Modifying expression of the *engrailed* gene of *Drosophila melanogaster*

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Summary

The *engrailed* gene is required for segmentation of the *Drosophila* embryo and is expressed in cells constituting the posterior developmental compartments. In mutant embryos lacking *engrailed* function, portions of the cuticular pattern in each segment are deleted, resulting in fusion of adjacent denticle bands. Using P-element-mediated transposition, we generated flies that express the *engrailed* gene under the control of an *hsp70* promoter, and found that ectopic, heat-shock-induced, *engrailed* expression caused pattern defects similar to those in embryos lacking *engrailed* function. Sensitivity to heat shock was only during the cellular blastoderm and early gastrulation periods. This window of sensitivity corresponds to the time when wild-type *engrailed* protein localizes into segmentally reiterated stripes and represents only a small portion of the normal period of *engrailed* gene expression.

Key words: segmentation gene, *engrailed*, compartments, *Drosophila melanogaster*.

Introduction

Among the regulatory genes directing *Drosophila* development, three general categories have been identified. These are: (a) maternal effect genes, which are expressed during oogenesis and which specify the structure and the spatial polarities of the egg (Nüsslein-Volhard, 1979; Schüpbach & Wieschaus, 1986); segmentation genes, which determine the number and polarity of the segments (Nüsslein-Volhard & Wieschaus, 1980); and homeotic genes, which are responsible for the identity of each segment (Garcia-Bellido, 1977; Lewis, 1978). Such groupings successfully categorize the function of more than 40 genes whose mutant phenotypes suggest a role in regulating the development of the fruit fly. However, the *engrailed* gene, which is unusual for its varied roles and for its persistent expression throughout the life of the fly, does not fit easily into any of these single categories.

The *engrailed* gene was first identified in 1926 by a spontaneous mutation, *en*¹ (Eker, 1929). Recent genetic studies using chemical mutagens, X-rays and hybrid dysgenesis have yielded a large number of point mutant, breakpoint mutant, insertion and deletion alleles (Nüsslein-Volhard & Wieschaus, 1980; Kornberg, 1981a; Eberlein & Russell, 1983; Gubb, 1985; Gustavson, Ali & Kornberg, unpublished data). These mutants define a single lethal complementation group with a lethal period during late embryogenesis. *engrailed* mutant embryos have a variety of defects, with the most striking cuticular abnormalities being the deletion of the naked cuticle between denticle bands, resulting in the fusion of adjacent denticle bands and loss of clear segmental subdivisions. Thus, *engrailed* helps to establish the segmental organization during embryogenesis, and can be classified among the segmentation genes.

Subsequently, in the primordia of the adult integument, the *engrailed* gene helps to maintain the segmental borders and also provides a posterior compartment identity to a portion of the cells within each of the imaginal segments (Garcia-Bellido & Santamaria, 1972; Morata & Lawrence, 1975; Kornberg, 1981a,b; Lawrence & Struhl, 1982). Cells lacking *engrailed* function develop normally in the anterior compartments, but in the posterior compartments they grow across the compartment and segment boundaries which normally restrict their growth, and they do not form normal posterior structures. Occasionally they produce anterior structures in posterior locations. These phenotypes suggest a homeotic transformation.

In addition to its role in segmentation and in establishing posterior compartment identity, wild-type *engrailed* function is essential during the precel-
lular stages of embryogenesis, when rapid nuclear divisions produce the somatic, germ cell and yolk nuclei (Karr et al. 1985). engrailed mutant embryos have abnormal distributions and numbers of these nuclei. It is not clear how this very early engrailed function relates to its later roles.

The engrailed gene has been isolated in recombinant form (Kuner et al. 1985; Poole et al. 1985; Fjose & Gehring, 1985). It has a single small transcription unit (Drees et al. 1987) that encodes a homeobox-containing nuclear protein (Poole et al. 1985; DiNardo et al. 1985). The engrailed gene is expressed in the posterior compartment cells of the embryonic ectoderm and of the hindgut, in a portion of the cells in the nervous system and in the posterior compartment cells of the primordia of the adult cuticle (Kornberg et al. 1985; Weir & Kornberg, 1985; DiNardo et al. 1985; Brower, 1986; Hama & Kornberg, unpublished data).

