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The *engrailed* Locus of D. melanogaster Provides an Essential Zygotic Function in Precellular Embryos

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Summary

Early embryonic development in Drosophila depends on genes expressed during oogenesis or after zygote formation. We show that the *engrailed* gene is needed for the processes that organize the embryo during the nuclear divisions that precede cellularization. During the precellular blastoderm stages *engrailed* mutant embryos show several notable anomalies: the pole cells form at a position slightly displaced from the posterior pole; yolk nuclei continue to divide after the tenth nuclear division cycle, when wild-type yolk nuclei have stopped dividing mitotically; and somatic nuclei are not positioned uniformly along the embryo periphery and do not undergo mitotic divisions in regular waves. This early requirement for engrailed does not appear to be a maternal function, and only genetically *engrailed* embryos displayed these precellular phenotypes. Synthesis of a 2.7 kb poly(A)$^+$ transcript of the *engrailed* region was found in precellular embryos.

Introduction

Early embryogenesis in Drosophila is designed for an unusually rapid and accelerated developmental program. The first 13 mitotic divisions occur synchronously with a cycle time of less than 20 min, and generate three types of nuclei: about 100 polyploid yolk nuclei in the embryo interior, 6000 somatic nuclei along the surface, and 45 pole-cell nuclei at the posterior end (Rabinowitz, 1941; Sonnenblick, 1950; Zalokar and Erk, 1976; Turner and Mahowald, 1976; Foe and Alberts, 1983). During the 14th nuclear division cycle, nuclei at the embryo periphery cellularize (cellular blastoderm formation), and the newly formed cells adopt commitments to particular developmental pathways and express some genes in remarkably precise patterns (Illmensee, 1978; Lohs-Schardin et al., 1979; Underwood et al., 1980; Wieschaus and Gehring, 1976; Hafen et al., 1984). Although many genes have been identified that regulate development after cellularization (Wieschaus et al., 1984; Nusslein-Volhard et al., 1984; Jurgens et al., 1984), few are known to affect earlier development.

Evidence of several types supports the hypothesis that blastoderm formation depends solely upon maternal inheritance of stored mRNA and protein. No known zygotic-lethal mutations arrest or affect morphogenesis prior to the formation of the syncytial blastoderm (Wright, 1970). Oogenesis provides the egg with a rich endowment of RNA (Anderson and Lengyel, 1979). Cleavage nuclei have an interphase period of only 3.4 min, and embryos do not synthesize RNA in detectable amounts until the cellular blastoderm forms (Zalokar, 1976; Lamb and Laird, 1976). However, such evidence does not exclude a role for zygotically expressed functions required in only small amounts.

The *engrailed* gene is one of the zygotic genes whose mutant phenotype suggests an important regulatory role in development (Garcia-Bellido and Santamaria, 1972). An embryonic lethal (Kornberg, 1981a; Nusslein-Volhard and Wieschaus, 1980), this gene is expressed and is required only for the development of the posterior compartment cells of each insect segment (Morata and Lawrence, 1976; Kornberg, 1981a, 1981b; Lawrence and Struhl, 1982; Kornberg et al., 1985; Fjose et al., 1985). Analysis of genetically mosaic flies containing patches of *engrailed* cells has revealed that engrailed functions both to maintain compartment and segment boundaries (Lawrence and Morata, 1976; Kornberg, 1981b) and to form normal patterns in posterior compartments. Mutant phenotypes and expression of the gene have not been observed in anterior compartment cells. This discontinuous expression forms a zebra-like pattern of expressing and nonexpressing cells in embryos (Kornberg et al., 1985), and points to a role for engrailed as a molecular switch "selecting" a posterior over an anterior developmental state (Garcia-Bellido, 1975).

Previous studies have not established when during embryogenesis engrailed functions or if engrailed participates in the subdivision process that generates the reiterated pattern of anterior and posterior compartments. The developmental stage in which compartments and segments subdivide the embryo into discrete units is also not known, but functional and molecular studies suggest that segmentation and compartmentalization have occurred by the cellular blastoderm stage. Clones of cells marked by somatic recombination in cellular blastoderm embryos are restricted to grow within either the anterior or the posterior compartments (Wieschaus and Gehring, 1976; Lawrence and Morata, 1977), and by the end of the 14th division cycle, the pattern of expression of a number of genes, including *engrailed*, occurs in stripes with a segment or compartment interval (Hafen et al., 1984; Kornberg et al., 1985). If *engrailed* is involved in compartment formation, mutations in the gene might be expected to affect morphogenesis at this embryonic stage.

We describe in this report that the engrailed function, encoded by a zygotic-lethal, non-maternal-effect gene, is essential for the development of the cellular blastoderm. These findings demonstrate that not all precellular functions of the Drosophila embryo are provided maternally, and suggest that engrailed is responsible in part for organizing and patterning the early embryo, a function that precedes its known role in maintaining posterior compartment identity.

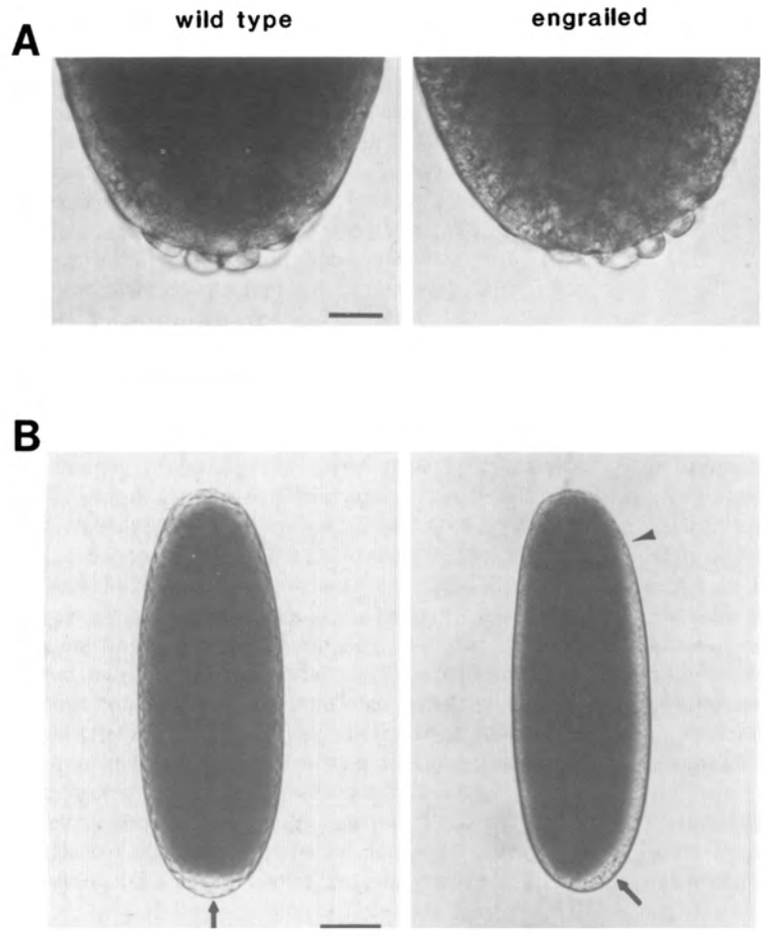

Figure 1. Bright-Field Micrographs of Living Wild-Type and *engrailed* Mutant Embryos

