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Early *even-skipped* stripes act as morphogenetic gradients at the single cell level to establish *engrailed* expression

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SUMMARY

***even-skipped* (*eve*) has been proposed to set up parasegment borders at the anterior edge of each of its seven stripes by providing a sharp expression boundary, where *engrailed* is activated on one side and *wingless* on the other. By expressing bell-shaped early *eve* stripes without the sharp boundary provided by narrow, late stripes, we find that the early gradient is sufficient for generating stable parasegment borders. Based on several lines of evidence, we propose that the anterior portion of each early stripe has morphogenic activity, repressing different target genes at different concentrations. These distinct repression thresholds serve to both limit and subdivide a narrow zone of *paired* expression. Within this zone, single cell rows express either *engrailed*, where *runt* and *sloppy-paired* are repressed, or *wingless*, where they are not. While the early *eve* gradient is sufficient to establish parasegmental borders without refined, late expression, late *eve* expression has a role in augmenting this boundary to provide for strong, continuous stripes of *engrailed* expression. In addition, we show that the early *eve* gradient is sufficient, at its posterior edge, for subdividing the *ftz* domain into *engrailed* expressing and non-expressing cells.**

Key words: segmentation, morphogen, *Drosophila*, homeodomain, transcriptional repression, *even-skipped*

INTRODUCTION

Segmentation in the *Drosophila* embryo begins in a syncytial blastoderm with the actions of three groups of genes, most of which encode transcription factors (for reviews, see Akam, 1987; Ingham, 1988; Ingham and Martinez-Arias, 1992). First, maternal effect gene products establish the basic anteroposterior coordinate in the form of gradients. These gradients are then used by zygotic gap genes to establish their region-specific expression. The maternal effect and gap gene products, in turn, activate periodic expression of pair-rule genes, usually in seven stripes in the blastoderm embryo. Pair-rule genes themselves are organized hierarchically into at least two groups. The primary pair-rule genes, *hairy*, *runt*, *even-skipped* (*eve*), and possibly *paired* (*prd*), establish periodic stripe expression and regulate secondary pair-rule genes. Following cellularization of the blastoderm, the periodic cues of these pair-rule genes are used to establish expression of segment-polarity genes, usually in 14 stripes, leading to the formation of 14 parasegments. The products of the segment polarity genes have a much less restricted range of function. In addition to transcription factors, they include diffusible factors, membrane proteins, and members of intracellular signaling cascades (Ingham and Martinez-Arias, 1992). Previous studies of the expression patterns of pair-rule genes and the consequences of alterations in these patterns have been crucial to the elucidation of the segmentation cascade. However, the expression patterns of many pair-rule genes, like those of most other segmentation genes, undergo changes with time, thereby altering their relative phase relationships. This renders a static analysis imprecise. Therefore it would be useful to create an embryo in which only part of the normal temporal pattern of a pair-rule gene is expressed, so that one can examine how different temporal and spatial aspects of expression relate to function. This is the primary motivation of the work presented here.

Two pair-rule genes, *eve* and *fushi tarazu* (*ftz*) play critical roles in defining parasegments, as reflected in their expression patterns. *eve* expression starts as three broad bands that cover most of the trunk region. At precellular blastoderm, these resolve into seven stripes that center on the odd-numbered parasegmental primordia (Harding et al., 1986; Macdonald et al., 1986; Frasch et al., 1987). In a roughly complementary pattern, *ftz* stripes start broadly and come to coincide with the seven even-numbered parasegmental primordia (Carroll and Scott, 1985; Hiromi et al., 1985; Lawrence et al., 1987). After cellularization, both patterns undergo ‘refinement’, in which stripes narrow from about four cells to about two cells by loss of expression from the posterior, with a concomitant increase of expression in the anterior-most cells, thereby sharpening the anterior borders. During this period, the segment polarity genes *engrailed* (*en*) and *wingless* (*wg*) begin to appear in the anterior-most and the posterior-most row of cells, respectively, in each parasegmental primordium (Lawrence et al., 1987; Ingham et al., 1988). In particular, the sharpened borders of *eve* and *ftz* stripes coincide, cell by cell, with the anterior