Unresolved at present is the relationship between the different patterns of engrailed expression and the pleiotropic engrailed phenotype. Included among the several different types of engrailed mutations are deficiencies that completely remove the engrailed region (Kornberg, 1981a; Eberlein & Russell, 1983; Gubb, 1985), nonsense alleles that encode truncated engrailed proteins (Gustavson & Kornberg, unpublished data), and breakpoint mutations that separate portions of the engrailed regulatory sequences from their structural gene (Kuner et al. 1985). The majority of these lesions reduces wild-type engrailed function (Weir, Ali & Kornberg, unpublished data) and generates embryos in which the naked cuticle between alternate denticle bands is deleted (e.g. Fig. 3A). In the most severely affected embryos, the naked cuticle between each segment is deleted, resulting in embryos with a lawn of denticle hairs on the ventral surface. The regions deleted represent the posterior compartments and the caudal portion of the preceding anterior compartments, and include the anterior/posterior compartment borders (Hama & Kornberg, unpublished data). Since the engrailed gene is expressed only in the posterior compartment of each segment, the regions missing in engrailed mutant embryos extend beyond the realm of engrailed expression.

The engrailed gene is one of a number of genes that in mutant embryos result in defects in the cuticular pattern (Nüsslein-Volhard & Wieschaus, 1980). For the pair-rule class of segmentation genes (such as fushi tarazu (ftz) and hairy (h)), the regions in which the genes are expressed in wild-type embryos give rise to the portion of the cuticular pattern deleted in mutant embryos, suggesting that expression in these regions is required for normal patterning (Hafen et al. 1984; Ingham et al. 1985). For either of these genes, induction of ectopic expression in early development leads to cuticular pattern deletions approximately reciprocal to those caused by lack of expression of the genes (Struhl, 1985; Ish-Horowicz & Pinchin, 1987). This suggests that lack of expression in the cells in which the genes are normally not expressed is also important for generation of the cuticular pattern (Struhl, 1985; Ish-Horowicz & Pinchin, 1987; Ish-Horowicz, this volume).

A similar comparison of the phenotypes resulting from lack of expression and from ectopic expression should help to clarify the role of the engrailed gene. For instance, since the pattern deletions caused by lack of engrailed expression include the posterior compartments, ectopic expression might be expected to delete all or part of the anterior compartments. In this communication, we demonstrate the importance of the precise quantitative and position-specific control of the engrailed gene by regulating engrailed expression with a heat-shock promoter. Induction of engrailed expression with this heterologous promoter affects both segmentation and viability, and the pattern defects that result are not reciprocal to, but instead are similar to, those of engrailed mutant embryos.

**Ectopic expression of the engrailed gene**

To evaluate the role of engrailed function, we have expressed the engrailed protein at times and places that normally lack engrailed expression. Fly strains containing engrailed coding sequences under the control of an inducible promoter (the Drosophila hsp70 heat-shock promoter) were generated using P-element-mediated germline transformation (Spradling & Rubin, 1982). The transforming DNA was a modified Carnegie-20 P-element vector (Rubin & Spradling, 1983) with an insert of approximately 4·4 kb consisting of a 0·4 kb hsp70 promoter fragment (provided by H. Stellar), a 2 kb EcoR1 fragment from the engrailed cDNA clone c2.4 (Poole et al. 1985), and a genomic DNA fragment containing the engrailed polyadenylation region (Fig. 1). The downstream EcoR1 site in the cDNA clone is the upstream site in the genomic polyadenylation fragment, thereby reconstructing the engrailed 3' untranslated region and polyadenylation site. Five independent transformed lines were obtained. One of these lines, designated hs-en3, contains a single P-element insertion on the second chromosome and is homozygous viable. The hs-en3 strain was analysed in detail, but similar results were obtained with a second independent line, hs-en41. These strains also contain endogenous wild-type engrailed alleles on their second chromosomes.

To establish that the fusion hs-engrailed gene is
Heat shocking embryos aged nuclear cycle 13 or earlier strongly reduced viability of both the parental and hs-en3 embryos. Because the control embryos were affected, we have not attempted to determine whether the presence of the hs-engrailed gene had specific effects. For the parental strain, heat-shock treatment at cellular blastoderm or later stages had no effect on either viability or cuticular patterns. However, heat-shock treatment of similarly aged hs-en3 embryos had substantial effects, and the nature and severity of the effects depended upon the time at which the embryos were heat shocked.

