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Multiple modes of *engrailed* regulation in the progression towards cell fate determination

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Abstract

The *engrailed* gene product of *Drosophila* specifies the fate of a subset of cells in each segment. Our studies of *engrailed* regulation suggest that fate determination is an elaborate, multistep process. At the time in embryogenesis when the *engrailed*-dependent cell fate is probably determined, four modes of control act in an overlapping progression to govern *engrailed* expression. After activation by *pair-rule* genes, both an extracellular signal, *wingless*, and autoregulation are required for *engrailed* expression. Autoregulation graduates to *wingless* independence, but is transient, and is superseded by an *engrailed*-independent mode of maintenance.

Two levels of commitment have been defined for the choice of cell fate¹. Early in development, fate can be instructed by environmental cues. This fate specification is flexible and allows fate to change in response to extracellular signals. But cells can undergo a transition from this type of specification to one which is determined and therefore irreversible. Because a determined cell maintains a particular fate independent of its environment, the transition to a determined state requires that development comes under cell-intrinsic, rather than cell-extrinsic control. The identification of selector genes in *Drosophila* provides an opportunity to study the basis of both types of cell-fate control. Selector genes encode regulators whose function defines the fate of a cell, and whose continued expression stably maintains cell fate^{2,3}. Where the choice of cell fate is initially dependent on the environment, selector gene expression must be sensitive to extracellular signals. By contrast, once cell fate is determined, expression of these genes must be governed exclusively by cell-intrinsic factors.

The *engrailed* (*en*) gene is a selector gene that distinguishes two populations of cells in each developing embryonic segment, the progenitors of the anterior and posterior compartments^{3,4}. This distinction can be seen as a lineage restriction when individual embryonic cells are marked by mitotic recombination^{5,6}. These give rise to clonal patches in the adult that never span the boundary between the anterior and posterior compartments, demonstrating that, from the time of marking, the progeny of a given cell only contribute to one compartment. This restriction requires expression of *en* by posterior cells^{3,4,7}. But it seems that the onset of localized *en* expression in early embryos is not sufficient to irreversibly determine posterior compartment identity because *en* requires at least one cell-extrinsic input at this time.

This input is provided by the *wingless* (*wg*) gene, expressed in segmentally repeated stripes of cells that lie next to *en*-expressing cells^{8,9}. The *wg* protein, which is homologous to the product of the proto-oncogene *Wnt-1* (ref. 10), is secreted and appears to be taken up by *en*-expressing cells⁹. In *wg* mutant embryos, *en* expression is initiated in a normal striped pattern but soon stops, indicating that continued *en* expression in wild-type embryos requires *wg* in

neighbouring cells^{11,12}. Here we show that *en* expression becomes independent of this extracellular influence and comes to rely on positive autoregulation. But autoregulation is transient and so does not supply a mechanism for determination of the *en*-cell fate. We show that autoregulation is followed by a still-later-acting mode of *en* control. These transitions may be the progression from a stage in which cell fate is influenced by interactions with neighbours to a later stage in which cells are stably determined.

Transition to *wg* independence

If anterior and posterior compartments become determined, then the selector gene *en* must become independent of the extracellular *wg* influence. Previous experiments with a temperature-sensitive(*ts*) *wg* allele demonstrated that, during embryogenesis, there are changes in the requirement for *wg* in body patterning¹³. To test whether *wg* becomes dispensable for *en* expression, *wg^{ts}* embryos were shifted from permissive to restrictive temperature at half-hour intervals beginning at cellular blastoderm (2.5 h). After further development at restrictive temperature, the embryos were fixed and stained with anti-*en* antibodies (Fig. 1). When *wg^{ts}* embryos are shifted to nonpermissive temperature before 4 h of development, *en* expression decays prematurely in the thoracic and abdominal ectoderm (compare mutant expression in Fig. 1a with wild type in Fig. 2b). Loss of *en* parallels that seen in *wg* null mutants^{11,12}. (We do not consider the regions of the embryo where *en* expression does not involve *wg* control: the head, the first three *en* stripes and the central nervous system (CNS) in each segment.) Between 4 and 5 h, inactivating *wg* has progressively less effect on *en* expression. For example, when *wg* is inactivated at about 4.5 h, a few cells in each segment persist in expressing *en* (Fig. 1b). After 5 h, removing *wg* function has no apparent effect on *en* expression (Fig. 1c). The striped pattern is indistinguishable from that of *wg^{ts}* embryos raised at permissive temperature. We conclude that *en* expression becomes independent of the *wg* signal at about 5 h of development. Thus, different regulators maintain *en* past this time.