borders of *en* stripes, thus demarcating parasegmental boundaries (Lawrence et al., 1987). Consistent with the regulatory relationships suggested by this coincidence, even-numbered *en* stripes are missing in *ftz* mutants (Howard and Ingham, 1986; DiNardo and O'Farrell, 1987), and even-numbered parasegments are not formed (Wakimoto et al., 1984; Martinez-Arias and Lawrence, 1985). Similarly, odd-numbered *en* stripes and odd-numbered parasegments are not formed in *eve* hypomorphic mutants (Nüsslein-Volhard et al., 1985; Frasch et al., 1988). However, *eve* null mutants lack both even- and odd-numbered *en* stripes (Harding et al., 1986; Macdonald et al., 1986), and no segmentation occurs in the trunk region (Nüsslein-Volhard et al., 1985). Thus, *eve* is required for expression of all *en* stripes, but low levels of activity suffice for even-numbered stripes. It is not known what aspects of the *eve* pattern are important for this regulation: *eve* could act as a local morphogen for downstream genes; if so, the overall level and shape of the expression pattern may be the most important qualities (Lawrence, 1987; Lawrence and Johnston, 1989). Alternatively, *eve* may act in combination with other genes to provide distinct signals in different parts of each stripe. In this case, the extent of expression at different times relative to that of other pair-rule genes may be more important (DiNardo and O'Farrell, 1987; Ingham et al., 1988). Therefore, it is of considerable interest to relate *eve*'s changing expression pattern to its role in activating *en* and *wg*.

Transgenic studies showed that a 6.4 kb fragment from the upstream region of *eve* is sufficient to drive expression of stripes 2, 3, and 7 (Goto et al., 1989; Harding et al., 1989). Further dissection of the fragment uncovered two classes of regulatory elements: stripe-specific 'early elements' which have been shown to translate the non-periodic cues of gap genes, stripe by stripe, into the periodic pattern of *eve*, and an element which governs expression of all late, narrowed stripes (Goto et al., 1989; Harding et al., 1989; Small et al., 1991; Jiang et al., 1991). This latter element has been shown to respond to early *eve* expression, providing autoregulatory feedback. By removing this autoregulatory contribution, we have recently shown that the late element is in fact responsive to other pair-rule genes in the absence of feedback (M.F. and T.G., unpublished data). Thus, it carries out refinement of the *eve* pattern in response to the primary pair-rule genes (Goto et al., 1989). In this work, we used transgenes to create embryos that express only unrefined early stripes, or both unrefined early stripes and refined late stripes. This allowed us to study the changing roles of *eve* expression before and after refinement. Our results show that early and late *eve* expression have distinct roles in regulating downstream genes. Notably, only early expression is required for the activation of both even- and odd-numbered *en* stripes, while late *eve* stripes strengthen expression of the odd-numbered *en* stripes. A model is proposed for how early *eve* expression, acting as a bell-shaped, concentration-dependent morphogenetic field, biased by combinatorial interactions with other primary pair-rule genes, subdivides each of its domains into multiple functional subregions. These findings add to the previous discoveries of embryo-length morphogenetic gradients, composed of maternal gene products, and the more localized gradients of gap gene products, and show that *Drosophila* uses the same principle again to further refine pattern information to the single cell level.

MATERIALS AND METHODS

Drosophila strains

eve transgenes based on pCaSpeR3 (Thummel et al., 1988) were constructed as follow; E+L-*eve* carries an *eve* genomic DNA fragment from −6.3 kb (*Nde*I restriction site) to +1.85 kb (*Mlu*I site) relative to the transcription start site, which includes the late element, early stripe 2, 3, and 7 elements, the protein coding sequence, and the polyadenylation site. E-*eve* contains genomic DNA from −4.95 kb (*Kpn*I) to +1.85 kb (*Mlu*I site). L-*eve* has a fragment from −6.3 kb to −4.95 kb combined with a fragment from −0.27 kb (*Sfi*I) to +1.85 kb. These fragments were inserted into pCaSpeR3 with the direction of transcription opposite to that of the *white* gene. The RVΔSac construct is an E+L-*eve* construct with the 5′ deletion endpoint at −5.65 kb (*Eco*RV site) containing an internal deletion from −5.50 kb, a site downstream of the MAS (Jiang et al., 1991), to −5.01 kb (*Sac*I site). A construct having the same 5′ endpoint but no internal deletion is fully active as a late element (Goto et al., 1989).