Orientation of cycle 10 (A) and cycle 12 (B) embryos is posterior down, viewed from the dorsal side. In wild-type embryos (left), pole cells (arrow in B) are at the posterior end and the peripheral clearing is regular. Notice the displaced pole cells in both en^{LA4}/en^{LA7} mutant embryos (right, A and B, arrow) and the nonuniform clearing near the anterior end of the cycle 12 mutant embryo (arrowhead in B, right). Nomenclature for embryonic stages is from Foe and Alberts (1983). Cycles 1–9, cleavage stages: exponential and synchronous multiplication of the nuclei in the interior; polar nuclei appear at the surface of the posterior end at stage 9. Cycles 10–13, syncytial blastoderm stages: stage 10, yolk nuclei stop dividing and remaining nuclei appear on the surface and divide in near synchrony four times. Cycle 14, cellular blastoderm stage: ~ 1.5 hr period of mitotic quiescence during which plasma membranes form between the peripheral nuclei and gastrulation begins. Upper bar = 25 μm; lower bar = 100 μm.

Results

Pole Cell Placement and Cortical Morphology in *engrailed* Embryos

The role of the *engrailed* gene during early embryonic development was examined by comparing the morphology of living wild-type and *engrailed* mutant embryos. Standard techniques of bright-field and Nomarski–differential interference contrast (DIC) light microscopy were used. All *engrailed* alleles examined in this study are recessive lethal mutations that arrest embryonic development after secretion of the embryonic cuticle. In their terminal state, *engrailed* embryos have fused and aberrant segment patterns and have abnormal head morphology (Kornberg, 1981a).

During the cleavage divisions that precede the arrival of nuclei at the embryo periphery, opaque yolky material is evenly distributed throughout the volume of the embryo. With the arrival of nuclei at the posterior end, pole cells form symmetrically about the anterior–posterior axis in the posterior pocket between the embryonic and vitelline membranes (Mahowald, 1962). Soon after the somatic nuclei arrive at the surface, a clearing of the peripheral periplasm can be seen as a lightening around the edges of the embryo (Sonnenblick, 1950; Bownes, 1975). This "periplasmic clearing" (Counce, 1973) is common among developing insect embryos and reveals complex nuclear and cytoplasmic events that accompany subsequent division cycles. Although it has not been previously noted, these two features—pole cell position and periplasmic clearing—are both abnormal in *engrailed* mutant embryos.

Wild-type and *engrailed* mutant embryos can be distinguished at nuclear cycle 10 by comparing the relative positions of their pole cells. The position of the pole cells in wild-type embryos is highly regular: among 100 embryos examined, all 96 embryos that survived to the blastoderm stage formed pole cells symmetrically about the anterior–posterior axis. However, in mutant embryos, the pole buds are displaced laterally (Figure 1, top; see legend for description of nuclear cycles and relevant nomenclature). Embryos from heterozygous *engrailed* parents ($en^{LA4}/+$ ♀ and $en^{LA7}/+$ ♂) were collected, dechorionated, and scored for pole bud position under a dissecting microscope. They were then allowed to complete embryonic development and their genotypes were evaluated. Of 49 embryos with normal pole cell position, 2 died before cuticle secretion, 45 were wild type, and 3 were *engrailed*. Of 12 embryos with aberrant pole cell morphology, 11 survived until cuticle secretion, and all of these

Table 1. Living *engrailed* Embryos Identified during Nuclear Cycle 12

engrailed Alleles[a]				
♀	♂	Total	*engrailed*[b]	*engrailed*+[b]
LA4	LA7	64	62	2
LA7	LA4	17	17	0
LA4	LA10	19	17	2
LA4	LA11	23	21	2
LA7	LA10	18	17	1
LA7	LA11	18	16	2
LA4	SF31	66	63	3
SF31	SF31	46	45	1
C2	SF31	37	35	2
SF49	SF31	35	33	2
IM199	T84	23	23	0
IM199	B24	10	10	0
T84	B24	21	21	0
		397	380	17

[a] Each parent carried one *engrailed* and one Oregon R wild-type second chromosome.
[b] The genotypes of the embryos were scored after 24 hr.

were *engrailed*. Thus, 14/58 (24%) or one-fourth of the progeny (the expected Mendelian proportion) were *engrailed*, and pole cell position is a reliable indicator of *engrailed* embryos at cycle 10.

By division cycle 12, both elements of the engrailed phenotype, pole cell position and periplasmic clearing, are apparent. Pole cells were displaced from the posterior pole, and periplasmic clearing was not uniform around the embryo periphery. In the example shown (Figure 1B, right), peripheral clearing on the right side of the embryo is not uniform. Although the extent of clearing irregularities varied as each mutant embryo developed and also varied between different embryos, a good correlation between abnormal pole cell position, irregular periplasmic clearing, and the *engrailed* genotype was found. Many combinations of *engrailed* alleles (involving en^{LA4}, en^{LA7}, en^{LA10}, en^{LA11}, en^{B24}, en^{IM199}, en^{T84}, $In(2R)en^{C2}$, $In(2R)en^{SF49}$, and $Df(2R)en^{SF31}$) were examined. Of 63 embryos from these crosses with normal syncytial blastoderm morphology, 2 died before cuticle secretion, and 61 developed into normal larvae. Of 431 embryos from these crosses with abnormal morphology, 34 died before cuticle secretion and could not be scored, and 380 (96%) of the embryos that developed displayed the segmental and head abnormalities characteristic of the engrailed phenotype (Table 1). All *engrailed* alleles studied could be detected with this dissecting microscope assay of precellular blastoderm embryos. The alleles include apparent point and break-point mutations, and those induced with both alkylating mutagens and X-rays in several different genetic backgrounds.

To examine the engrailed phenotype further, living embryos were viewed under DIC optics to reveal details of nuclear behavior and cellularization that bright-field optics cannot resolve. Wild-type and mutant embryos were selected from crosses of en^{LA4} and en^{LA7} flies and were observed from nuclear cycle 12 until gastrulation approximately 1.5–2 hr later (Figures 2 and 3).

