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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 & Weischaus, 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 & Weischaus, 1980; Kornberg, 1981*a*; Eberlein & Russell, 1983; Gubb, 1985; Gustavson, Ali & Kornberg, unpublished data).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, 1981*a*,*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, 1981*a*; 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 *Eco*R1 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 *Eco*R1 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

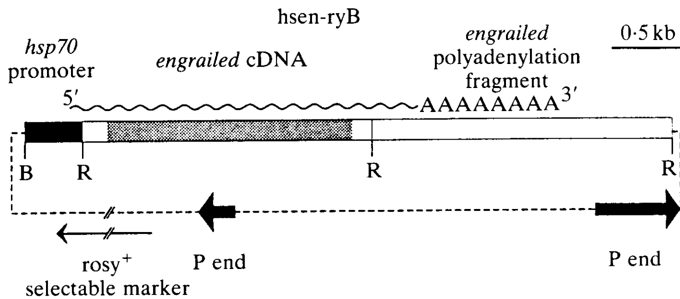

Fig. 1. Structure of the hs-*engrailed* P element used for transformation. A map of the DNA used to generate the hs-*engrailed* transformed flies is shown. The 0·4 kb *Bam*H1–*Eco*R1 fragment containing the *hsp70* promoter (gift of H. Stellar) was fused upstream of the 2 kb *Eco*R1 fragment from *engrailed* cDNA clone c2.4 (Poole *et al.* 1985) containing the *engrailed* protein-coding sequences. Downstream from this was fused the 2·1 kb genomic *Eco*R1–*Bam*H1 fragment containing the *engrailed* polyadenylation site. This hs-*engrailed* fusion gene was inserted into the *Hpa*l site of the P-element vector Carnegie-20 (Rubin & Spradling, 1983). The wavy line represents the *engrailed* transcript originating from the *hsp70* promoter. The *engrailed* open reading frame is shown in light grey. The 10·8 kb Carnegie-20 vector (dotted line) is not drawn to scale. This vector contains P-element terminal repeats (arrows) and *rosy*$^+$ selectable marker. B, *Bam*H1 site; R, *Eco*R1 site. There are additional *Bam*H1 and *Eco*R1 sites that are not shown.

heat inducible in the transformed lines, embryos 0–12 h postfertilization from either hs-en3 or the parental strain were heat shocked, and RNA and protein were isolated and probed for the presence of the hs-*engrailed* gene product. Relative to the parental strain, the hs-en3 embryos produced greater amounts of both *engrailed* RNA (Fig. 2A) and protein (Fig. 2B) upon heat-shock induction. RNase protection assays confirmed that this *engrailed* RNA initiated from the hsp70 promoter (not shown). When heat-shocked hs-en3 embryos were immunostained with an anti-*engrailed* antibody, low levels of *engrailed* protein were detected in all nuclei in addition to the usual *engrailed* stripes (not shown). Thus, after heat-shock treatment embryos carrying the hs-*engrailed* gene produce *engrailed* protein in excessive amounts and at inappropriate locations.

Induction of the hs-*engrailed* gene disrupts segmentation

To determine how the heat-induced *engrailed* gene expression affects embryonic development, hs-en3 embryos at different stages were heat shocked and returned to 25 °C to continue development. Embryos of the same age from the parental stock were treated similarly. Embryos were separated by hand into three classes: cellular blastoderm (embryos in nuclear cycle 14 with visible cell membrane furrows), gastrulating (embryos with cephalic and ventral furrows) and germ band extension (in the process of germ band extension or fully extended). Embryos of each class were heat shocked at 36 °C for 30 min and then placed on agar plates at 25 °C for an additional 24 h. The numbers of embryos that failed to hatch were noted, and hatched larvae and unhatched embryos were examined for cuticular phenotypes.

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 bands 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

