentation of the posterior dorsal surface. Magnification: (A)–(F), 120×; (G) and (H), 150×.

dant embryonic transcript is 2.7 kb, and it is present also during the larval instar periods. A cDNA copy of this transcript has been isolated and its sequence determined (Poole et al., 1985). To construct a probe of high specific radioactivity containing extensive homology with *engrailed* transcripts, a 1.4 kb portion of this cDNA lacking 350 nucleotides at the 3′ end and ~1 kb from the 5′ end was recombined into the pUC 9 plasmid vector (Vieira and Messing, 1982). This portion of the cDNA clone contains a region with partial homology to the homeo box sequences of other homeotic genes (McGinnis et al., 1984), but is otherwise devoid of any repeated elements detectable by analysis of genomic Southern blots (data not shown). To localize the regions of *engrailed* expression, this highly labeled recombinant plasmid (p9-1.4) was hybridized to either frozen sections of Drosophila embryos and larvae or to whole imaginal discs. Sites of hybridization were revealed by autoradiographic detection of bound probe.

Embryonic Expression

Within hours after fertilization, rapid mitotic division and cell movements transform the Drosophila egg into a multilayered embryo, with anatomically distinguishable epidermis, nervous system, and primitive digestive track. Autoradiographic evidence of *engrailed* transcription was obtained from sections of embryos approaching or having just initiated gastrulation. Sections of earlier stages, either during the first 13 divisions of the syncytial preblastoderm nuclei or during formation of the cellular blastoderm, did not reveal any pattern of hybridization that could be distinguished above background (not shown).

In early gastrulating embryos (3–4 hr, Figure 1, A–F), autoradiographic grains were found to be most prominent just posterior to the cephalic furrow invagination, but are also generally found in the cells of the embryo posterior to the cephalic furrow. (The cephalic furrow is a deep oblique groove of cells dividing the embryo into an anterior third that contains the primordia for all of the head segments but one, and a posterior two-thirds that will generate the remaining head, thoracic, and abdominal segments.) In addition, a repeating pattern of hybridized cells posterior to the cephalic furrow was consistently observed. In some cases these bands of hybridization spanned no more than a single cell along the longitudinal axis of the egg (see insert, Figure 1E). In some embryos, grains were also observed over the posterior row of cells in the cephalic furrow itself (Figure 1, G and H). At this time