Both low and high magnification views reveal the asymmetric positioning of pole cells and the abnormal shape of the posterior ends of mutant embryos. The pole cells are displaced toward the lateral surface when compared to wild type (Figures 2a, 2e, 3a, 3f). Perhaps as a result, yolk cytoplasm and somatic nuclei were observed in the region of the posterior pocket between the embryonic and vitelline membranes, where the pole cells normally reside (Figures 1a, 1b, 3a, 3f). Pole cell morphology was not obviously abnormal in mutant embryos (Figure 3), nor was the disposition of the somatic nuclei that underlie the pole cells (Figures 3a, 3f). No irregularities of cellularization were noted (Figures 3c, 3h). Although these DIC photographs fail to resolve additional abnormalities in a definitive way (the packing density of the somatic nuclei along the ventral surface of the embryo in Figure 2f may not be uniform), significant changes in the somatic nuclei, not obvious from light microscopy, are detectable by fluorescence microscopy (see below).

Gastrulation in *engrailed* Embryos

Gastrulation movements in Drosophila start following the maximum inward extension of the cleavage furrows of cellularization. The earliest movements are the invagination of the ventral and cephalic furrows, and the extension of the germ band and pole cells around the posterior pole up onto the dorsal surface (Sonnenblick, 1950; Turner and Mahowald, 1976). Viewed from the ventral surface (Figure 2g) the pole cells move out of the plane of focus as the ventral furrow moves around the posterior end (Figures 2c, 2d, 3d, 3e). These morphogenetic movements in the wild type are highly symmetric with respect to the anterior–posterior axis (Figures 3d, 3e).

With the onset of gastrulation, the mutant phenotype had four distinguishing features: the timing of the cephalic furrow invagination was consistently early relative to germ band extension (Figures 2c, 2g); the lateral displacement of the pole cells persisted during germ band extension (Figure 2g); the cephalic furrow was also laterally asymmetric (arrowheads, Figure 2g); and gastrulation was invariably shifted to the same side to which the pole cells had been displaced (Figures 2h, 3j).

Aberrant Mitotic Synchrony and Nuclear Spacing in *engrailed* Precellular Embryos

To investigate the engrailed phenotype in precellular embryos still further, the fixation and staining techniques recently developed to examine blastoderm nuclei and associated cytoskeletal elements were used (Mitchison and Sedat, 1983; Karr and Alberts, submitted). Embryos were dechorionated, identified by their wild-type or engrailed morphology, fixed, and stained with both a DNA-specific dye, DAPI, and a monoclonal antitubulin antibody to reveal DNA and microtubule structures. Two observations about the wild-type pattern are pertinent: the relative spacing of the blastoderm nuclei over the surface is remarkably even and uniform; and the variation in the stage of mitosis between adjacent nuclei is small, i.e., abrupt regional changes in mitotic state were not observed (Figures 4a, 4b; Foe and Alberts, 1983).

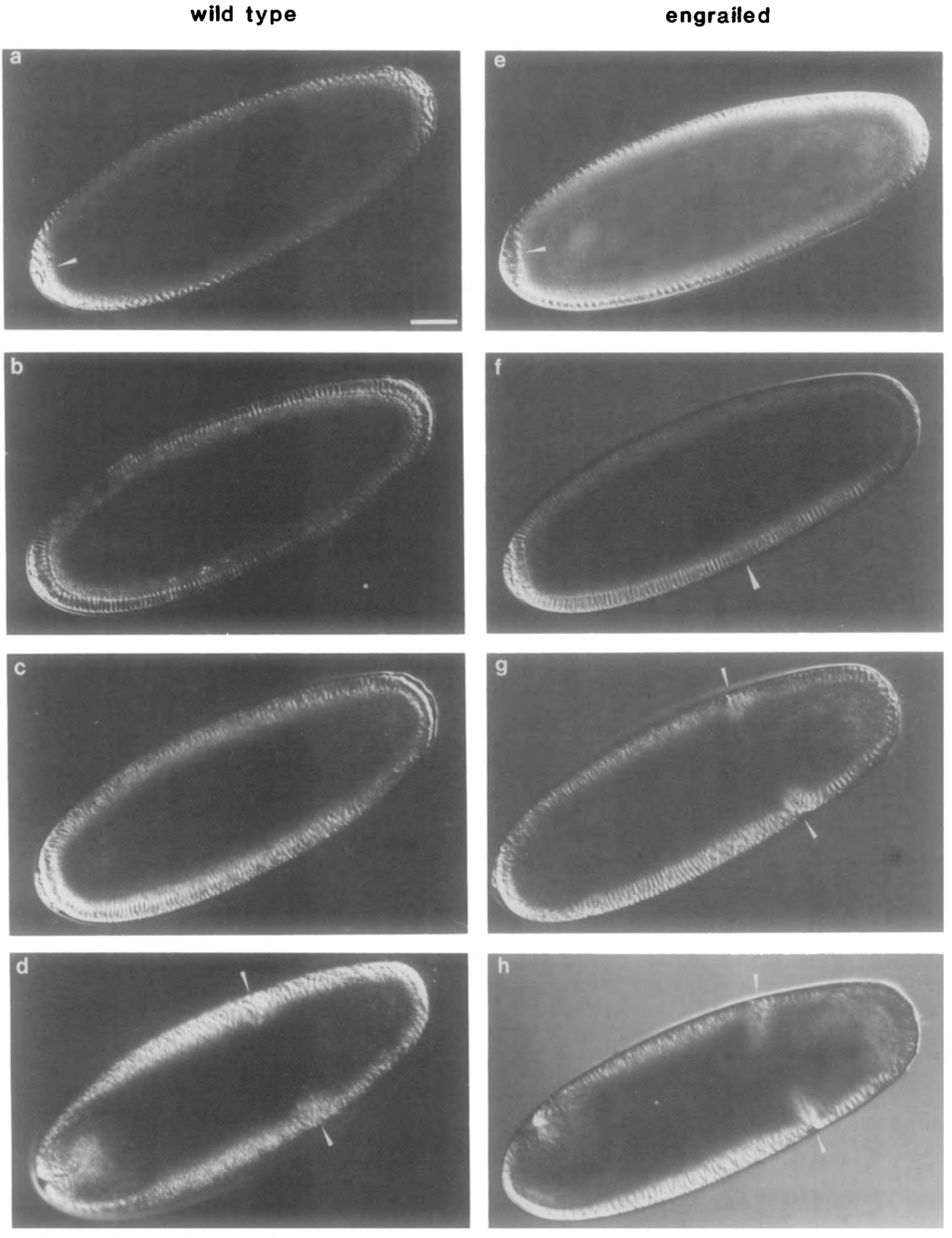

Figure 2. Chronological DIC Micrographs of Living Wild-Type and *engrailed* Mutant Embryos during Formation of the Cellular Blastoderm

Orientation is anterior right, viewed from the ventral side. Arrowheads point to pole cells in (a) and (e), to uneven packing of cellularizing nuclei (f), and to cephalic furrow invaginations in (d), (g), and (h). Elongation of the nuclei and concomitant growth of cell membranes inward from the periphery can be seen in both the wild-type embryos (left panels, b and c) and en^{LA4}/en^{LA7} embryos (right panels f and g). Abnormalities in the mutant include: asymmetric position of the pole cells (e, f, g), abnormal shape of the posterior end (e, f), non-uniform packing of nuclei (arrowhead, f), and timing of gastrulation. Bar = 50 μm.