Flies of the genotype *w*;*Df(eve)*/*CyO*;+/+ were used as host for injection of pCaSpeR-*eve* constructs. Transgenic flies were crossed with a marked balancer strain (*y w;Sco/CyO P[hb-lacZ];D/TM3)* and screened for *Df(eve)/CyO P[hb-lacZ]*, so that *Df(eve)/Df(eve)* embryos were identifiable by staining for β-galactosidase.

mRNA and protein localization

In situ hybridization to whole mount embryos using digoxigenin-(DGG) labeled probes was performed as described (Tautz and Pfeifle, 1989). DGG-labeled anti-sense mRNA probes were used. Those probes were visualized using alkaline phosphatase-conjugated anti-DGG antibody (Boehringer Mannheim). In double-staining, in situ hybridization with an RNA probe was followed by antibody staining, with either anti-Engrailed (Patel et al., 1989; kindly provided by Steve DiNardo) or anti-Ftz (kindly provided by Dianne Mattson and Ian Duncan) monoclonal antibodies (Kellerman et al., 1990). Biotinylated secondary antibodies were detected using avidin-conjugated peroxidase (VectorLabs). Embryos were mounted in Fluoromount.

Cuticle preparations

Cuticles were prepared as described (Wieschaus and Nüsslein-Volhard, 1986). Embryos were collected on grape juice plates, aged for 18 to 24 hours at 25°C, dechorionated with 50% bleach, and devitellinized with methanol/heptane. After incubation at 60°C for 40 minutes in PBS, the embryos were mounted in Hoyer's medium/lactic acid (1:1) and incubated at 60°C until cleared.

RESULTS

Localized rescue patterns

Previous studies showed that a 4.8 kb fragment from the upstream region of *eve* contains stripe-specific 'early elements' ('Es') sufficient to drive the expression of broad, unrefined early stripes 2, 3, and 7 during blastoderm. In contrast, a 6.4 kb fragment expresses not only these early stripes but also all seven refined, late stripes in gastrulating embryos (Goto et al., 1989; Harding et al., 1989). This difference in *cis*-regulatory activity was attributed to the presence in the 6.4 kb fragment of a 'late element', or 'L', which can direct late stripe expression of a reporter gene in wild-type embryos. Since these early and late stripes are different in both the timing of their appearance and their shape, they may have distinct functions in vivo. We investigated this possibility by examining the phenotypic consequences of expressing Eve (the *eve* protein) driven by either the 4.8 kb fragment (E-*eve*) or the 6.4 kb fragment (E+L-*eve*) in the absence of endogenous *eve*. This was conveniently carried out