The viability of cellular blastoderm hs-en3 embryos was markedly decreased after heat shock. In independent experiments, the percentage of larvae that hatched from the vitelline membrane varied from 5 to 35% (the variability is presumed to be a consequence of slight differences in the heat-shock treatments or to slight variations in the age of the embryos; most hatched larvae also showed similar cuticle defects). A wide variety of defects were observed among the unhatched embryos that had been heat shocked at the cellular blastoderm stage (Fig. 3). First, most of the embryos had defects in the head region. These defects included missing mouthpart structures and misshapen heads, and have not been characterized in detail. Second, >95% of the embryos had abnormal denticle belts. Types of defects varied considerably, ranging from the absence of all or part of one or two abdominal denticle bands (Fig. 3B,C), to apparent fusions of one or more adjacent pairs of abdominal denticle bands (Fig. 3D), to a complete absence of segmentally arranged denticle belts in small severely disrupted embryos having only a short lawn of denticles or patches of denticles (Fig. 3E,F). In many embryos, defects were not symmetric about the anterior–posterior axis. In most, the abdominal denticle bands were disrupted, although due to the severity of the effects, it was usually not possible to identify definitively which of the denticle bands were missing or fused.

Hs-engrailed induction in gastrulating embryos results in engrailed-like pair-rule defects

Gastrulating hs-en3 embryos were also affected by
heat-shock treatment. As with the younger embryos, the majority failed to hatch. Of the embryos that failed to hatch, almost all had a variety of cuticular defects. These defects included abnormal head structures and defects in denticle belt patterns. However, in contrast to the irregular phenotypes produced by heat-shocked cellular blastoderm embryos, gastrulating embryos gave rise to less variable effects. Embryos that had been heat shocked at gastrulation exhibited a series of phenotypes ranging from fusions of a single adjacent pair of abdominal denticle belts, to more extreme pair-rule type fusions of several pairs of denticle belts. The pair-rule phenotypes were in many cases similar to the effects of strong engrailed alleles (Fig. 4). For example, in enLA4 mutant embryos, thoracic denticle bands T3 and abdominal band A1 fuse, as do denticle bands A2 and A3, A4 and A5, and A6 and A7 (Fig. 4A). Heat-shocked hs-en3 gastrulae produced segment fusions with similar patterns (i.e. A2/3, A4/5 and A6/7, with the A6/7 fusion being the most prevalent) (Fig. 4B,C). In the most extreme cases, the abdominal denticle bands fused into a narrow lawn of denticles (Fig. 4D).

**A narrow time window of sensitivity to hs-engrailed induction**

In wild-type embryos, strong engrailed gene expression can first be detected just prior to gastrulation in a single stripe of cells in the posterior compartment of parasegment 2 (Weir & Kornberg, 1985; DiNardo et al. 1985; Karr et al. in press). During early gastrulation, the gene is expressed in a rapidly changing series of striped patterns, culminating during germ band extension in strong expression in all posterior compartments along the length of the embryo. The temperature-sensitive period of hs-en3 embryos for cuticular pattern defects was restricted to the cellular blastoderm stage and the beginning of
Fig. 3. Heat-shock induction of hs-en3 embryos at cellular blastoderm. Hs-en3 flies were allowed to lay eggs on freshly yeasted agar plates for several hours. Embryos were collected and dechorionated with bleach. Embryos at cellular blastoderm stage were selected, and transferred in a small volume of liquid to a prewarmed small glass dish supported in a 36°C water bath and containing prewarmed NaCl/Triton solution. The dish was covered and incubated for 30 min with occasional agitation. At the end of the heat-shock period, the embryos were transferred to a small agar plate. Excess liquid was blotted away, and the agar plate was covered and placed in a humidified chamber at 25°C for 24 h. The vitelline membranes were removed from unhatched embryos before mounting in Hoyer’s lactic acid (1:1).