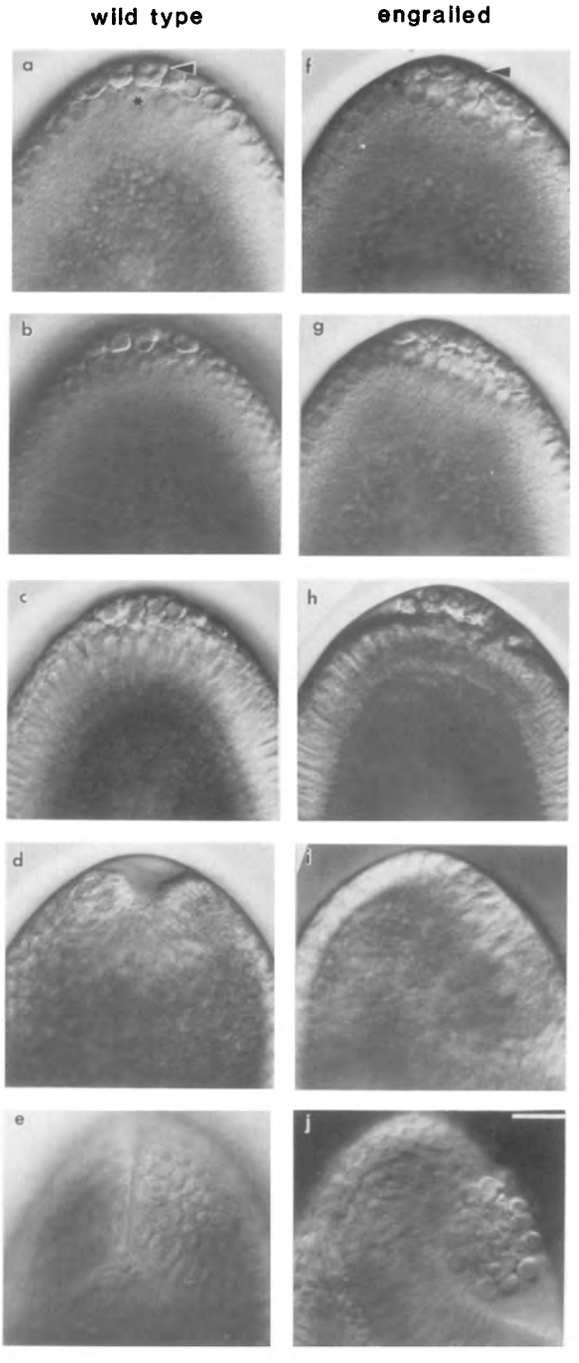

Figure 3. Chronological DIC Micrographs of the Posterior End of Living Wild-Type and Mutant Stage Embryos Beginning at Division Cycle 13

Orientation is posterior up, viewed from the ventral side. Pole cells (noted with arrowheads) in wild-type embryos (left) and en^{LA4}/en^{LA7} mutant embryos (right) are prominent at the posterior end until they invaginate in a pocket of the ventral furrow during germ band extension. Asterisks in (a) and (f) are in peripheral somatic nuclei. Bar = 25 μm.

Both the nuclear spacing and the mitotic patterns were consistently altered in *engrailed* mutants, and several examples are shown (Figures 4c–4f). In one case, nuclei in interphase near the posterior and anterior ends with a spacing characteristic of nuclear cycle 13 are separated by nuclei that are clearly in cycle 12 metaphase (Figures 4c, 4d). In another case, an *engrailed* embryo with uneven spacing of surface nuclei is seen in which the nuclear densities in two regions of this embryo differ precisely by a factor of 2 (Figure 4e). The final example displays an embryo with both an abrupt break in mitotic staging and an uneven spacing of nuclei (Figure 4f).

Table 2. Yolk Nuclear Number and Position in Wild-Type and *engrailed* Embryos

	Total Yolk Nuclei[a]	Avg. Nuclear Density[b]	Avg. Nuclear Density in Posterior Region[c]
Wild Type	102 114	6.5 ($n = 22$)	1.7
engrailed	196 244	9.75 ($n = 16$)	6.7

[a] Whole fixed embryos stained with DAPI as described in Experimental Procedures were mounted under coverslips that gently flattened the embryos, facilitating the examination of the internal yolk nuclei. Total yolk nuclear counts were determined by optically sectioning through four focal planes of each embryo.

[b] Averages were determined by counting the total number of yolk nuclei in 2100 μm^2 fields randomly chosen at four positions along the anterior–posterior axis. *engrailed* embryos were hand-selected during the syncytial blastoderm stages and fixed immediately as described in Experimental Procedures.

[c] Average number of yolk nuclei in a 2100 μm^2 field located at the posterior end of each embryo. Fields were chosen such that the square graticule approached, but did not overlap, the somatic nuclei at the posterior end.

Yolk Nuclear Number and Placement in *engrailed* Embryos

A small proportion of cleavage stage nuclei remains in the yolk-rich interior while the majority of the nuclei migrate to the periphery (Rabinowitz, 1941; Sonnenblick, 1950). These yolk nuclei stop dividing after the mitosis of nuclear cycle 10 and become polyploid as their DNA replication continues (Foe and Alberts, 1983). Examination of yolk nuclear number, morphology, and behavior revealed significant differences between wild-type and *engrailed* embryos (Figure 5 and Table 2).