Requirement for *en*

Studies of expression of mutant *en* alleles suggest that *en* undergoes autoregulation. The *en^{CXI}* allele is a rearrangement (R. Holmgren, personal communication) that truncates the *en* transcription unit (data not shown) and produces a cytoplasmic protein. For this reason, and because *en^{CXI}* shows a severe *en* phenotype (S.D., unpublished observation), we consider this a nonfunctional protein. We can monitor expression of this allele using an anti-*en* antibody and distinguish it from the nuclear wild-type *en* product because *en^{CXI}* is cytoplasmic. The initial activation of *en^{CXI}* expression occurs normally (compare Fig 2a with c). But in embryos homozygous for *en^{CXI}*, striped expression in the ectoderm stops during full germ-band extension (Fig. 2d), a time when *en* is easily detectable in wild-type embryos^{7,14} (Fig. 2b). Thus, the normal persistence of *en* expression past this stage is directly or indirectly dependent on the action of *en* itself.

A second mutant *en* allele, *en^{IM}*, has a stop codon in the homeodomain that truncates the protein upstream of the DNA binding motif (Gln at position 497 mutated to the stop codon TAG; J. Little and P.O'F., unpublished data). RNA *in situ* hybridization shows that *en^{IM}* mutant embryos fail to maintain *en* transcripts (data not shown). The behaviour of these mutant alleles demonstrates that maintaining *en* expression requires *en* function.

Autoregulation of *en*

To test whether *en* activates its own expression we constructed transgenic flies that carry the *en* gene driven by a heat-shock promoter (Fig. 3a). Embryos that carry this construct (*hs-en*) produce *en* transiently in all cells after a brief heat shock (Fig. 3b). We examined the effect of this protein in cells that do not normally express *en*. Inducing global *en* expression by heat

shock had a different effect early, during the *wg*-dependent period for *en*, than it did later, at *wg*-independent stages.

Our standard early heat-shock protocol consists of heat-shocking embryos at 3–3.5 h of development. On recovery, the globally induced *en* expression resolves into stripes that are abnormally broad as a consequence of new *en* expression in the ventral and lateral regions (compare Fig. 3c and d). This new expression pattern is stable and persists to at least 12 h of development when cells begin to differentiate (data not shown).

To verify that the novel *en* pattern results from activation of the endogenous *en* gene, we used *en*^{CXI} as a marker for activity of the *en* promoter. Embryos with one copy of *en*^{CXI} and one copy of wild-type *en* are phenotypically wild type except for the cytoplasmic accumulation of truncated *en*^{CXI} protein (compare insets in Fig. 3c and e). When heat-shocked, *en*^{CXI} heterozygotes that carry a *hs-en* element express the cytoplasmic *en* protein ectopically, resulting in broadened stripes (Fig. 3e). Therefore, the pulse of *en* produced from the heat-shock promoter results in transactivation of the *en* promoter.

If stable *en* expression after heat shock results from activation of an autoregulatory circuit, then maintaining ectopic *en* expression should require a functional endogenous *en* gene. Indeed, in *en*^{CXI} homozygous mutant embryos the early heat-shock protocol does not give stable ectopic expression. The *en*^{CXI} mutants with or without the *hs-en* insert are nearly indistinguishable, as both lose ectodermal *en* expression (not shown). Thus, maintenance of expression from the endogenous *en* promoter, once activated in new cells by heat-shock-produced *en*, requires the activity of the endogenous *en* gene. We conclude that *en* is subject to positive autoregulation. Because *en* is a transcription factor¹⁵, one simple model for autoregulation is that *en* directly acts at its own promoter. But autoregulation might be indirect and could even involve cell-extrinsic signalling.

Another consequence of heat shock is repression of *wg*. Although the *wg* RNA pattern is almost normal immediately after heat shock (data not shown), on 1 h of recovery from heat shock, *wg* expression is drastically reduced (compare Fig. 4a and b). At this time, *en* is expressed in the broadened stripes shown in Fig. 3c. The stripes of *wg* RNA have completely disappeared by 2 h after heat shock (Fig. 4c). The *wg* decay is still consistent with its requirement for *en* expression; *wg* RNA is detectable until the end of the *wg*-dependent period as defined with *wg*^{ts}.

The loss of *wg* after heat shock requires the presence of *hs-en*, thus it appears that *wg* is repressed by either the pulse of *en* from the heat-shock promoter or ectopic expression of the endogenous *en* gene. The repression of *wg* by *en* when the two genes are expressed in the same cell may have a function in normal development. This mechanism could assure that the expression of these two genes is established in immediately adjacent but nonoverlapping stripes.

Activation of autoregulation by *wg*

Ectopic *en* expression can only be induced during the *wg*-dependent window (Fig. 3f, described below). We therefore considered the possibility that the initiation of autoregulation requires *wg* input by examining the effect of *hs-en* in *wg*-mutant embryos (*hs-en; wg* null). After recovery from standard early heat shocks, *en* expression decays in *hs-en; wg* null embryos just as it does in *wg* mutant embryos without *hs-en* (data not shown). Therefore, *wg* function is required for autoregulation at this stage.

As *en* expression normally requires *wg* input transiently in development, this experiment does not exclude the possibility that the *wg* signal is only required before autoregulation is initiated.