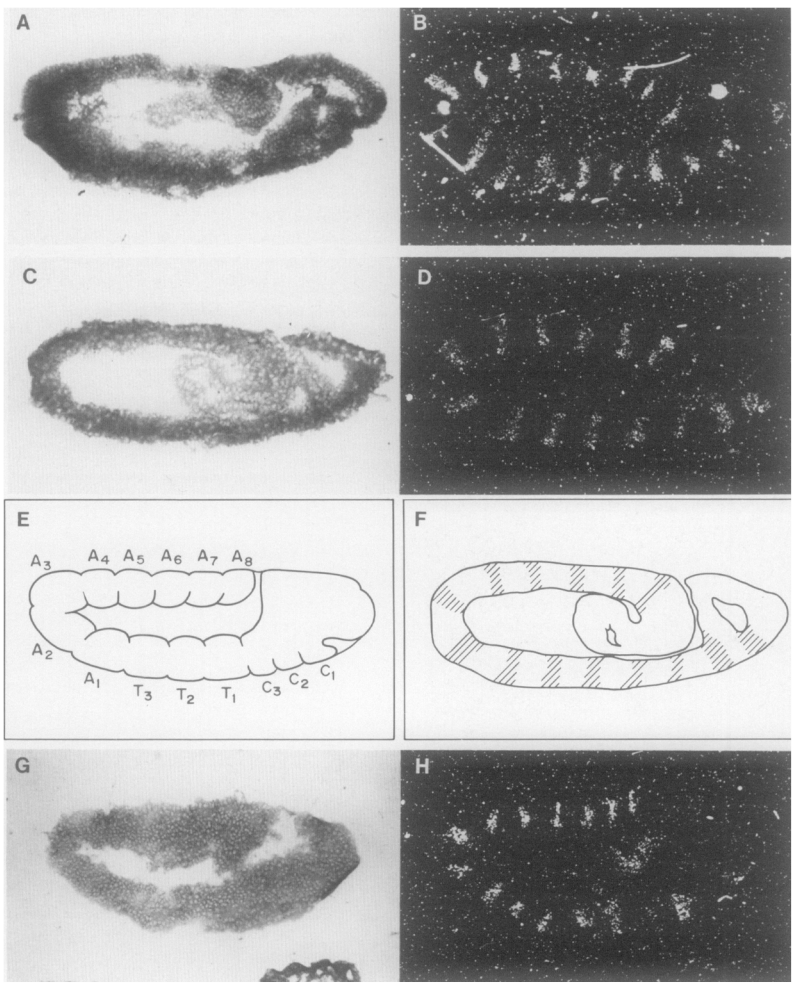

Figure 2. Localization of *engrailed* Transcripts in Sections of 5–8 hr Embryos

Bright-field (A, C, G) and corresponding dark-field (B, D, H) micrographs are sagittal sections of extended germ-band embryos and are oriented dorsal up, anterior to the right. (E) depicts a lateral view, drawn by Poulson (1950) of a whole embryo similar in age to the embryo in (C) (1950). In (F), the embryo in (C) has been drawn, with regions of hybridization shaded. (G) and (H) show an embryo in which the hybridized cells include only those within ~5 cell diameters of the embryo surface. Note that the posterior midgut rudiment is labeled, seen as a V-shaped wedge of grains in the central portion of the embryo, about one-third from the anterior pole. Magnification, 150×.

in development, segments are not yet distinguishable as anatomical subdivisions.

During invagination of the cephalic furrow, two other major developmental events take place—invagination of the primitive mesoderm along a ventral longitudinal groove (the ventral furrow), and extension of the ventral cells of the embryo, moving the germ band anteriorly from the posterior pole along the dorsal surface (germ-band extension, see Fullilove et al., 1978 for details and micrographs). By 6.5 hr after fertilization the most posterior cells of the germ band have nearly reached to the cephalic furrow on the dorsal surface and have transferred the primordial germ cells and the primordium for the posterior midgut into the interior. The germ band subsequently retracts (8.5 to 9 hr after fertilization) to return the most posterior somatic cells to the posterior pole. During this period the head segments involute and the thoracic segments expand to form an acephalic embryo entirely covered with a sheet of epidermal cells.

Identification and localization of the cells containing *engrailed* transcripts highlight many of these dramatic events of early Drosophila development (Figure 2). With the extension of the germ band, the intensity of the hybridization signal in embryo sections increased. As the posterior cells reach over to the dorsal surface (Figure 1, G and H; Figure 2, A and B) bands of hybridization along the ventral surface extended inward for the full extent of the ventral furrow invagination, encompassing the mesodermal precursor cells. The maximum number of bands of hybridization observed at this stage was 17 (Figure 2, A, B) and some hybridization was also evident at the most anterior portion of the head. At completion of germ-band extension, RNA homologous to the *engrailed* probe was most abundant. Remarkably, the anatomical description of this embryonic stage (Figure 2E, obtained from Poulson, 1950) and the hybridization signal (Figure 2, C, D, and F) showed a direct correspondence: the probe dramatically delineates each segmental subdivision. *engrailed* RNA was observed in all eight abdominal segments, the three thoracic segments, and in the head. Within each segment, the ratio of band width of cells devoid of *engrailed* transcripts to cells containing *engrailed* transcripts was approximately 2:1.

A slightly older embryo (Figure 2, G, H) revealed that *engrailed* transcripts were no longer present over the full extent of the internalized cells but were now localized to the outermost five cells. Hybridization was also observed among the cells of the posterior midgut rudiment, a tissue not normally thought to be segmented. The amount of hybridization decreased over the course of germ-band