In the wild type, yolk nuclei present during nuclear cycle 13 are mostly deep in the interior of the embryo. In contrast, the yolk nuclei in *engrailed* embryos were consistently more numerous and dispersed. Several methods illustrate these differences. Among the wild-type and mutant embryos shown in division cycle 13, the greater dispersion and number of yolk nuclei in the mutant are apparent at low magnification (Figures 5a, 5b). The total number of yolk nuclei was determined, and the density of nuclei in the embryo interior was assessed by counting yolk nuclei in small, similarly located optical fields (Table 2). By both methods, the mutant embryos have approximately twice as many nuclei. Although an initial recruitment of abnormally large numbers of nuclei to the yolk region cannot be eliminated as a contributing factor, an additional mitotic division appears to be involved: yolk nuclei containing mitotic figures were observed in division cycle 12-13 mutant embryos. In the two examples shown (Figures 5c,

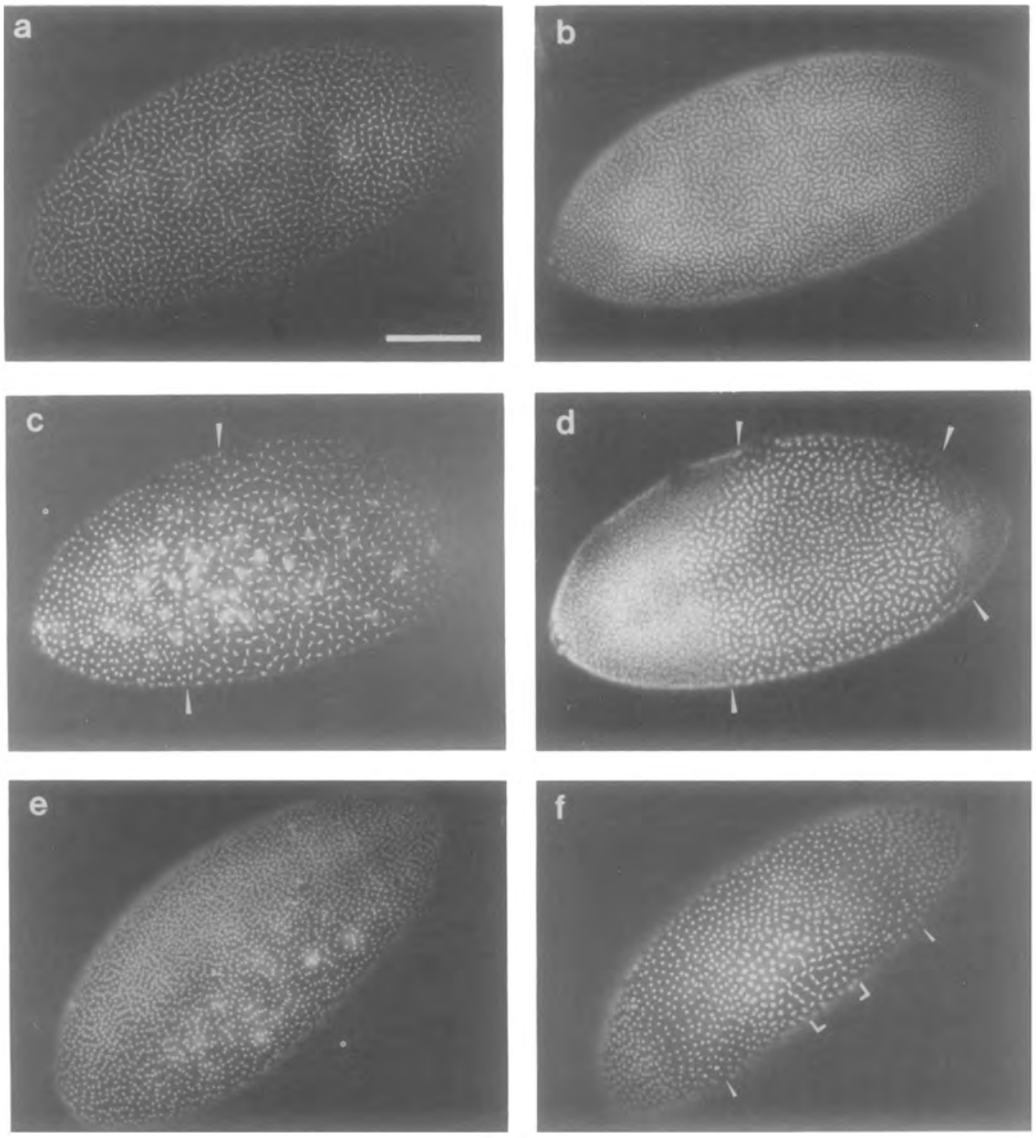

Figure 4. Fluorescence Micrographs of Fixed Wild-Type and Mutant Embryos

Orientation is anterior right, viewed from the lateral surface. Embryos were stained with either DAPI (a, c, e, f) or second antibody labeled antitubulin antibody (b, d). Uniform arrangement of nuclei in the wild-type embryos (a, b) is contrasted with the non-uniform arrangement of nuclei in en^{LA4}/en^{LA7} (c, d), en^{LA4}/en^{LA10} (e), and en^{LA4}/en^{LA11} (f) embryos. Non-uniform areas are indicated with arrowheads and brackets. Bar = 100 μm.

cycle 12 embryo; 5d, cycle 13 embryo), the mitoses were located among nondividing polyploid nuclei deep within the yolk interior.

Another aspect of the greater dispersion of yolk nuclei in mutant embryos is illustrated by the morphology of the posterior polar region. Few yolk nuclei at this stage have been seen in this portion of the interior of wild-type embryos; their density near the posterior pole was consistently high in mutant embryos (Figures 5a, 5b).

The Early *engrailed* Function Is Not Maternally Encoded

Two lines of evidence indicate that the abnormal morphology of blastoderm stage *engrailed* mutant embryos reflects an early requirement of zygotic expression. The precellular phenotype was characteristic only of embryos that received mutant *engrailed* alleles from both parents, indicating that the paternal genome can provide the engrailed function prior to cellularization. This would not be expected for a phenotype dependent solely upon maternal endowment.