(A) Cuticle of a wild-type embryo for comparison. (B–F) Representative cuticle preparation of hs-en3 embryos heat shocked at cellular blastoderm. A wide variety of pattern defects can be seen. Most embryos had misshapen heads and missing head structures. Denticle band pattern defects ranged from irregular holes in the denticle bands and fusions of denticle bands (B,C,D), to patchy lawns of denticles (E,F).
gastrulation. This is the period during which the *engrailed* protein localizes to stripes in posterior compartments. Hs-en3 embryos that were heat shocked during germ band extension, when the striped pattern of wild-type *engrailed* expression has already been established, were less affected than were younger embryos. 75% of germ band extended embryos hatched after heat shock. Most of the hatched larvae had a wild-type cuticular pattern, although a few larvae had a single pair of denticle bands fused on one side. Of the embryos that failed to hatch, ~30% appeared to have normal denticle patterns, although some of these also had head defects. Among the remaining embryos, a range of increasingly severe pair-rule fusions were seen. As with gastrulating embryos, the registration of segment fusions was similar to that of *engrailed* mutants.

To define the window of heat-shock susceptibility more precisely, hand-selected hs-en3 embryos just beginning to invaginate the cephalic furrow were either immediately heat shocked or allowed to age 30 or 60 additional minutes at 25°C before heat-shock treatment. As before, most hs-en3 embryos treated at the cephalic furrow stage failed to hatch, and they developed with head defects and a distribution of increasingly severe fusions of pairs of denticle bands in the same registration as *engrailed* mutants; the most strongly affected embryos had only a short and
narrow lawn of denticles. However, embryos heat shocked only 30 min later in development had essentially normal denticle belts, although there was still significant lethality and a high proportion of head defects. Heat-shock induction 60 min after cephalic furrow formation yielded similar results.

In wild-type embryos, the engrailed gene is expressed throughout development in the progenitors of the adult integument (Kornberg et al. 1985; Drees et al. 1987; Brower, 1986; Hama & Kornberg, unpublished data). Nevertheless, heat-shock treatments of hs-en3 animals had no discernable effects on larval or adult morphology. Adult flies that eclosed after daily 30 min heat-shock treatments throughout the larval and pupal periods had no detectable decrease in viability, nor did they have any noticeable defects. Thus, there is only a narrow time window in development during which hs-en3 embryos are sensitive to ectopic expression of the engrailed gene.

Conclusion

These experiments describe the consequences of changing the highly position-specific and time-specific program that controls expression of the engrailed function. engrailed expression was induced from the hsp70 promoter, a promoter that is not cell-type specific. During a brief period of embryogenesis (cellular blastoderm and very early gastrulation), segmentation was severely disrupted. We will discuss two aspects of these results that were unexpected: first, that the effects on segmentation were similar to the consequences of loss of function mutations in the engrailed gene and, second, that the temperature-sensitive period was much narrower than the period of wild-type engrailed expression in posterior compartments.

The hs-en phenotype

Among the extant engrailed mutants, three types fail to produce active engrailed protein: point mutants that truncate the engrailed protein (e.g. the nonsense mutants enLA4, enLA7 and enLA10), deletions that lack the entire coding sequence (e.g. enSFX31, enA and enB), and breakpoint mutants that separate the coding sequence from its upstream promoter elements (e.g. In(2R)enSF49, T(2;3)enSF42 and T(2;3)enSP62). These mutants are embryonic lethals and their segmentation is disrupted to variable extents. In the most extreme examples, cuticle morphology has little segmental character and the denticles form a lawn covering much of the ventral surface. Embryos affected less severely have the naked cuticle of alternate segments deleted and appear as if alternate pairs of denticle bands have fused together in a pair-rule fashion. The registration of fusion is thoracic segment T1 with T2, T3 with abdominal segment A1, A2 with A3, A4 with A5, and A6 with A7. Hs-en3 animals induced by heat treatment during the very beginning of gastrulation have fused segments with the same registration.

How is it that loss of engrailed expression and constitutive overexpression of engrailed have similar effects? This observation is particularly puzzling in light of similar experiments carried out on animals transformed with hs-fz (Struhl, 1985) or hs-h (Ish-Horowicz & Pinchin, 1987) fusion genes. In these cases, heat treatment affected segmentation in a manner complementary to the respective mutant phenotypes.

The consequences of engrailed under- or over-expression are perplexing. Although engrailed mutants have lost engrailed function in their posterior compartment cells (Gustavson, Weir, Ali and Kornberg, unpublished data), it is not clear how the mutant phenotype – deletion of the naked cuticle in alternate segments or every segment – actually arises. Moreover, with respect to hs-en3 overexpression, it is not clear whether these same phenotypes arise from expression of the hs-engrailed gene in anterior cells (which do not normally express the gene), or from overexpression of engrailed RNA or protein in posterior compartment cells (which normally do express the engrailed gene, but presumably at lower levels). There are a number of ways to interpret the effects of the hs-engrailed gene induction.