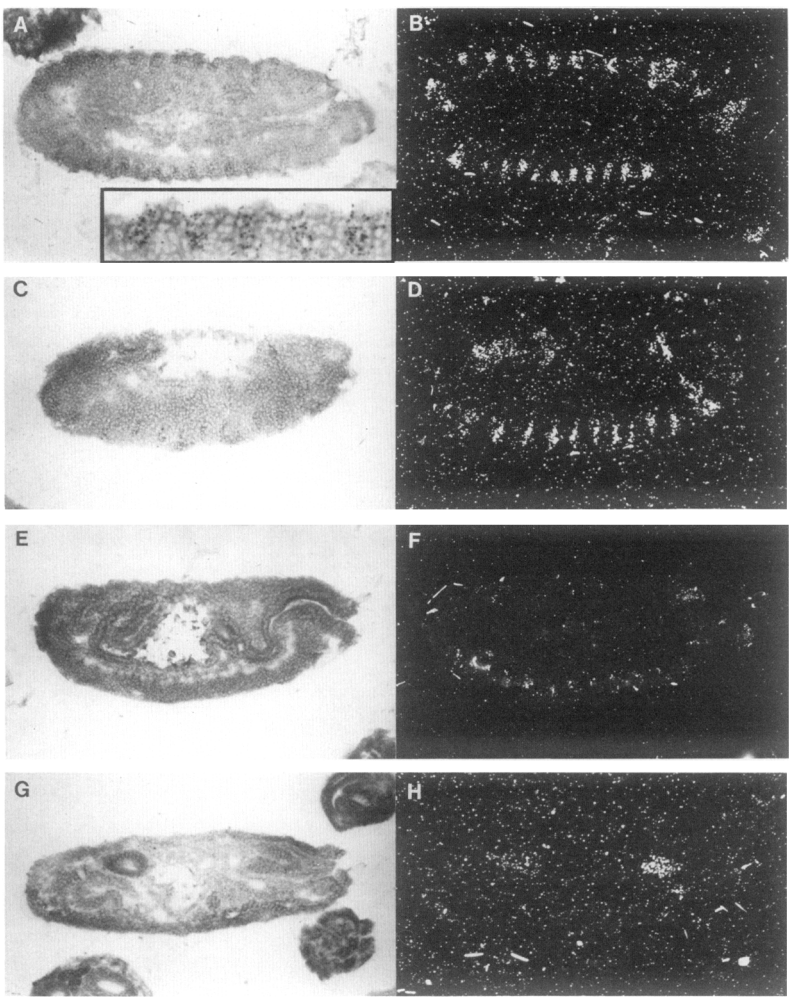

Figure 3. Localization of *engrailed* Transcripts in Sections of 8–13 hr Embryos

Bright-field (left) and corresponding dark-field (right) micrographs of embryos are oriented with anterior to the right. (A) is a frontal section of an 8–9 hr embryo. Inset is an enlargement of a portion of the peripheral region showing grains over the posterior portion of each anatomical segment. (C) is a sagittal section of an 8–9 hr embryo in the process of germ-band contraction. Note its peripheral labeling. (E) is a sagittal section of a 10–10½ hr embryo. The nervous system extends the length of the embryo and is not labeled. The neuropile of the segmental ganglia are visible as a repeating pattern of lightly staining spheres near the ventral surface. (G) is a slightly oblique frontal section of a 13 hr embryo. Grains cluster over the cephalopharyngeal apparatus, located toward the anterior end, and the posterior midgut toward the posterior end. Magnification, 150×.

contraction in older embryos. Autoradiographic grains were observed in a pattern similar to the earlier stages, but only the more peripheral cells of the embryo contained hybridizing RNA. This RNA was greatly reduced. With maturation of the embryos, the morphology delineating each individual segment becomes more pronounced and the correlation between anatomical segments and hybridization to the *engrailed* probe was easily visible. Grains were found over the posterior third of each segment (insert, Figure 3A). Mesodermal cells, and the neural ectodermal cells of the ventral nerve chord, contained little or no hybridizable RNA (Figure 3, A–F). Older embryos contain little *engrailed* RNA; hybridization was limited to a portion of the cephalopharyngeal apparatus and the posterior midgut (Figure 3, G and H).

Larval Expression

During the pupal period, the integument for the adult (or "imago") is secreted by nests of cells, the "imaginal discs," that had grown logarithmically throughout the larval periods. By the late third-instar larval period, these imaginal discs are sufficiently large and sturdy and, except for a slender stalk, are free of connections to other organs so that isolation is possible. In addition, the disc derivatives of each segment have a characteristic morphology that permits them to be identified either in section or after isolation. It was of particular interest to monitor *engrailed* expression in the imaginal discs for two reasons: analysis of the adult cuticle has demonstrated a requirement for *engrailed* function in the posterior compartments of each of the adult segments (Lawrence and Morata, 1976; Kornberg 1981a, 1981b; Lawrence and Struhl, 1982), and fate mapping (Bryant, 1975) and clonal analysis (Brower et al., 1981) have accurately mapped the location of the anterior and posterior compartments in the wing imaginal disc. In these organs, therefore, a direct correlation between *engrailed* function and expression can be made.

Hybridization of the p9-1.4 probe to serial frozen sections of third-instar larvae identified imaginal disc cells containing *engrailed* RNA (Figure 4). Labeling of the cells appeared in many instances to be predominantly cytoplasmic (insert, Figure 4A). Hybridization to imaginal disc adepithelial cells or to larval digestive track, hypoderm, muscle, salivary gland, or ventral ganglia was not detected (not shown).