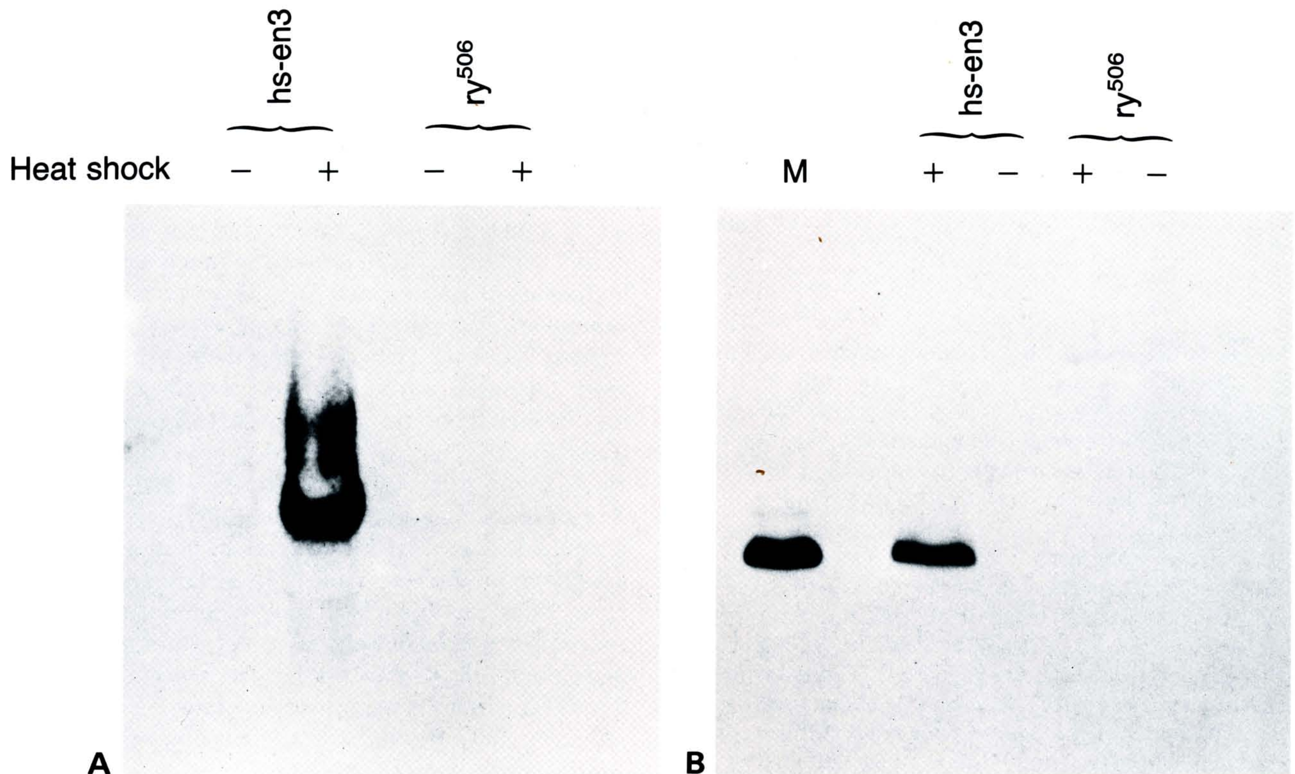

Fig. 2. Heat-shock induction of hs-*engrailed* RNA and protein in hs-en3 embryos. (A) Hs-en3 or parental strain ry^{506} embryos from an overnight collection were dechorionated with bleach and heat shocked in 0·7 % NaCl, 0·04 % TritonX100 in small covered glass dishes in a water bath at 37°C for 30 min ('+' lanes) or maintained under the same conditions at 25°C ('–' lanes). A Northern blot of RNA isolated from these embryos was probed with an *engrailed* cDNA clone that will recognize transcripts originating from both the endogenous *engrailed* genes and the hs-*engrailed* genes in hs-en3 flies. Both transcripts run as the same size bands on Northern blots. Upon heat shock, large amounts of *engrailed* RNA are induced in the hs-en3 embryos compared with the ry^{506} embryos. On longer exposures, the endogenous *engrailed* RNA can be seen in the ry506 lanes. (B) Hs-en3 or ry^{506} embryos from an overnight collection were heat shocked ('+') or maintained at 25°C ('–'). Protein extracts were electrophoresed on an SDS–polyacrylamide gel and transferred to nitrocellulose. *engrailed* protein was detected with an anti-*engrailed* monoclonal antibody (Karr *et al.* in press) and ^{125}I-secondary antibody. Upon heat shock, large amounts of *engrailed* protein are induced in the hs-en3 embryos. Lane M, *engrailed* protein expressed in engineered Schneider 2 cells run as a marker.