The precellular phenotype has been described for *engrailed* embryos produced by heterozygous adults with one wild-type and one mutant allele. If the early phenotype were partially dependent on abnormally low expression of the *engrailed* locus during oogenesis, two wild-type copies of the *engrailed* gene in the female might be expected to suppress the phenotype in mutant progeny. Conversely, embryos from females whose germ line contained only mutant *engrailed* alleles might be expected to have more severe abnormalities. Therefore mutant embryos produced by females with three copies of the *en-*

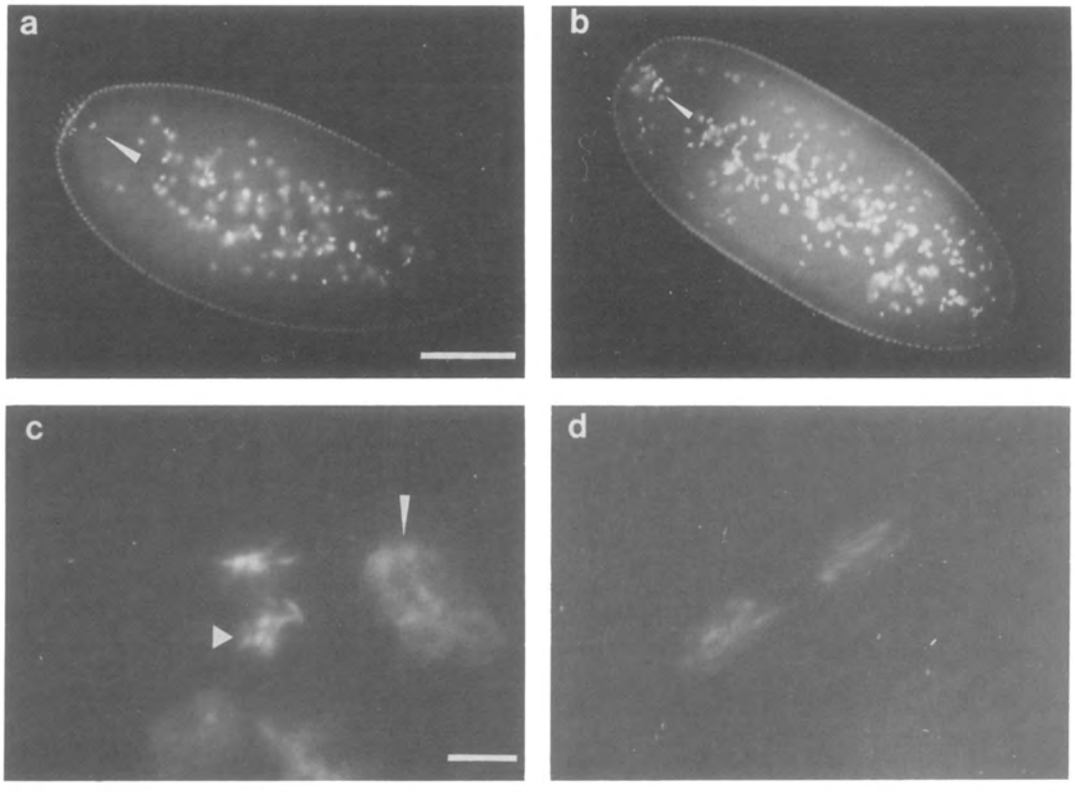

Figure 5. Optical Sections of Fixed Wild-Type and Mutant Embryos

Embryos were fixed, stained with DAPI, and photographed under epifluorescence optics to visualize the interior yolk nuclei. Orientation of low magnification views of wild-type (a) and en^{LA4}/en^{LA7} mutant (b) stage 13 embryos is anterior right. These optical sections reveal the peripheral surface nuclei and the larger (and more brightly staining) polyploid yolk nuclei in the central region. Arrowheads point to yolk nuclei in the posterior polar region. Yolk nuclei of cycle 12 (c) and 13 (d) mutant embryos are seen in mitosis. Condensed chromosomes, indicative of the mitotic state (triangle in c), are readily distinguished from the decondensed, nondividing interphase nuclei (arrowhead in c). In (d), a dividing mitotic chromosome pair during anaphase movement reveals the separating, condensed chromosome strands. Bar = 100 μm (a, b) and 5 μm (c, d).

grailed locus (two of which were wild type and the other mutant—en^{SF7} or en^{28}), by heterozygous females with one wild-type and one mutant copy, were compared with embryos produced by females with germ lines homozygous for either en^{LA4} or en^{LA7}. As expected of a strictly zygotic function, the morphology of cycle 10, cycle 12, cellular blastoderm, or mature mutant embryos from females with these different genetic constitutions was unaffected by maternal genotype.

Expression of the *engrailed* Locus in Syncytial Blastoderm Embryos

Previous studies have demonstrated the presence of a 2.7 kb poly(A)$^+$ engrailed transcript in the embryonic and imaginal tissues that require the *engrailed* gene for their normal morphogenesis (Poole et al., 1985; Drees and Kornberg, unpublished results). The finding that the engrailed function is also essential in precellular embryos suggests that transcription of the gene may be activated during these early developmental stages as well. To test this possibility, RNA extracted from wild-type adult ovaries and from young embryos was subjected to Northern analysis. Oocyte RNA contained no detectable sequences homologous to the *engrailed* gene (Figure 6a). In contrast RNA homologous to the *engrailed* gene was detected in the early embryonic stages examined. One hundred embryos during cleavage (before syncytial blastoderm formation), in division cycles 10, 12, and 14, and in gastrulation were identified under a dissecting microscope, and their RNA was extracted for analysis on Northern blots. A 2.7 kb RNA with homology to a portion of the *engrailed* gene was present in cycle 12 embryos, and it became more abundant with the advancing age of the developing embryos (Figure 6, lanes labeled 12, 14, and early gastrula). The amounts of engrailed RNA in earlier embryos, either during cleavage or in cycle 10, were significantly lower (Figure 6b, lanes pre-9 and 10). The accuracy with which the embryos were staged was separately monitored by fixing and staining identical pools of embryos for examination under fluorescence optics. Fewer than 2% were older than designated and these did not differ by more than one division cycle. Therefore, the RNA detected demonstrates that the *engrailed* gene is expressed during these developmental stages.

Discussion

The *engrailed* locus is active and required during the precellular stages of Drosophila development. The evidence is molecular and morphological: transcripts of the

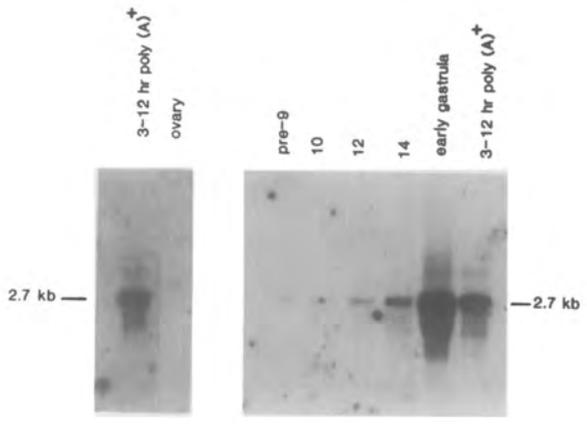

Figure 6. Transcription of the *engrailed* Locus in Ovaries and in Early Embryos

RNA was extracted from hand-picked wild-type embryos before the appearance of pole cells at the posterior end (pre-9), at cycles 10, 12, and 14, and 15 min after commencement of gastrulation (gastrula), and from hand-dissected ovaries. RNA from 50 ovaries and from 100 embryos of each stage was fractionated in a formaldehyde–agarose gel along with poly(A)$^+$-selected RNA (70 ng) that had been prepared from mass collected 3-12 hr embryos. After transfer to nylon, RNA was detected with a 237 bp single-stranded probe from a nonrepetitive, transcribed portion of the *engrailed* gene. (A Xho II–Bam HI restriction fragment at position 840–1066 (Poole et al., 1985) was inserted into M13 mp8 and copied with the large fragment of E. coli DNA polymerase I.) Exposure was for 14 days.

locus were detected in cleavage stage embryos and throughout the syncytial blastoderm stages, and abnormalities were seen in the position of the pole cells and the patterned arrangements of the yolk and peripheral nuclei in *engrailed* mutant embryos. Such pervasive and general abnormalities associated with the absence of a zygotic function in precellular embryos are both unexpected and unprecedented.