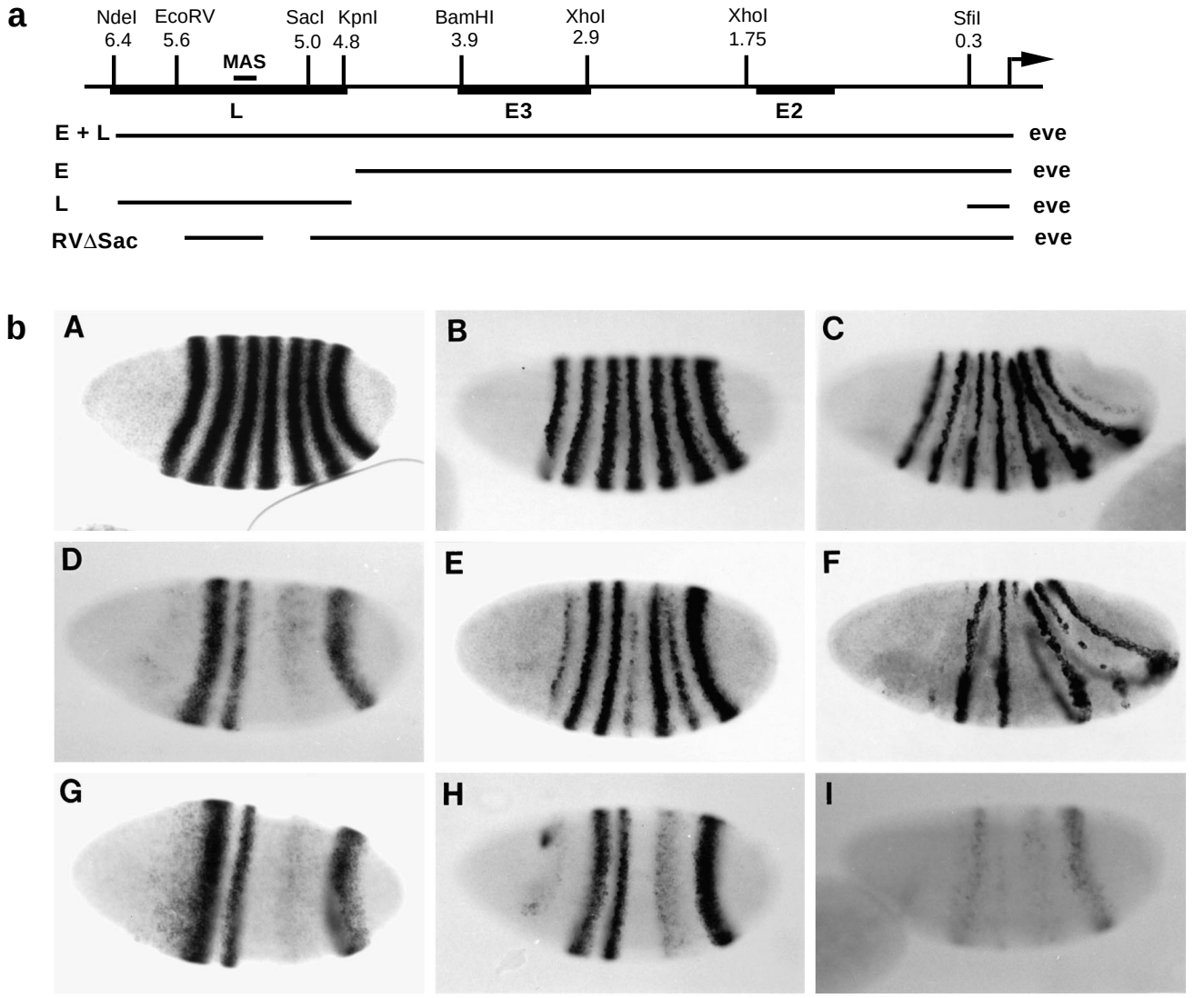

Fig. 1. Localized rescue of an *eve* deficiency mutation. (a) Map of the 5′ upstream region of the *eve* gene and sequences used in *eve* transgenes. L is the late element, and E2 and E3 refer to stripe-specific, early elements. MAS is the minimal autoregulatory sequence defined by Jiang et al. (1991). E+L-*eve* and E-*eve* were used, in a *Df(eve)* background, to generate EL embryos and E embryos, respectively. (b) Expression of *eve* in wild-type and locally rescued embryos. Whole mount in situ hybridization using a digoxigenin-labeled *eve* probe was carried out to wild-type embryos (A,B,C), EL embryos (D,E,F), or E embryos (G,H,I). (A,D,G) Early cellular blastoderm stage; (B,E,H) late cellular blastoderm; (C,F,I) gastrulation. At the early stage (D,G), E and EL embryos express early stripes 2, 3, and 7, and a broad, weak stripe in the region of normal stripes 5 and 6. Later, EL embryos initiate expression of all seven late stripes (E), but, in the absence of early stripes 1 and 4, late stripes 1 and 4 are not maintained (F). Early stripe 5/6 does lead to rescue of a somewhat broad, late stripe 5 and, frequently, a weak, late stripe 6.

by transforming an *eve* deficiency mutant, *Df(eve)*. The construction of these two transgenes, E-*eve* and E+L-*eve*, is diagrammed in Fig. 1a. Since these transgenes reproduce only part of the normal *eve* pattern in an *eve* deficiency host, we will refer to this approach as ‘localized rescue’.