To identify the region of hybridization more precisely and to relate such hybridization to the known fate map of intact discs, a method was developed to visualize the

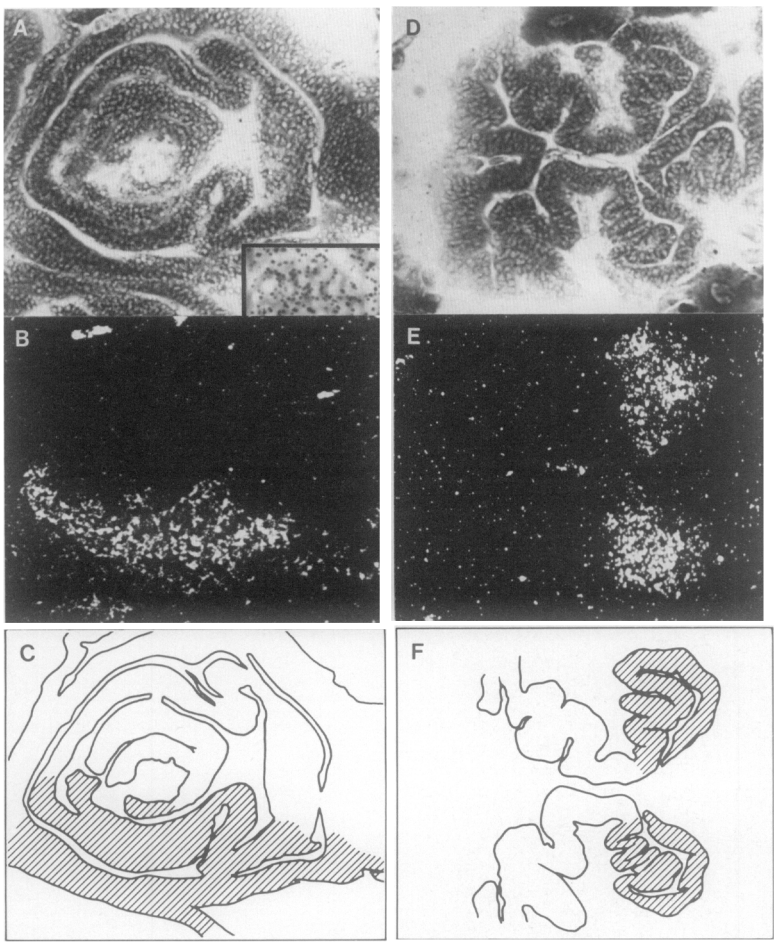

Figure 4. Localization of *engrailed* Transcripts in Sections of Late Third-Instar Larvae

(A) is a frontal section of a mesothoracic leg disc (bright-field illumination). Inset is an enlargement from a similar section showing cytoplasmic localization of grains. (B) is a corresponding dark-field micrograph and (C) a drawing of this section with the shaded area representing the region of hybridization. (D), (E), and (F) are a similar series of photographs of a sagittal section of a pair of prothoracic leg discs. Magnification, 215×.

binding of probe to whole imaginal discs (see Experimental Procedures). Fixed imaginal discs that had been exposed to the p9-1.4 probe were dehydrated and dried onto slides for autoradiography. Grains were observed concentrated over approximately one-half of each imaginal disc. Where sufficient integrity of the morphological structures was retained, identity of the imaginal disc could be established and a characteristic pattern of hybridization could be associated with the different types of imaginal discs (Figure 5). Comparison of the hybridization signal in a wing imaginal disc to the location of the anterior–posterior compartment border that had been previously identified by clonal analysis (Brower et al., 1981) reveals a striking correspondence. Grains were clearly clustered over the posterior compartment (Figure 6).

Discussion

This study has demonstrated that expression of the *engrailed* locus is spatially restricted in a pattern that reflects the basic segmental organization of Drosophila. In the posterior portion of each segment, cells express the *engrailed* locus; these are the cells of the posterior compartments. These results indicate that the early Drosophila embryo is made up of alternating bands of tissue in a "zebra-like" fashion, which do or do not express the *engrailed* locus (Kornberg, 1981, 1981b). Examples of spatially restricted gene expression are numerous and well documented (e.g., pancreatic RNAase; the contractile proteins of muscle). Such products reflect the specialized functions of their respective tissues. The spatially restricted expression of the *engrailed* locus differs significantly. The function of the *engrailed* locus is related directly to position-dependent expression and is independent of the specific roles of the particular tissue in which it is expressed.