Therefore, we tested whether the *wg* requirement is concurrent with autoregulation using *wg^{ts}* embryos carrying *hs-en* (*hs-en; wg^{ts}*). Heat shock has two effects on *hs-en; wg^{ts}* embryos raised at permissive temperature: it provides a pulse of *en* to all cells and simultaneously removes *wg* function. We carried out standard early heat shocks in the *wg*-dependent window, then let the embryos develop further at a temperature that is restrictive for the *wg^{ts}* allele. As with heat shock in a *wg* null background, *hs-en; wg^{ts}* embryos failed to give stable ectodermal *en* expression (data not shown). Thus, in early embryogenesis, the cell-extrinsic *wg* signal is required during or after the initiation of autoregulation.

Autoregulation independent of *wg*

Although *en* autoregulation requires *wg* early, normal *en* expression continues after the end of the *wg*-dependent period. To discover whether there is a later stage of autoregulation that is *wg*-independent, we heat-shocked *wg* mutant embryos aged beyond the *wg*-dependent period. Recall that, after about 5 h, *wg* mutant embryos show no *en* expression in the ectoderm at any position posterior to the fourth *en* stripe^{11,12} (Fig. 5a). Surprisingly, in *hs-en; wg* null embryos, a late heat-shock protocol induced stable, albeit variable, *en* expression in the ectoderm (Fig. 5b). Thus, *wg* function is required for autoregulation only early in development; there is a later stage of autoregulation that acts independently of *wg* (Fig. 8a). Perhaps a different positive regulator substitutes for *wg* later, or alternatively, *wg* may counteract an early repressor of *en* that is not present late (Fig. 8b).

The second stage of *en* autoregulation is restricted to fewer cells. The pattern generated in *hs-en; wg* null embryos after late heat shock is different from that seen with earlier heat shocks in wild-type *hs-en* embryos. Although late heat shock induces *en* in a variable number of cells, the cells are distributed in a narrow stripe. These stripes line up with underlying *en*-expressing cells of the CNS, strongly suggesting that these are posterior compartment cells. It appears that, once *en* expression becomes *wg*-independent, only the cells of the posterior compartment are capable of *en* autoregulation (Fig. 8b). We will return to this point below.

Restricting autoregulation

Autoregulation of *en* does not operate in all cells, as globally induced *en* resolves into patterned expression after heat shock (compare Fig. 3b and c). Two possibilities, which are not mutually exclusive, might explain this restriction. First, necessary coactivators may be localized. Second, there may exist repressors that block autoregulation in some cells.

We have identified at least one repressor that restricts autoregulation. In embryos mutant for the segment polarity gene *naked* (*nkd*), *en* is expressed in a broadened stripe pattern¹² (Fig. 6a, compare with Fig. 3d (wt)). Thus, *nkd* directly or indirectly represses *en* in certain cells of the embryo. We tested whether *nkd* activity restricts *en* autoregulation using *nkd* embryos carrying *hs-en* (*hs-en; nkd*). After the standard early heat shock, these embryos stably express *en* in almost all ectodermal cells, except the head and telson (Fig. 6b). This global expression involves more cells than the sum of broadened *en* expression in *nkd* (Fig. 6a) and broadened *en* in *hs-en* (Fig. 3c), at least in the lateral and dorsal regions. Therefore, *nkd* function restricts early *en* autoregulation. But autoregulation is restricted by different factors later in development. Although early heat shocks of *hs-en; nkd* embryos produce global *en* expression, later in development *nkd* mutants are indistinguishable with and without heat shock (data not shown).

The pattern to which *en* autoregulation is restricted after heat shock also changes during development. As shown above, late autoregulation, detectable as reinduction of *en* in *wg* mutants (Fig. 5b), is not activated in cells outside the normal *en* domain. This is also evident

from a comparison of early and late heat shocks in wild-type embryos. Although early heat shocks in wild type induce stable *en* expression in cells outside the normal *en* stripe, heat shock after 4 h does not generate stable ectopic *en* expression, demonstrating a block to autoregulation in cells outside the posterior compartment (Fig. 3f). Using a similar construct, Poole and Kornberg reported the effect of heat-shock-produced *en* on the larval body pattern¹⁶. Only early heat shocks disrupted the pattern, consistent with our observation that the *en* expression pattern is undisturbed by later heat shocks.

Autoregulation becomes dispensible

In addition to the two stages of autoregulation, we have identified one further mode of *en* regulation. Between 1 and 2 h after the transition to *wg* independence, *en* expression also becomes independent of *en* function. The evidence for later, *en*-independent regulation comes from late heat shocks of *en* mutants carrying *hs-en*. The early heat-shock protocol does not induce stable expression of the endogenous *en* locus in *en^{CXI}* homozygous embryos (see above), presumably because the mutant *en* protein cannot autoregulate. But later heat shocks (between about 4 and 7 h) result in reactivation of the *en^{CXI}* locus (Fig. 7a). The mutant *en* protein is expressed in a pattern of partial stripes in cells at the position expected for *en* expression. The position of the original *en* cells can be identified by a stable *lac-Z* protein expressed under the control of *en* regulatory sequences¹⁷. Though the initial expression of the endogenous *en^{CXI}* gene decays (Fig. 2d), the more stable β -galactosidase persists to the time when heat shock has reinduced *en^{CXI}* expression. The newly induced *en^{CXI}* expression is in a subset of the cells marked with *lac-Z* (data not shown).