One possibility is that during cellularization and early gastrulation, posterior compartment cells require a precisely regulated level of engrailed expression for viability. In engrailed mutant embryos, lack of engrailed function in these cells inhibits growth and, in hs-en3 embryos, overexpression of engrailed in the same cells does so as well. In both cases, the same cuticular phenotype would then result from lack of growth of the posterior compartment cells.

A second possibility is that levels of engrailed expression later in development are sensitive to the amount of engrailed present during the temperature-sensitive period for hs-engrailed induction, when wild-type engrailed protein first localizes into stripes. Thus, overexpression in posterior compartment cells during early embryogenesis may repress the endogenous engrailed genes (or genes controlled by the endogenous engrailed genes) later, resulting in a phenotype similar to engrailed mutants. It has been suggested that engrailed expression is controlled by separate regulatory systems during early and late embryogenesis (DiNardo et al. 1988). In addition to being regulated by other segment polarity genes (DiNardo et al. 1988; Martinez Arias et al. 1988), it is
possible that the later regulation is sensitive to earlier levels of engrailed protein or RNA.

Finally, it has previously been proposed that juxtaposition of engrailed-expressing (posterior compartment) and engrailed non-expressing (anterior compartment) cells is of paramount importance in establishing and maintaining the segmental pattern (Kornberg, 1981b). What engrailed loss of function mutants and hs-en3 animals have in common is the loss of clear distinctions between engrailed-expressing and non-expressing cells. In the mutants, the difference between anterior and posterior compartment cells diminishes due to loss of engrailed function in the posterior cells. In hs-en3 animals, engrailed expression in the anterior cells renders both compartments en+. If engrailed acts to define borders between groups of cells, and does so by juxtaposing cells with different states of engrailed expression, then the similar phenotypes arising from over- and under-expression may be understood as similar outcomes when the engrailed-dependent differences between anterior and posterior cells are blurred.

Time dependence of the hs-en phenotype
The hs-engrailed effects on the cuticular pattern could only be elicited during the cellularization and early gastrulation period. This was unexpected, since the endogenous engrailed genes continue to be expressed in posterior compartment cells after gastrulation (Kornberg et al. 1985; DiNardo et al. 1985). In addition, the engrailed protein pattern in patched mutant embryos is initially normal, but at germ band extension a new stripe of engrailed protein appears in each segment and the cuticles of patched mutant embryos have duplicated segment borders (DiNardo et al. 1988; Martinez Arias et al. 1988). Assuming that the duplicated pattern elements are a direct result of the extra bands of engrailed expression in patched mutant embryos, why should expression induced by heat-shock treatment have little effect during germ band elongation when expression in the same cells of patched mutant embryos has such extreme consequences? It is possible that the failure of the hs-engrailed gene to affect development after early gastrulation is a consequence of the transient nature of the heat-shock induction. The half-life of the engrailed protein is less than 15 min in early gastrulae (Weir et al. 1988), and engrailed expression in heat-shocked embryos may not persist as long as in patched embryos. The transient nature of heat-shock induction may also explain why daily 30-min heat-shock treatments of hs-en3 animals during larval and pupal growth had no discernable effects on development of the adult pattern.

It should be noted that a 20-min heat shock caused embryos carrying a heat shock―Antennapedia con-

struct to produce extra denticle bands, and that the period of sensitivity extended to embryos 5 h old (Gibson & Gehring, 1988). In addition, 1–2 h heat-shock treatments caused a transformation of antenna to leg in larvae carrying this construct (Schnewly et al. 1987; Gibson & Gehring, 1988). It may be that the unusual and extreme time dependence of hs-engrailed correlates with a changing role for the engrailed function during embryogenesis. For example, there may be different requirements for establishment (during cellular blastoderm) and maintenance of engrailed expression (after gastrulation commences), and the consequences of ectopic expression may change with time. Thus, by germ band extension, the anterior compartment cells may not be competent to respond to the new engrailed expression induced by heat shock, or the posterior compartment cells may have become insensitive to the induced changes in level of engrailed expression.

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References