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 en^{LA4} 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

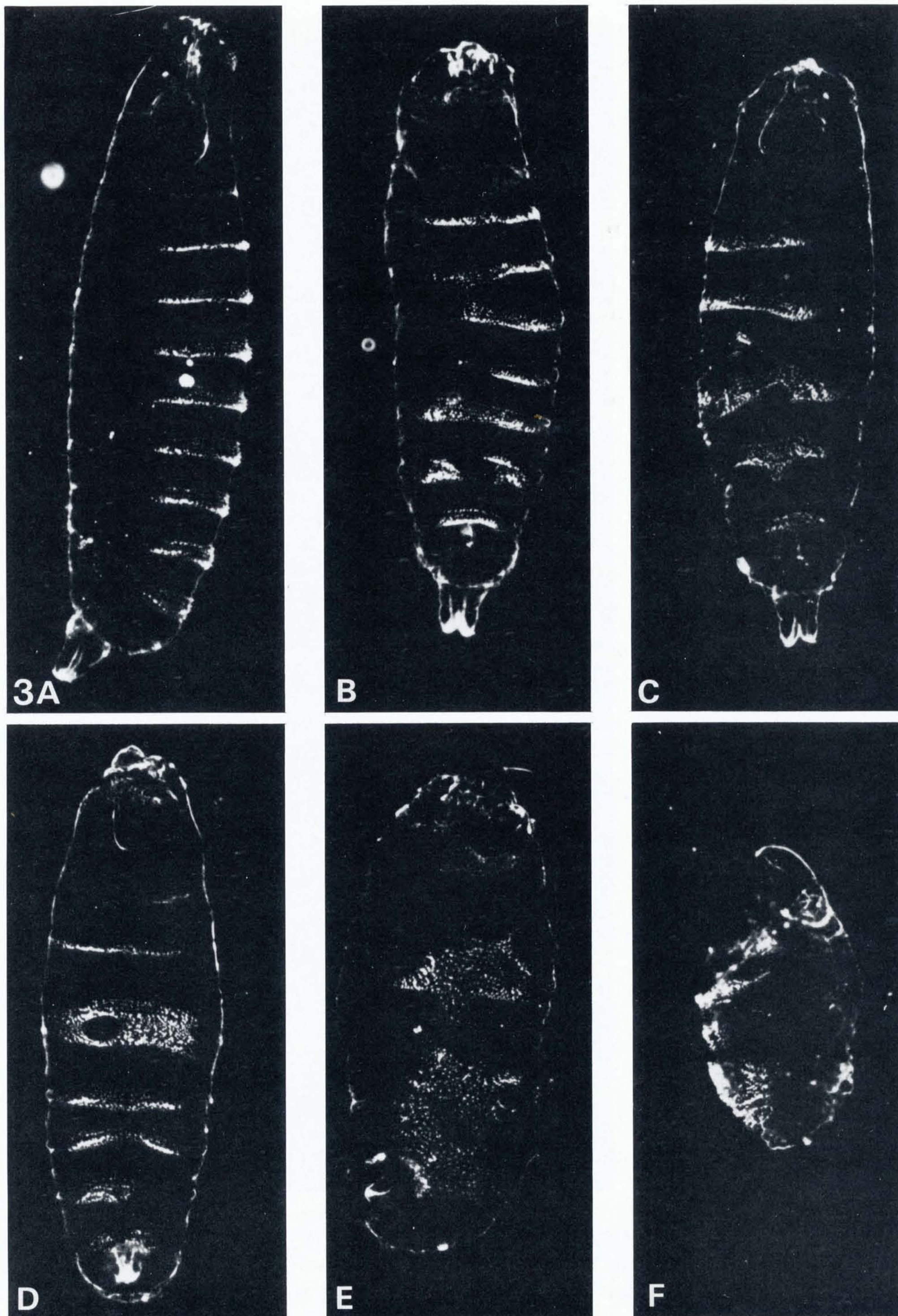

Fig. 3. Heat-shock induction of hs-en3 embryos at cellular blastoderm. Hs-en3 flies were allowed to lay eggs on freshly yeasted agar plates for several hours. Embryos were collected and dechorionated with bleach. Embryos at cellular blastoderm stage were selected, and transferred in a small volume of 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).