The late *en^{CXI}* expression initially requires *en* function as it is reactivated by the pulse of *en* produced by heat shock. As reactivation is in the appropriate cells, it seems that the factors that restrict autoregulation to these cells act independently of *en* function. Heat-shock-produced *en* quickly decays, but *en^{CXI}* expression is maintained throughout embryogenesis in the absence of *en* function. This identifies a late-acting, *en*-independent maintenance programme for *en* expression.

Autoregulation not only becomes dispensible for *en* maintenance, but seems to stop functioning near the time that these new regulators come into play. As described above, heat shock of *en* mutant embryos between 4 and 7 h can induce expression that lasts well beyond 7 h. But heat shocks done still later, after 7 h, do not initiate new *en* expression. The inability of the *hs-en* protein to activate the endogenous *en^{CXI}* allele shows that *en* autoregulation is not sufficient to activate *en* after 7 h.

A second indication that *en* autoregulation stops near 7 h comes from looking at the period of expression of a construct in which *en* regulatory sequences drive *lac-Z* expression in the *en* pattern¹⁷ (Fig. 7c). The construct appears to include an *en* autoregulatory element. When wild-type embryos carrying both the *en-lac* fusion gene and *hs-en* are given an early heat shock, expression of *lac-Z* is induced in novel cells in parallel with induction of the endogenous *en* gene (Fig. 7b). Hence, this construct responds to *en* autoregulation. If *en* autoregulation operates continuously, expression of this *en*-responsive construct should always parallel expression of *en*. But in wild-type embryos, *lac-Z* expression stops at about 7 h¹⁷ even though *en* expression persists. Taken together, the data indicate that *en* autoregulation does not function beyond 7 h and that *en* expression is maintained past this time by new regulators.

Progression to determination

Regulation of *en* expression is unexpectedly complex during embryogenesis. Over a 4 h period, there are four distinguishable modes of *en* activation (Fig. 8a). These regulatory programmes act in an overlapping progression. The pair-rule regulatory programme initiates striped *en*

expression at cellular blastoderm^{18,19}, and we infer that it remains active until about 4.5 h, as *en* expression is maintained until this time in *wg* mutants^{11,12}. Even before 4.5 h, *wg* provides an activating signal from the neighbouring cell. This is inferred from the earliest time that *wg*-dependent ectopic *en* induction can be observed, at about 3 h. In contrast to the redundancy between pair-rule and *wg* control, *en* autoregulation is dependent on the *wg* signal during their period of overlap.

The initial dependence of autoregulation on the extracellular *wg* signal indicates that the activation of *en* expression does not immediately constitute a determined state. Instead, the period of *wg* dependence seems to represent a phase in development during which the choice of cell fate is responsive to neighbouring cells. Such a stage might provide a proofreading step to correct mistakes in the position of *en* activation. This would not be possible if *en* autoregulation were sufficient for cell-intrinsic stabilization of expression as soon as *en* is activated.

Temperature shift experiments suggest that the end of *wg* involvement in *en* expression is about 5 h. Recently, others have arrived at a similar conclusion (A. Besjovec and A. Martinez Arias, manuscript submitted). Despite the initial dependence on the *wg* signal, the *en* autoregulatory loop continues to function beyond the *wg*-dependent period, as demonstrated by the induction of *en* expression in *wg* mutants after late heat shocks. There are two likely explanations for the transition to *wg* independence (Fig. 8b). First, if *wg* acts positively, *wg*-independent *en* autoregulation could arise because another positively acting factor takes over at 5 h. Alternatively, *wg* might be required to counteract an early acting repressor of *en* autoregulation. If the repressor normally decays at 5 h, the requirement for *wg* would be lifted. Whether *wg* acts positively or counteracts negative regulators of *en* autoregulation, the factors that influence *en* autoregulation do change near the time that *en* becomes *wg*-independent. This is indicated by the observation that late autoregulation is active in only a subset of the cells that are capable of early autoregulation.

Evidence for positive autoregulation has been obtained for several genes involved in the determination of cell fate, such as the *Drosophila* sex-determination gene, *Sex-lethal*²⁰, the homeotic gene, *Deformed*²¹, and a vertebrate myoblast determination gene, *myoD*²². Here, we have shown that the selector gene *en*, responsible for the determination of posterior compartments^{3,4}, undergoes positive autoregulation. Surprisingly, *en* autoregulation does not persist throughout the period that *en* is expressed. We define the end of autoregulation as the time after which heat shock cannot induce the endogenous allele in *en* mutants, and the time that an *en*-responsive reporter gene loses expression in a wild-type background. As autoregulation is transient, it does not supply a mechanism for stable determination of the *en* cell fate.