Developmental compartments are discrete areas of tissue produced exclusively by all of the descendants of a small group of founder cells that contribute only to this structure (Garcia-Bellido, 1975). Cells in the embryo are recruited to their respective compartments to form the founder population as a consequence of their position and are related to each other only by their common location. Once demarcated as common members of a compartment, however, these chosen cells retain their common identity throughout development. Area and lineage are thus the two properties that characterize compartments from their creation in the young embryo to their ultimate resolution in the adult. These ideas are inferred from the studies using x-ray-induced somatic recombination to generate large cell clones in the adult epidermis, and generalization of these concepts to the development of

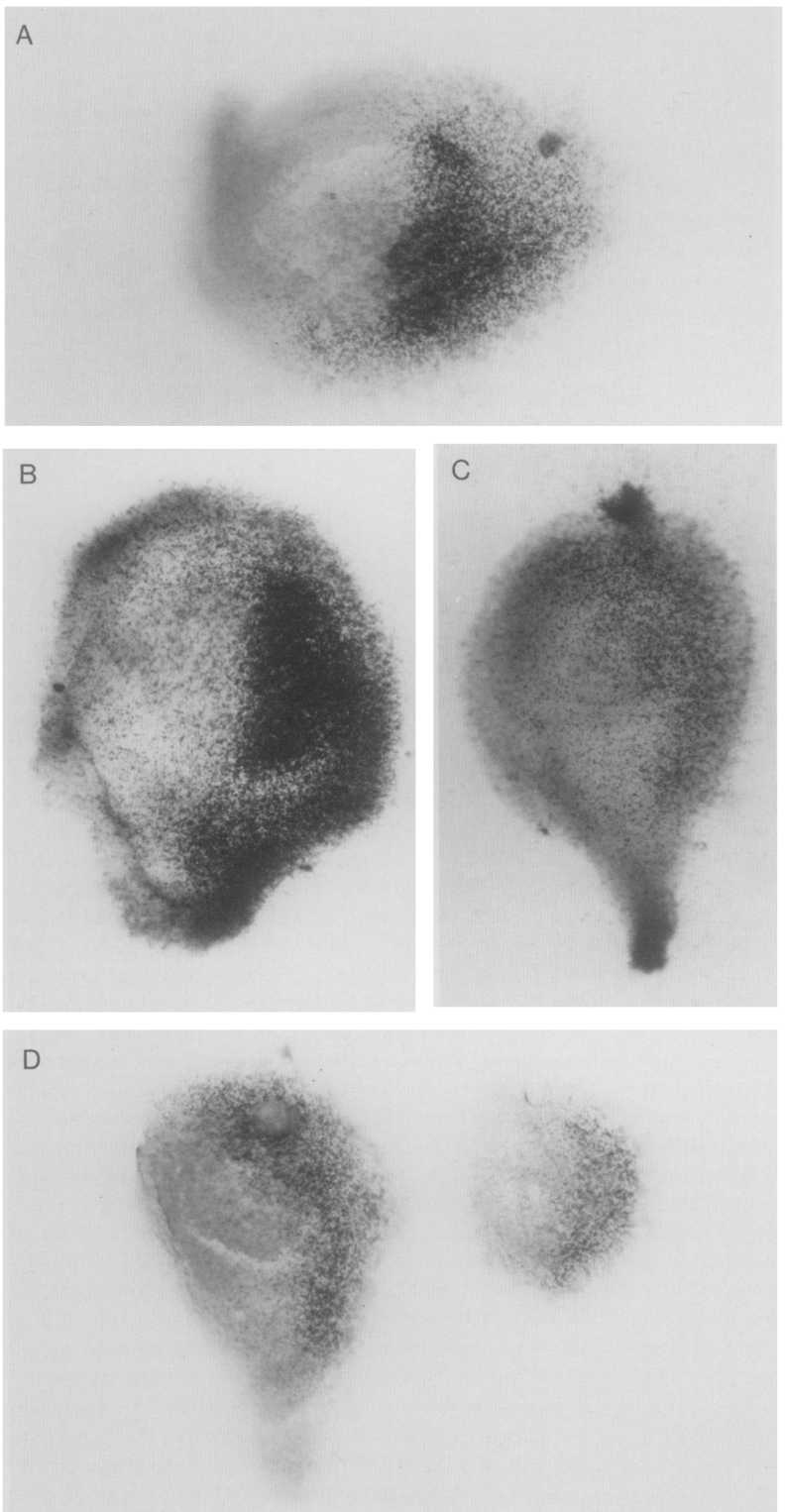

Figure 5. Localization of *engrailed* Transcripts in Whole Imaginal Discs

Mass-isolated discs were hybridized in solution and autoradiographed after drying onto microscope slides. The probe was labeled with ^{35}S-dATP. Similar results were obtained with probe labeled with ^{3}H-nucleotides. (A) mesothoracic leg disc; (B) antennal disc; (C) prothoracic leg disc; (D) haltere disc (left) and labial disc (right). Magnification, 250×.

other tissues remained only conjecture. Despite much effort (Ferrus and Kankel, 1981; Lawrence, 1982), cell-lineage analysis of the tissues of earlier stages and of internal tissues has failed to identify developmental compartments.