This study of *engrailed* mutants is the first to report precellular effects of a zygotic mutant. Yet there have been other indications of early function of the zygotic genome. A maternal-effect mutant, *abo*, has a preblastoderm arrest phase that can be rescued by the paternal complement (Pimpinelli et al., 1985). Also, deletions of whole chromosome arms can arrest embryogenesis prior to nuclear migration (Poulson, 1940; Scriba, 1969), an effect ascribed to gross chromosomal behavior rather than to the absence of essential genes. Should the *engrailed* gene prove to be just one of a class of genes essential for blastoderm formation, then reevaluation of such effects will be needed.

The precellular engrailed phenotype is not associated with or dependent upon maternal engrailed function. Previous studies recorded no increase in the severity of the embryonic cuticular phenotype in embryos produced by females with *engrailed* mutant gene lines (Lawrence et al., 1983). The present findings show that the early mutant phenotype is also independent of the maternal genotype. Only genetically *engrailed* zygotes were found to be morphologically defective, and no expression of the *engrailed* locus has been detected in RNA isolated from ovaries. Thus, at least the paternally derived genome must be expressed during precellular development; maternal information alone appears to be inadequate for organizing the early embryo.

Inasmuch as the early engrailed function is not provided during oogenesis, when is the *engrailed* gene expressed and used in early embryogenesis? We used the dissecting microscope to identify mutant embryos that were developing abnormally as early as division cycle 10. Although earlier abnormalities are not detectable with such light microscopy, earlier expression of the *engrailed* locus is probable for several reasons. Since all break-point and apparent point mutations cause the early phenotype, it seems that it is not simply the gene sequence or transcript, but rather the translation product, that is responsible for the early engrailed function. Sufficient time for making the needed quantities of the engrailed translation product must therefore be provided before the stage where, for lack of it, the mutant embryos become abnormal. Given the 10–12 min mitotic cycle time of the cleavage nuclei, the first expression of the *engrailed* locus must be considerably earlier than division cycle 10.

Evidence for such early transcription was obtained. By staging embryos individually and isolating RNA for analysis with high specific activity probes, these studies of engrailed transcription have detected expression during the syncytial and cellular blastoderm stages and perhaps before cycle nine. Cleavage stage engrailed transcription would represent the earliest direct demonstration of zygotic expression in Drosophila and it would be consistent with the early requirement for engrailed function. Although indications of transcription prior to formation of the syncytial blastoderm have been obtained previously in autoradiographs of embryo sections (Zalokar, 1976) and in spreads of chromatin on electron microscope grids (McKnight and Miller, 1976), interpretations of these earlier results have been equivocal. In contrast, transcription during cleavage stages has been demonstrated in other animals, including other insects (Schenkel and Schnetter, 1979), echinoderms (Rinaldi and Monroy, 1969), amphibians (Brown, 1964), and mammals (Mintz, 1964). For Leptinotarsa (Coleoptera), autoradiographs of embryos have revealed α-amanitin-sensitive RNA synthesis in all stages after formation of the zygotic nucleus (Schenkel and Schnetter, 1979). Our work suggests that the early developmental program of Drosophila also expresses the zygotic genome during the cleavage stages. In Drosophila, the early engrailed phenotype indicates that such expression is functionally significant.

What is the mechanism of *engrailed* gene activation in the zygote? Although our findings do not identify the activating signal, they do suggest that the information is not in the cortex of the embryo. The engrailed phenotype is evident as the pole nuclei bud from the embryo surface at division cycle 10, and the abnormalities are likely to be due to a lack of earlier engrailed expression when the nuclei were more centrally located. Also centrally located and abnormal in *engrailed* embryos are the patterns and mitotic behavior of the yolk nuclei. The hypothesis that cortical determinants designate the fates of naive nuclei

upon their arrival at the embryo periphery does not fit with the observation that the early organization of the embryo depends on the expression of internal nuclei. Whatever the mechanisms that activate the *engrailed* locus and respond to its function at these early stages, it seems reasonable to assume that they do not operate at so great a distance.

Zygotic expression is apparently also critical for the cytoskeletal organization of the embryo, since blastoderm nuclei and their surrounding cytoskeletal structures are perturbed over the surface of mutant embryos. It is noteworthy that the appearance of the individual cytoskeletal domains in all stages of the mitotic cycle is normal, but their patterned arrangement is not. This aspect of the early engrailed phenotype is consistent with later effects of *engrailed* mutants. In the posterior compartments of the later stages, the structures such as hairs, bristles, and various other sensory organs produced by *engrailed* cells are normal but their patterns are not.

An intriguing feature of the *engrailed* locus is the positional specificity of its function: only cells of the posterior compartments depend on it (Lawrence and Morata, 1976). Thus the locus has been seen as a binary developmental switch, selecting a developmental pathway for the posterior compartment cells (Garcia-Bellido, 1975). How is the precellular requirement and expression of the *engrailed* locus to be reconciled with its later, position-specific role? The precellular mutant phenotype does not appear to be confined to isolated portions of the embryo. Rather, the patterns of all three types of nuclei in all parts of the embryo are abnormal. Also, probes for the engrailed transcript have failed to resolve any pattern of *engrailed* expression by in situ hybridization to sections of precellular embryos (Weir and Kornberg, 1985). Thus, we must consider the possibility that the early role of engrailed is not a function of regional localization and that its regulatory roles differ in this respect before and after cellularization.

Conclusion

The *engrailed* gene functions earlier than had been anticipated for this or any zygotic gene. For *engrailed*, a role in regulating pattern formation generally, and not simply in the development of the posterior compartments, is indicated. For other regulatory loci, their participation in the processes that organize the blastoderm warrants further investigation.