We have detected a yet-later-acting tier of *en* maintenance that is independent of autoregulation (Fig. 8a). After the *wg*-dependent period, the transient production of functional *en* through heat shock promotes the stable expression of the *en*^{CXI} mutant protein in homozygous embryos. Thus, after an autoregulatory phase, *en* is controlled by new regulators that maintain expression independent of *en* function. This program may provide maintenance of expression and not activation as *en*^{CXI} expression is not reactivated at late stages without heat shock. Although it has not yet been tested directly, it may be this level of *en* regulation that provides the stable, cell intrinsic control that characterizes a determined cell. Candidates for later acting *en* regulators, both positive and negative, have been identified among the *trithorax* and *Polycomb* gene classes. Mutations in these genes have been found to affect maintenance of expression patterns of homeotic selector genes^{23–25}, and members of the *Polycomb* class have been shown to affect *en* expression as well (D. Moazed and P.O'F., unpublished data)^{26–28}

The classically defined stages of reversible and irreversible cell fate commitment lead us to expect changes in the mode of *en* regulation during development. But the elaborate progression that we have uncovered is difficult to rationalize in all its detail. Although the transition to *wg* independence suggests a move away from cell-extrinsic control of identity, the subsequent transition, from autoregulatory activity to a still later stage of control, has no ready explanation. Identifying the later-acting regulators and the time at which the *en* cell fate becomes irreversibly determined may help explain these transitions.

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References

1. Slack, JMW. Egg to Embryo: Determinative Events in Early Development. Cambridge University Press; Cambridge: 1983.
2. Garcia-Bellido A. CIBA Fdn Symp 1975;29:161–182.
3. Lawrence PA, Morata G. Devl Biol 1976;50:321–337.
4. Kornberg T, Siden I, O'Farrell P, Simon M. Cell 1985;40:45–53. [PubMed: 3917856]
5. Garcia-Bellido A, Ripoll P, Morata G. Nature 1973;245:251–253.
6. Garcia-Bellido A, Ripoll P, Morata G. Devl Biol 1976;48:132–147.
7. Kornberg T. Proc natn Acad Sci USA 1981;78:1095–1099.
8. Baker NE. EMBO J 1987;6:1765–1773. [PubMed: 16453776]
9. van den Heuvel M, Nusse R, Johnston P, Lawrence PA. Cell 1989;59:739–749. [PubMed: 2582493]
10. Rijsewijk F, et al. Cell 1987;50:649–657. [PubMed: 3111720]
11. DiNardo S, Sher E, Heemskerk-Jongens J, Kassis JA, O'Farrell PH. Nature 1988;332:45–53.
12. Martinez-Arias A, Baker N, Ingham PW. Development 1988;103:157–170. [PubMed: 3197626]
13. Baker NE. Devl Biol 1988;125:96–108.
14. DiNardo S, Kuner JM, Theis J, O'Farrell PH. Cell 1985;43:59–69. [PubMed: 3935318]
15. Jaynes JB, O'Farrell PH. EMBO J 1991;10:1427–1433. [PubMed: 1673924]
16. Poole SJ, Kornberg TB. Development suppl 1988;104:85–93.
17. Kassis JA. Genes Dev 1990;4:433–443. [PubMed: 2110923]
18. DiNardo S, O'Farrell PH. Genes Dev 1987;1:1212–1225. [PubMed: 3123316]
19. Howard K, Ingham P. Cell 1986;44:949–957. [PubMed: 3955654]
20. Cline T. Genetics 1984;107:231–277. [PubMed: 6735170]
21. Kuziora MA, McGinnis W. Cell 1988;55:477–485. [PubMed: 2902926]
22. Thayer MJ, et al. Cell 1989;58:241–248. [PubMed: 2546677]
23. Wedeen C, Harding K, Levine M. Cell 1986;44:739–748. [PubMed: 3081265]
24. Struhl G, Akam M. EMBO J 1985;4:3259–3264. [PubMed: 2419125]
25. Mazo AM, Huang DH, Mozer BA, Dawid IB. Proc natn Acad Sci USA 1990;87:2112–2116.
26. Busturia A, Morata G. Development 1988;104:713–720. [PubMed: 2908325]
27. Dura JM, Ingham P. Development 1988;103:733–741. [PubMed: 2907878]
28. Smouse D, Goodman C, Mahowald A, Perrimon N. Genes Dev 1988;2:830–842. [PubMed: 2905316]
29. Spradling AC, Rubin GM. Science 1982;218:341–347. [PubMed: 6289435]
30. Tautz D, Pfeifle C. Chromosoma 1989;98:81–85. [PubMed: 2476281]
31. Baker NE. Development 1988;103:289–298. [PubMed: 3224555]
32. Poole SJ, Kauvar LM, Drees B, Kornberg T. Cell 1985;40:37–43. [PubMed: 3917855]

33. Pirotta, V. Vectors, A Survey of Molecular Cloning Vectors and their Uses. Rodriguez, RL.; Denhardt, DT., editors. Butterworths; Boston: 1988. p. 437-456.