Shown in Fig. 1b are the *eve* mRNA patterns at three developmental stages in wild-type embryos (WT; A,B,C) and in *Df(eve)* embryos locally rescued with either E+L-*eve* (EL embryos; D,E,F) or E-*eve* (E embryos; G,H,I). The patterns of the two transgenes at pre-cellular blastoderm, consisting of three early stripes, are essentially identical to each other (D,G), and are also similar to the β-galactosidase (β-gal) pattern previously observed with the equivalent *lacZ* construct (Goto et al., 1989; Harding et al., 1989). However, in addition to the fact that they have only three stripes, these patterns differ from the wild-type *eve* pattern in two respects. First, stripe 2 is considerably stronger than stripe 3, which appears slightly narrower than in wild-type embryos, and stripe 2 has an anterior trailing edge that is somewhat more intense than that seen in either the endogenous *eve* pattern or the β-gal pattern (D,G). Both the disparity of stripe intensities and the anterior trailing disappear gradually during blastoderm, although stripe 3 remains somewhat weaker than stripe 2. Second, an aberrant, weak ‘stripe’ is observed that covers the early stripe 5 and 6 domains. It is not clear whether this ‘stripe 5/6’ reflects normal regulation of stripe formation in this part of the embryo, but it has significant bearing on the regulation of downstream genes in this region by the transgenes, as described below.

After cellularization, stripes in the wild-type embryo become narrow, with their anterior borders sharply demarcated (B). The patterns in E and EL embryos have diverged markedly by this stage. In E embryos, stripes have begun to fade and become somewhat narrower (H), perhaps due to uniform loss of expression. In EL embryos, stripes 2, 3, and 7 undergo refinement after cellularization, and then fade as the germband extends; that is, they behave normally (E,F). The other late stripes are also initiated, but in the absence of early stripes, late stripes 1 and 4 fade rapidly. However, late stripes 5 and 6 persist: stripe 5 is strong and somewhat broader than normal; stripe 6 is much weaker and 'patchy'. The behavior of these stripes is likely to be linked to the earlier expression of stripe 5/6. Their locations are more posterior than normal, which may reflect the fact that the level of expression of stripe 5/6 is considerably weaker than normal (see below and Discussion). In summary, the expression pattern of E+L-*eve* is more or less normal in the domains of stripes 2, 3, and 7, while the regions of stripes 1 and 4 remain *eve* deficient, and the domains of stripes 5 and 6 are aberrant due to the expression of an early but weak stripe 5/6.

Localized rescue of *en* and *wg* expression by E-*eve* and E+L-*eve*

The E and EL embryos provide an opportunity to dissect differences in early and late *eve* function. Since a major part of *eve*'s role in segmentation is in the initiation of *en* expression, we examined *en* expression patterns in the transformants (Fig. 2). We were particularly interested in testing the idea that broad, early stripes are involved in activating (even-numbered) *en* stripes in neighboring *ftz* domains, while narrow, late stripes activate (odd-numbered) *en* stripes in their own domains (Goto et al., 1989).

In EL embryos, *en* stripes 2, 3, 4, 5, 6, 9, 11, 13, and 14 are expressed (Fig. 2E), although stripe 11 is often weak and incomplete. The identities of these stripes have been confirmed by En/*wg* double staining (data not shown). The appearance of most of these stripes can be ascribed to expression of specific *eve* stripes: *en* stripes 3, 5, and 13 are in the domains of *eve* stripes 2, 3, and 7, while *en* stripes 4, 6, and 14 are in the *ftz* domains immediately posterior to these *eve* domains. These correlations suggest that stripes 2, 3, and 7 expressed by E+L-*eve* function normally in initiating *en* expression. (*en* stripe 5 is slightly shifted posteriorly, and stripe 6 is weaker than in wild type and may also be slightly shifted anteriorly. These features correlate with early stripe 3 being somewhat weaker and/or narrower than in wild type; see Discussion.) *en* stripes 9 and 11 are in the domains of *eve* late stripes 5 and 6 which, as noted above, are apparently induced by early stripe 5/6; these *en* stripes also appear to be shifted posteriorly (the anteriorly adjacent w*g* stripes are expanded, Fig. 2F). Eve early stripe 5/6 does not rescue *en* stripes 10 and 12, which would be in the *ftz* domains just posterior to early *eve* stripes 5 and 6, although in some embryos weak stripe-like expression is visible just posterior to stripe 9, which may correspond to a weak stripe 10. The expression of *en* stripe 2 is unexpected (see Discussion). Finally, *en* stripes 1, 7, and 8 are completely absent, correlating with the complete absence of *eve* early stripes 1 and 4.