Experimental Procedures

Fly Culture

Flies were grown under standard conditions at 25°C either in half-pint milk bottles or in population cages. Strains with *engrailed* mutations (Kornberg, 1981a; Nusslein-Volhard and Wieschaus, 1980; Eberlein and Russell, 1983) were outcrossed to an Oregon R wild type, and the heterozygous *engrailed* progeny were crossed inter se for analysis of the embryonic phenotype. All alleles had normal chromosome morphology except as indicated. Flies were transferred to yeasted food vials for 2–4 hr; the freshly laid embryos were dislodged from the food under water with a paint brush, transferred by pipette to a wire mesh screen, and washed extensively; and the chorion membranes were removed by immersion in a dilute solution of commercial bleach (active ingredient 2.6% sodium hypochorite). Embryos were washed extensively with a Triton X-100 solution (containing 0.03% Triton X-100 and 0.7% NaCl) to remove the bleach and were subsequently transferred in the same solution to petri dishes for microscopic observation.

Germ-line clones were generated by exposing instar II larvae of the genotype $en^{LA4}/FS(2)D$ or $en^{LA7}/FS(2)D$ (Yarger and King, 1971; Schupbach, 1982) with 1000 rad X-rays. Individual females were mated to $en^{LA7}/SM5$ or $en^{LA4}/SM5$ males, respectively, and the progeny of fertile females were collected and examined. Duplication-bearing females were en^{LA4} or $en^{LA7}/+/Dp(2;3)en^{28}$ or $en^{LA4}/en^{LA7}/Dp(2;3)en^{28}$.

Microscopy

Living dechorionated embryos were examined with a dissecting microscope at 50× magnification to identify embryos displaying the mutant phenotype. In addition, selected embryos were placed on a slide between two number 0 microscope coverslips and covered with an 18 mm diameter number 1 coverslip. The edges around the coverslips were carefully sealed with Halocarbon oil (series 27; Halocarbon Products Corporation, Hackensack, N.J.) to prevent evaporation in a manner that allowed oxygen exchange to continue. The embryo was oriented with the dorsal side facing up by rolling the embryo under the number 1 coverslip until the micropyle was facing up. (See Fullilove and Jacobson, 1978, for a description of the external morphology of the Drosophila embryo.) The embryo was observed with Nomarski–DIC optics using Zeiss 16× or 40× objectives, and the images were recorded on Kodak Technical Pan 2415 film. Embryonic development was typically recorded for 2–3 hr or until gastrulation had begun. Embryos were then carefully removed from the slide, placed on agar plates for overnight incubation, and scored for the engrailed cuticular phenotype (Kornberg, 1981a).

Fixed Embryo Preparations

Wild-type and selected *engrailed* embryos were fixed by one of two methods for examination under fluorescence optics. The formaldehyde/methanol fixation method initially described by Mitchison and Sedat (1983) and subsequently modified by Karr and Alberts (submitted) was employed with some additional modifications. Ten to fifteen embryos were pipetted into a 10 ml screw-top tube that contained 2.5 ml of 0.1 M Pipes, .001 M $MgSO_4$, and .001 M EGTA (pH 6.9), supplemented with 1 μM taxol; 2.5 ml of heptane was immediately added, and the solution was rapidly shaken for 20–25 sec; 0.5 ml of a 20% formaldehyde solution was then added, and vigorous shaking was continued for an additional 15 min. Embryos were removed from the heptane–buffer interface with a pipette and were ejected into a mixture of heptane:methanol (50:50; the methanol solution was 90% methanol, 10% H_2O, and .05 M Na_3EGTA), that had been previously cooled to −70°C with dry ice. This suspension was shaken vigorously for 10 min. The embryos were freed of their vitelline membrane by rapidly warming the heptane–methanol mixture to 37°C in a water bath. Embryos without vitelline membranes fell to the bottom of the tube. Alternatively, selected embryos were placed directly into heptane that had previously been shaken with a 25% glutaraldehyde solution as originally described by Zalokar (1976), and the vitelline membranes were removed manually.

Embryos without vitelline membranes were transferred into PBS and prepared for indirect immunofluorescence to reveal microtubule structures as described by Karr and Alberts (submitted) or were stained with DAPI to reveal nuclear morphology as described by Foe and Alberts (1983). Fixed devitellinized embryos were incubated for 1 hr with a primary antitubulin monoclonal antibody (Blose et al., 1984), and following a 2 hr rinse, the embryos were treated with rhodamine-labeled goat anti-mouse IgG for 1 hr. Embryos were rinsed extensively to remove unbound fluorescent antibodies and were stained with 1 μg/ml DAPI (4′,6-diamidino-2-phenyl-indole) for 5 min. Embryos were rinsed a final time in PBS and mounted under coverslips in Fluoromount G (Southern Biotechnologies) and examined with epifluorescence optics using a Zeiss 16×, 25× Plan-Neofluor, or 100× Neofluor objective.

Analysis of Embryonic RNA

Embryos were staged after dechorionation by scoring with a dissecting microscope the lack of pole cells (pre-cycle 9), the presence of pole cells and appearance of peripheral nuclei (cycle 10), clearing of the peripheral cytoplasm and smaller more numerous pole cells (cycle 12), the onset of cellularization (cycle 14), and the formation of the cephalic

furrow (gastrula). Pre-cycle 9 embryos were at least 1 hr after fertilization and were predominantly cycle 7 and 8 embryos.

Total RNA was prepared essentially according to the procedure of Cheley and Anderson (1984). In our adaptation, ovaries or dechorionated embryos were homogenized using a 2 ml Teflon Dounce homogenizer in a total volume of 1 ml 7.8 M guanidine–HCl, 0.1 M potassium acetate. After 5–10 strokes, 0.6 volumes of ETOH were added and the nucleic acid was allowed to precipitate overnight at −20°C. The precipitates were collected by a 10 min centrifugation at 12,000 × g and resuspended in 0.4 ml DEPC treated 10 mM Hepes (pH 7.0), 1.0 mM EDTA with 62.5 μg E. coli tRNA/ml added as carrier. The nucleic acid was again precipitated with 2 volumes ETOH for 1 hr at −70°C. The pellets were resuspended in sample buffer (50% formamide, 2.2 M formaldehyde, 1× running buffer) and the RNA was fractionated in agarose gels containing formaldehyde.

Electrophoresis and blotting conditions were as described by Poole et al. (1985), except that gels were blotted onto MS1 MAGNA NYLON 66 (0.45 μm). Blotting conditions were as described in MS1 Technical Service Bulletin #103. All RNA blots were separately monitored for sequences homologous to a probe for cytoplasmic actin mRNA.

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