The function of the *engrailed* locus has been deduced from the phenotypes of mutant whole animals and mutant cell clones generated by somatic recombination. The requirement for *engrailed* function in the posterior compartments for maintenance of the anterior–posterior compartment border suggested a direct role for the *engrailed* locus in establishing the identity of posterior cells

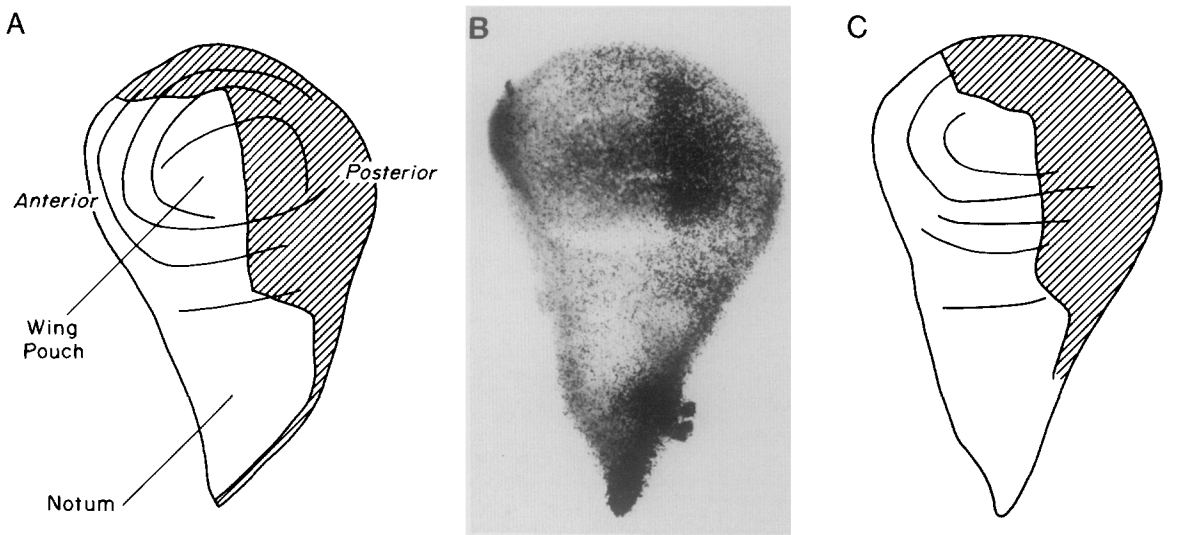

Figure 6. Localization of *engrailed* Transcripts in a Wing Imaginal Disc

(A) is a wing imaginal disc indicating the fate map locations of the adult wing and notum structures and the location of the anterior (open) and posterior (shaded) compartments. Lines indicate folds in the disc epithelium (from Brower et al., 1981). (B) is a bright-field micrograph of a wing imaginal disc after hybridization and autoradiography. Grains along lower right are due to binding of the ^{35}S-labeled probe to a tracheal fiber. Such binding was not observed with ^{3}H probe. (C) is a tracing of the disc in (B), with lines indicating the epithelial folds and the shaded area the region of hybridization. Magnification, 140×.

(Lawrence and Morata, 1976; Kornberg, 1981b; Lawrence and Struhl, 1982; Morata et al., 1983). It was proposed that the role of the *engrailed* locus is to choose between two alternative developmental pathways: either an active state in the posterior compartments or an inactive one in their anterior neighbors (Garcia-Bellido and Santamaria, 1972; Garcia-Bellido, 1975). In situ localization of RNA transcripts from the *engrailed* locus provides evidence that is pertinent for evaluating several of these proposals.

The juxtapositions of cells containing *engrailed* transcripts with nonexpressing cells are as abrupt as the discontinuities in developmental potential that have been mapped by clonal analysis. In the embryo the extent of an individual posterior compartment may be only the width of a single cell (insert, Figure 1E). In the mature imaginal discs, the border between expressing and nonexpressing (Figure 5B) cells is straight, as is the compartment boundary in the final structure. In the wing blade, for instance, the anterior–posterior compartment transits a remarkably straight course for almost the full length of the wing. The abrupt juxtaposition of hybridizing and nonhybridizing cells in the imaginal discs certainly reflects the precision with which the compartment border is maintained and indicates that the straight border in the final structure does not result from the expansion of a meandering boundary during invagination. The position of the discontinuity of hybridization signal corresponds in part with the anterior–posterior communication restriction border that has been observed after dye injection (Weir and Lo, 1982).