FIG. 1.

Expression of *en* becomes *wg*-independent after 5 h. Embryos are stained with an anti-*en* antibody¹⁴. Magnification 140×, anterior is left and ventral is down in all figures except when noted. Embryos are shifted from 18 °C to 29 °C (at different times, but age of embryos is corrected for effect of temperature and is given in hours after egg laying (AEL) as if embryos were raised at 25 °C. *a*, *wg*^{IL114ts/wg^{CX4} embryo grown at nonpermissive temperature (29 °C) from ~3.5 to 7.5 h AEL. Expression of *en* is indistinguishable from that seen in a *wg* null embryo at this stage^{11,12}. Only expression that is not under *wg* control persists: in the head, the first four ectodermal stripes (1–4), and a segmentally repeated subset of CNS cells.}

Expression has disappeared from the lateral ectoderm posterior to the fourth *en* stripe (for example, the normal position of stripe 7 is indicated by an arrow). In wild-type embryos at this stage, *en* striped expression is easily detectable (see Fig. 2b). *b, wg^{IL114ts}/wg^{CX4}* embryo grown at nonpermissive temperature from ~4.5 to 8 h AEL. Some ectodermal cells continue to express *en*, creating a partial stripe in each segment (for example, stripe 7 is indicated by an arrow). *c, wg^{IL114ts}/wg^{CX4}* embryo grown at nonpermissive temperature from ~5 to 8 h AEL. The *en* stripes are almost wild-type (for example, stripe 7 is indicated by an arrow). Some stripes are incomplete dorsally. This is the same phenotype seen when these embryos are grown exclusively at permissive temperature (18 °C). Immunocytochemistry was done as in refs 14, 18. The *wg^{IL114ts}* is described in ref. 13 and was obtained from the Tübingen stock collection. The *wg^{CX4}* is described in ref. 8.

FIG. 2.

Expression of *en* stops prematurely in *en* mutants. Embryos (120×) are stained with an anti-*en* antibody. *a*, Wild-type embryo, ~4.5 h AEL. *b*, Wild-type embryo, ~6.5 h AEL. *c*, An *en*^{CX1}/*en*^{CX1} embryo ~4.5 h AEL. The mutant cytoplasmic protein is expressed in a wild-type pattern. *d*, An *en*^{CX1}/*en*^{CX1} embryo, ~6.5 h AEL. The mutant protein has disappeared from the ectoderm at this stage. For example, the normal position of stripe 7 is indicated by an arrow (compare with *c*). Some cells of the CNS in each segment continue to express the *en*^{CX1} allele. The diffuse signal in other areas of the embryo is yolk autofluorescence. Loss of *en*^{CX1} is not the result of defects in the *cis*-acting control region because this allele is expressed normally when heterozygous with wild-type *en* (data not shown). The *en*^{CX1} stock was obtained from R. Holmgren.

FIG. 3.

Globally induced *en* activates the endogenous *en* gene in novel cells. Embryos (115×) are stained with an anti-*en* antibody. *b–e*, Embryos between 6 and 7 h AEL. *a*, Schematic diagram of *hs-en*. The *en* sequences consist of a 2-kb *EcoRI*–*SnaBI* fragment of the *en* cDNA clone c-2.4 (ref. 32). Transcription of *en* sequences (stippled box) is driven by the *hsp70* heat-shock promoter (*hsp*), contained in a 1.3-kb *SphI*/*PstI* fragment from pUCHSP 70 (provided by E. Gavis). The polyadenylation sequences (*pA+*) are from SV40. For transformation, the above sequences are inserted into the pCaSpeR vector³³ containing P element ends (ovals) and the *white* gene (*w+*) as a selectable marker. *b*, An *hs-en* embryo, heat shocked at ~6 h, then

immediately fixed and stained. The *en* expression induced by heat shock is detectable in all cells. *c*, An *hs-en* embryo, heat shocked at ~3 h AEL, aged 3.5 h, then fixed and stained. The *en* stripes are abnormally broad in the ventral and lateral regions compared with wild type (compare distance between arrowheads in *c* and *d*). Inset, 326× magnification of part of one stripe showing that the *en* antigen is confined to the nucleus. *d*, Wild-type embryo, showing the normal pattern of *en* expression. *e*, An *en^{CXI}/+;hs-en* embryo, heat shocked at ~3 h AEL, aged 3.5 h, then fixed and stained. The pattern of cytoplasmic *en^{CXI}* antigen is similar to the pattern of *en* in *c*. Inset is a 326× magnification of part of one stripe showing that the cytoplasmic antigen, characteristic of *en^{CXI}*, is produced in novel cells after heat shock. Both the wild-type nuclear and mutant cytoplasmic *en* antigens are expressed in these cells, although the nuclear signal is weak relative to the cytoplasmic signal. *f*, An *hs-en* embryo, heat shocked at ~5 h then fixed after 3 h recovery at 25 °C. Striped *en* staining is nearly wild type. There are isolated cells in the anterior compartment that express *en* (small arrowheads), as well as staining in the amnioserosa (large arrowhead), neither of which is seen in wild type.