In E embryos, *en* stripes 2, 3, 4, 5, 6, 13, and 14 are rescued by the early *eve* expression (Fig. 2G); in addition, as in EL embryos, a weak stripe 10 is often present. This pattern differs from that of EL in two ways. First, *en* stripes 9 and 11 are absent. This correlates with the absence of *eve* late stripes 5 and 6. Second, odd-numbered *en* stripes (particularly 3 and 5) are relatively weak and incomplete. Some rescue of odd-numbered *en* stripes is perhaps not surprising due to the temporal and

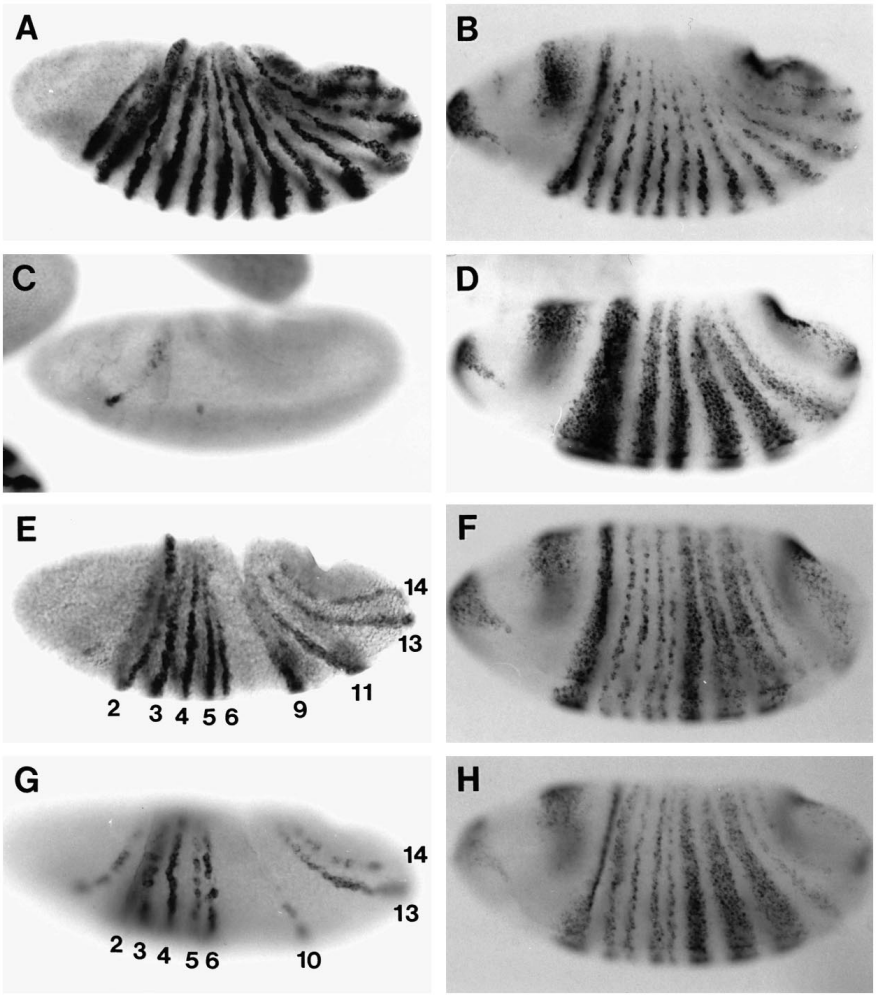

Fig. 2. *en* and *wg* expression in locally rescued embryos. Gastrulation stage embryos were hybridized with *en* probe (A,C,E,G) or *wg* probe (B,D,F,H). (A,B) wild type. (C,D) *Df(eve)*. (E,F) EL embryo. (G,H) E embryo. Expression of early *eve* stripes 2, 3, 5/6, and 7 is sufficient to activate *en* stripes 2 (partially hidden in this embryo), 3, 4, 5, 6, 13, 14, and occasionally 10 (G); however, odd-numbered stripes are relatively weak and incomplete. *en* stripe 5 is slightly posteriorly shifted, and stripe 6 may be slightly anteriorly shifted (see text). Normal *wg* expression is rescued in the regions of early *eve* stripes 2, 3, and 7, but not that of 5/6 (H). Addition of late expression restores normal expression of both *en* and *wg* more completely (E,F). Broad, derepressed *wg* stripes are 'split' where *eve* late stripes 5 and 6 are expressed (F).

spatial overlap of early and late expression, and the incompleteness of the rescue demonstrates the importance of late *eve* in establishing normal levels of *en*. Strikingly, however, this *en* expression is properly restricted to the anterior portion of the normal early stripes, showing that this aspect of regulation does not require the sharp, refined late expression that has been shown to coincide with these *en* stripes (see below and Discussion). In contrast, the normal expression of even-numbered *en* stripes in immediately posterior *ftz* domains indicates that early *eve* stripes are sufficient for this function. In summary, expression of an early *eve* stripe alone completely rescues the even-numbered *en* stripe in the immediately posterior *ftz* domain and rescues a weak version of the embedded odd-numbered *en* stripe, but late expression is additionally required for normal levels of the latter.