The phenotype of embryos deficient for *engrailed* function, pleitropic disruption of segmentation throughout the animal, suggests that the *engrailed* locus also plays a role during early development (Kornberg, 1981b). Analysis of cell clones induced during the embryonic period has indicated that the imaginal precursor cells of the mesothorax have been segregated into separate anterior and posterior compartments before hatching of the embryo (Weischaus and Gehring, 1976; Lawrence and Morata, 1977). If the functioning of the *engrailed* locus were essential for analogous reasons in the embryo and in the adult, then the existence of posterior compartments in the embryonic ectoderm and in the imaginal precursor cells of the embryo would be indicated. The compartment specificity of *engrailed* expression in the imaginal discs suggests that its expression in embryos is similarly delineated in only the posterior compartments and therefore demonstrates that developmental compartments do subdivide the segments of the early embryo.

It is noteworthy that there is a good correlation between indirect estimates of the relative size of the primordia for the imaginal posterior compartments and the proportion of cells in a segment that express *engrailed*. The size of the primordia at this early developmental period for the anterior and posterior compartments of the adult epidermis had been previously estimated from the frequency of mosaicism in male and female gynandromorphs and from the target size for induction of somatic recombination (Lawrence and Morata, 1977; Weischaus and Gehring, 1976). Both of these genetic methods indicated that the anterior compartment primordia is twice as large as the posterior compartment. *engrailed* hybridization, specific for posterior-compartment cells, was localized in a band over approximately one-third of each embryonic segment. Since the imaginal disc primordia constitute a small subset of embryonic ectoderm, it is ap-

parent that the mechanism designating the imaginal cells segregates groups in proportion to the size of the embryonic compartments.

In situ localization of *engrailed* transcripts demonstrates directly that expression of the locus is limited to the posterior compartment cells. Although we favor the hypothesis that regulation of transcription is responsible for this pattern of *engrailed* expression, the possibility exists that subsequent regulatory steps affect the stability of the *engrailed* transcripts in anterior compartment cells. The localization of *engrailed* transcripts to the posterior but not to the anterior compartments strongly supports the hypothesis of Garcia-Bellido (1975) that non-uniform requirement for *engrailed* function in the two compartments reflects the role of this gene in selecting the posterior developmental pathway. Proof of its role as such a developmental switch must await determination of the effect of *engrailed* expression in anterior-compartment cells.

Every segment examined to date has proved to be subdivided into anterior and posterior compartments. Therefore, as a biochemical probe for posterior compartment cells, *engrailed* expression has become a biochemical marker for segments. The number of bands of hybridization to *engrailed* RNA should therefore indicate the number of segments in the animal. Segment number has not been firmly established for Drosophila. Eight abdominal and three thoracic segments are clearly visible in the larva and adult, but there is uncertainty as to the number of head and terminal segments. In more primitive insects, the female and male genitalia are formed from the imaginal disc derivatives of the eighth and ninth abdominal segments, respectively. In Drosophila, there is evidence that the primordia for the female genitalia, male genitalia, and analia are integrated into a single genital imaginal disc that may represent the evolutionary fusion of primordia from separate abdominal segments (Dubendorfer, 1971). Anatomical ridges that may correspond to the ninth and tenth abdominal segments have been observed in scanning electron micrographs (Turner and Mahowald, 1977). Fate maps of the terminalia are also consistent with the existence of additional terminal segments (Schupbach et al., 1978). Segmentation of the Drosophila head is not readily apparent, in part because head involution during early embryogenesis internalizes the segments of the head and because several of the embryonic head segments may not contribute to the adult head or may fuse their anlage into a single imaginal disc. In the studies reported here, as many as 18 separately hybridizing regions were observed in gastrulating embryos (Figure 2, A and B). This observation suggests the existence of segments in addition to those visible in larvae and adults, but it does not establish their identity unequivocally. The position of the hybridized cells suggests that the posterior compartments in at least four head and as many as three terminal segments contain *engrailed* RNA.

In sections of embryos, hybridization revealed that although expression of the *engrailed* locus was uniform within a compartment, its presence in different areas was temporally regulated. Hybridizing cells appeared in the head later than elsewhere. *engrailed* transcripts were detected in the cells of the presumptive mesoderm of the ventral furrow, but not in the mesoderm of extended germband embryos. No expression was observed in the ventral nerve chord. Older embryos had more limited expression; in third-instar larvae, it was limited entirely to the imaginal tissues. Possibly, *engrailed* expression is specific for ectodermal cells, or perhaps some cells become committed to other developmental pathways after compartment identity has been established early. It should be noted, however, that the cDNA probe used in these studies does not detect the full spectrum of transcripts homologous with the *engrailed* region (Drees and Kornberg, unpublished data), and compartmental delineation of other organ systems may depend on the expression of other regions of the locus.