METHODS. Transgenic lines were established²⁹ using the p-wings clipped helper and *Df(1)w^{67c2}*,y as host. For heat-shock experiments, embryos were collected and aged at 25 °C on grape agar plates. At the appropriate stage, plates were put in moist chambers at 37 °C for 35 min. They were then returned to 25 °C for recovery. The novel expression pattern was detectable as soon as the *en* expressed from the heat-shock promoter decayed to background levels, ~45 min after heat shock. Similar results were seen with three different *hs-en* lines, one on chromosome III and two on chromosome II.

FIG. 4.

Decay of *wg* RNA after heat shock of *hs-en* embryos. Embryos (125×) are hybridized with a digoxigenin-labelled³⁰ *wg* probe. *a*, Wild-type embryo, ~5 h AEL, showing the normal pattern of *wg* RNA expression³¹. *b*, An *hs-en* embryo, heat shocked at ~3 h, then allowed to recover at 25 °C for 1 h. Although the patches of *wg* expression in the head are of normal intensity (arrowhead, compare with *a*), the striped expression in the ectoderm is dramatically reduced (for comparison, arrow points to the stripe that corresponds to the arrow in *a*). Signal persists mostly in the ventral ectoderm. *c*, An *hs-en* embryo, heat shocked at ~3 h and allowed to recover for 2 h at 25 °C. There is normal *wg* staining in the head (arrowhead), but all striped

ectodermal RNA has decayed (arrow shows the normal position of the stripe shown by arrows in *a* and *b*).

METHODS. Embryos were fixed and probed for *wg* RNA as in ^{ref.30}. The *wg* probe was made from a 1.3-kb genomic *EcoRI*–*HindIII* fragment containing the fourth and fifth exons (provided by N. Baker). In a collection of embryos from parents that were heterozygous for the *hs-en* insert, only 3/4 showed premature loss of *wg* RNA after heat shock, consistent with the loss resulting from *hs-en*, due to either heat-shock-produced *en* or stable ectopic *en*.

FIG. 5.

Late autoregulation is *wg* independent. Embryos (140×) are stained with an anti-*en* antibody. *a*, A *wg*^{CX4}/*wg*^{CX4} embryo⁸, ~8 h AEL. There is no striped *en* expression in the ectoderm posterior to the third *en* stripe. For example, the arrow points to the normal position of *en* stripe 7. The repeated staining in the ventral region is in the CNS¹¹. *b*, A *wg*^{CX4}/*wg*^{CX4};hs-*en* embryo, ~8 h AEL. Embryo was heat shocked at ~5 h then fixed after 3 h recovery at 25 °C. There are partial *en* stripes in positions that have no signal in a *wg* mutant without heat shock. For example, the arrow points to the partially rescued stripe 7. The staining phenotype varied. The embryo shown is representative of about half of the *wg*^{CX4}/*wg*^{CX4};hs-*en* embryos heat shocked

at this stage. The others had relatively fewer ectodermal cells staining. As with wild-type *hs-en*, there is staining in cells of the amnioserosa (arrowhead).

METHODS. Embryos were derived from parents that were heterozygous for *wg*^{CX4} on chromosome II and *hs-en* on chromosome III. Thus, 3 out of 16 of the embryos are homozygous for the *wg* mutation and carry at least one copy of *hs-en*. Consistent with this, 3 out of 16 of the embryos show the phenotype we interpret as *wg*^{CX4}/*wg*^{CX4}; *hs-en*, that is dramatically fewer than the wild-type number of *en* cells staining, due to lack of *wg*, and *en* staining in the amnioserosa, due to activity of the *hs-en* insert.

FIG. 6.

Autoregulation is repressed by *nkd*. Embryos (150×) are stained with an anti-*en* antibody. *a*, A *nkd*^{7E}/*nkd*^{7E} embryo, ~5 h AEL, showing abnormally broad *en* stripes compared with wild type¹². The broadening is due to abnormal *en* expression in cells posterior to the normal *en* stripe in *nkd* mutants (S.D., unpublished observation). *b*, A *nkd*^{7E}/*nkd*^{7E};hs-*en* embryo heat shocked at ~3 h and fixed after recovering for 2 h at 25 °C. Nearly all ectodermal cells stain except areas of the head and telson. In addition, some isolated areas in the ventral-lateral ectoderm do not express *en*. These cells vary somewhat in number and position from embryo to embryo, and thus do not appear to be cells of one specific type.

METHODS. The *nkd*^{7E}/*nkd*^{7E};hs-*en* embryos came from parents that were heterozygous for *nkd*^{7E} on chromosome III and homozygous for hs-*en* on chromosome II. Thus, 1/4 of the embryos were homozygous for *nkd* and carried hs-*en*, and 1/4 of the embryos showed global staining in the ectoderm after recovery from heat shock. The *nkd*^{7E} stock was obtained from the Tübingen stock collection.