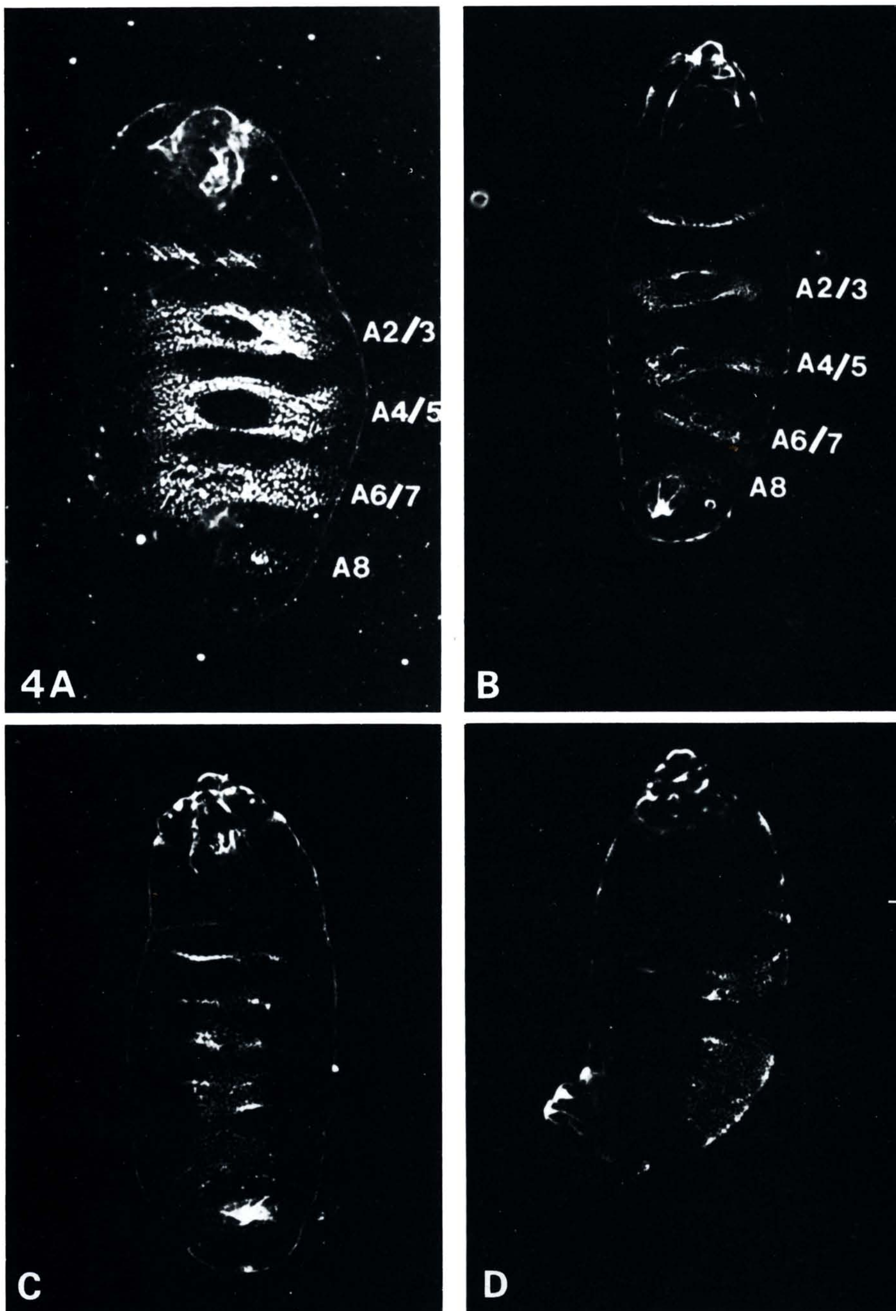

Fig. 4. Heat-shock induction of hs-en3 embryos during early gastrulation. Hs-en3 embryos were dechorionated, and embryos just beginning to invaginate the cephalic furrow were collected and heat shocked at 37°C.
(A) $en^{LA7}/Df(2R)en^{SFX31}$ mutant embryo showing the segment fusions typical of strong *engrailed* alleles. The naked cuticle of abdominal segments T3, A2, A4 and A6 is missing, resulting in the apparent fusions of segments T3/A1, A2/A3, A4/A5 and A6/A7.
(B–D) Typical cuticle preparations of hs-en3 embryos heat shocked during early gastrulation. Many showed a cuticle pattern similar to that seen in *engrailed* mutant embryos (compare A and B). The most severely affected embryos showed greater extents of segment fusions, particularly in the abdominal segments (C,D).

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 en^{LA4}, en^{LA7} and en^{LA10}), deletions that lack the entire coding sequence (e.g. en^{SFX31}, en^{A} and en^{B}), and breakpoint mutants that separate the coding sequence from its upstream promoter elements (e.g. In(2R)en^{SF49}, T(2;3)en^{SF42} and T(2;3)en^{SF62}). 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-*ftz* (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, 1981*b*). 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* construct 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 (Schneuwly *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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