Experimental Procedures

In situ hybridizations to embryos and larvae were performed essentially as described by Hafen et al. (1983). Embryos were permeabilized with heptane, fixed in 4% paraformaldehyde, and sectioned into 8 μm slices at −18°C on a Slee microtome. Climbing-stage, third-instar larvae were collected, washed, and transferred directly to O.C.T. embedding medium (Miles Laboratories) and then sectioned. Processing of the slides was after Hafen et al. (1983). Autoradiography was for one month at 4°C and the slides were developed at 15°C in Kodak D19 and fixed in Kodak Rapid Fix.

The hybridization procedure of Hafen et al. was modified to detect transcripts in whole imaginal discs. Mass-isolated imaginal discs generously provided by J. Fristrom (Fristrom, 1972) were allowed to settle into several washes of PBS (130 mM NaCl; 7 mM Na_2HPO_4; 3 mM NaH_2PO_4) in a conical polystyrene test tube. Fixation was for 15 min at 0°C in PBS + 4% paraformaldehyde and for 15 min at room temperature in PBS, 4% paraformaldehyde, 0.1% Triton X-100, 0.1% deoxycholate. Discs were washed five times with PBS for 5 min each. After a 20 min treatment with 0.2 N HCl, and five more washes in PBS, the discs were digested with pronase (0.15 mg/ml) in 1 ml of 50 mM Tris, pH 7.5, 5 mM EDTA for 5 min at room temperature. The concentration of pronase was one-fourth that used for the tissue sections and for only half as long. Digestion was half as much as would noticeably affect the disc morphology. After pronase treatment, glycine was added to 2 mg/ml (50 μl of 40 mg/ml solution) and the discs were again washed five times in PBS. After removal of PBS, 0.1 ml hybridization solution was added for 24 hr at 37°C. For the incubations with whole imaginal discs, equivalent results were obtained whether the p9-1.4 DNA was nick-translated in the presence of ^{35}S-dATP or ^{3}H nucleotides. Discs were washed ten times with PBS at 37°C over a 24 hr period and placed in 50 μl drops onto gelatin-subbed slides. After settling, they were dehydrated and fixed to the slides with 70% ethanol, added dropwise, and 95% ethanol. After air-drying, slides were dipped in emulsion for autoradiography. Exposure was for 2 weeks.

Radioactive probe was prepared by nick translation with DNA polymerase I (Rigby et al., 1977). Labeled nucleotides were from Amersham (^{3}H-dATP, 24.3 Ci/mmol; ^{3}H-dCTP, 62 Ci/mmol; ^{3}H-dTTP, 45 Ci/mmol; ^{35}S-dATP, 650 Ci/mmol). Plasmid DNA (40 μg/ml) containing an insertion of 1.4 kb of *engrailed* cDNA (Poole et al., 1985) was digested with pancreatic DNAase in 50 mM Tris-HCl, pH 8, 10 mM $MgCl_2$. DNAase levels were varied from 225 ng to 2.25 μg per μg of DNA. Digestions for 1 hr at 25°C were followed by inactivation of DNAase at 65°C for 15 min. Trial reactions (20 μl) with 0.4 μg of each digested DNA at 20 μg/ml with 100 μ*M* dCTP, dGTP, and dTTP and ^{35}S-dATP (15 μCi) and 2.5 U DNA polymerase I (Boehringer-Mannheim, nuclease-free) were for 1 hr at 16°C. A portion of the nick-translated DNA was fractionated on a 7 M urea–5% acrylamide gel and autoradiographed (Maniatis et al., 1982). DNA digested with DNAase to yield reaction products between 35 and 135 nucleotides long proved optimal for in situ hybridization. For the full reaction, an appropriate sample of predigested DNA (1 μg) was nick-translated with polymerase I for 1 hr at 16°C in the presence of 100 μCi each of two or three ^{3}H-deoxynucleoside triphosphates with the remaining nucleoside triphosphates

at 100 μM. Incubation was terminated by the addition of 50 μl 10 mM EDTA, 2 mg/ml denatured herring sperm DNA, and treatment at 65°C for 15 min. Unincorporated nucleotides were removed by spin dialysis through Sephadex G-50 (Maniatis et al., 1982). Specific activity for the DNA approached 10^8 cpm/μg. Immediately before use, the DNA was incubated at 100°C for 3 min and diluted 10-fold in hybridization solution (10 mM Tris, pH 7.5, 0.6 M NaCl, 1 mM EDTA, 0.25 mg/ml yeast tRNA, 1× Denhardt's solution, 10% filtered dextran sulfate, 50% Dowex AG deionized formamide).

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