FIG. 7.

A fourth mode of *en* control follows autoregulation. *a*, An *hs-en;en^{CXI}/en^{CXI}* embryo (110 $\times$), heat shocked at $\sim$ 5 h, aged 3 h, then fixed and stained with an anti-*en* antibody. The cytoplasmic *en^{CXI}* protein is expressed in partial stripes in each segment. *en^{CXI}* mutants normally show no expression by this stage. The number of cells that are rescued by heat shock varies, depending in part on the age at heat shock. This embryo is representative for a heat shock done at 5 h. Most rescued cells lie in the lateral and dorsal ectoderm and are a subset of the cells that initially express *en^{CXI}* in early embryos (see text). *b*, An *en-lac-E;hs-en* embryo (75 $\times$, ventral view, anterior left) heat shocked at $\sim$ 3 h, aged 2 h, then fixed and hybridized with a digoxigenin-labelled³⁰ *lac-Z* probe. Stripes of *lac-Z* RNA are abnormally broad compared with non-heat shocked control embryos (compare distance between arrowheads flanking the stripes in *b* and *c*). *c*, An *en-lac-E* embryo (75 $\times$, ventral view, anterior left), fixed at $\sim$ 5 h and hybridized with a digoxigenin-labelled³⁰ *lac-Z* probe. The *en-lac-E* contains 2.4 kb of *en* promoter/upstream sequences and the *en* first intron driving *lac-Z* expression¹⁷. Stripes of *lac-Z* RNA correspond to the position of *en* expression, although the level of *lac-Z* expression from this construct varies among the stripes.

METHODS. *a*, Embryos were derived from parents that were heterozygous for *en^{CXI}* on chromosome II and *hs-en* on chromosome III. Thus, 3/16 of the embryos are homozygous for the *en* mutation and carry at least one copy of *hs-en*. Consistently, 3/16 of the embryos show the phenotype we interpret as *en^{CXI}/en^{CXI};hs-en*, that is cytoplasmic stain in ectodermal stripes with fewer than the normal number of cells staining in each stripe. *b*, *c*, Embryos were fixed and probed for *lac-Z* RNA as in ^{ref. 30}. The *en-lac-E* stock was obtained from J. Kassis.

FIG. 8.

Integrating four modes of *en* regulation during embryogenesis. *a*, Approximate periods of action of the four modes of *en* regulation and their temporal overlap. The time line indicates hours AEL. 1, The start of the *pair-rule* period is defined as the time of the earliest appearance of *en* expression^{7,14}. The end is defined as the time when *en* expression stops in *wg* mutants¹¹, presumably a condition in which only *pair-rule* function activates *en*. 2, The start of *wg* influence is defined as the earliest time that heat shock stably induces *wg*-dependent ectopic *en* expression. The end of the *wg* period is defined as the latest time at which raising the temperature of *wg*^{ts} embryos has an effect on *en* expression. 3, The start of autoregulatory influence is taken as the earliest time that heat shock stably induces ectopic *en*. The end of the period during which *en* autoregulation operates is inferred from two observations: first, that *en*-responsive sequences cannot drive *lac-Z* expression past this time, and second, that heat shock does not activate expression in *en* mutants past this time. Stippling indicates the time during which autoregulation requires *wg* input. 4, The fourth mode of regulation (late control) begins when heat shock first results in stable expression in *en* mutants. It is not known whether this regulatory phase is responsible for maintaining *en* indefinitely or whether still later transitions occur. *b*, Two models to account for the onset of *wg*-independent *en* autoregulation. Cells spanning two segment primordia are schematized as eight open circles. Labelled cells represent those that normally express *en* or *wg*. Coactivation model: cells directly exposed to the *wg* coactivation signal are competent to autoregulate during the *wg*-dependent period. Autoregulation is allowed in whichever of these cells *en* is activated, either by *pair-rule* gene activity^{18,19} or by heat shock. Expression of *en* becomes *wg*-independent when another coactivator (A) takes over. This new coactivation is confined to posterior compartment cells, and *en* expression is thereby restricted to the posterior compartment after late heat shock. Antirepression model: *wg* prevents a general repressor of *en* (R) from acting on *wg* cells and their neighbours during the *wg*-dependent period. Autoregulation is allowed in whichever of these cells *en* is activated, either by *pair-rule* gene activity^{18,19} or by heat shock. Later, the

wg requirement is relieved because late repression (R') is confined to the anterior compartment. Autoregulation of *en* can now act in the normal *en*-expressing cells in the absence of the *wg* signal. Late heat shock cannot overcome the late repression in the anterior compartment and so does not induce ectopic autoregulation. It should be noted that, although we suggest that ectopically induced *en* expression occurs in the *wg*-expressing cells, we have not yet experimentally identified which cells induce *en* after heat shock. Additionally, *wg* is still expressed at late stages, but has not been indicated in the schematic because it is dispensable for *en* expression.