wg is another gene regulated by *eve* and *ftz* that is essential for segmentation. The previous observation that *wg* is ectopically expressed throughout the normal *eve* domain in *eve* mutants shows that Eve is a repressor of *wg* (Ingham et al., 1988; Fig. 2D). That this effect is direct is supported by the finding that *wg* is rapidly repressed following ectopic Eve expression (Manoukian and Krause, 1992). We found that in both E and EL embryos, *wg* expression is normal in the domains of *eve* stripes 2, 3, and 7, but remains derepressed in the domains of *eve* stripes 4-6 (Fig. 2F,H), as it is in *Df(eve)* mutants (Fig. 2D). Thus, early *eve* stripes are sufficient for the normal restriction of *wg* expression within *eve* domains. The failure of stripe 5/6 to repress *wg* in E embryos is likely to be due to its low level of expression. In contrast, late *eve* stripes 5 and 6 in EL embryos, which are expressed at higher levels, do repress *wg*, resulting in narrow stripes of cells devoid of *wg* expression within broad *wg* stripes in this region (F). These observations are consistent with Eve acting as a concentration-dependent morphogen (see Discussion).

Finally, we have investigated whether late *eve* expression alone can rescue odd-numbered *en* stripes in the absence of early expression. Embryos transformed with L-*eve* initiate expression of weak late stripes which are not sharply defined at the anterior border (data not shown). These stripes fade prematurely during gastrulation without rescuing *en* expression. Since this failure to rescue *en* may be due to a low level of Eve expression from the transgene, we also tested transgenes containing either a dimer or a trimer of L, which drive late *eve* expression at higher levels (see Jiang et al., 1991). For most cells, the outcome with an L multimer is the same as with a single L, although in a small number of cells there is enhancement and maintenance of *eve* expression and initiation of *en* to varying levels. These exceptional cells notwithstanding, the results clearly show that *eve* late stripes initiated without early stripes cannot properly organize the *eve* domain. The regulation of the late element in the absence of early *eve* expression will be described further elsewhere (M.F. and T.G., unpublished data).

Cuticle patterns confirm the roles of early and late *eve* expression

Although *en* stripes 3 and 5 in the E embryo are weak, their expression recovers to a certain degree in time (data not shown). This recovery is likely a consequence of the intercellular communication known to exist between *en*- and *wg*-expressing cells, which is required to maintain their expression (Ingham and Martinez-Arias, 1992). A consequence of this recovery is that the cuticle patterns of E and EL embryos (Fig. 3) are more similar to each other than might be expected from their initial *en* patterns (see Fig. 2E and G). Nonetheless, there are differences between

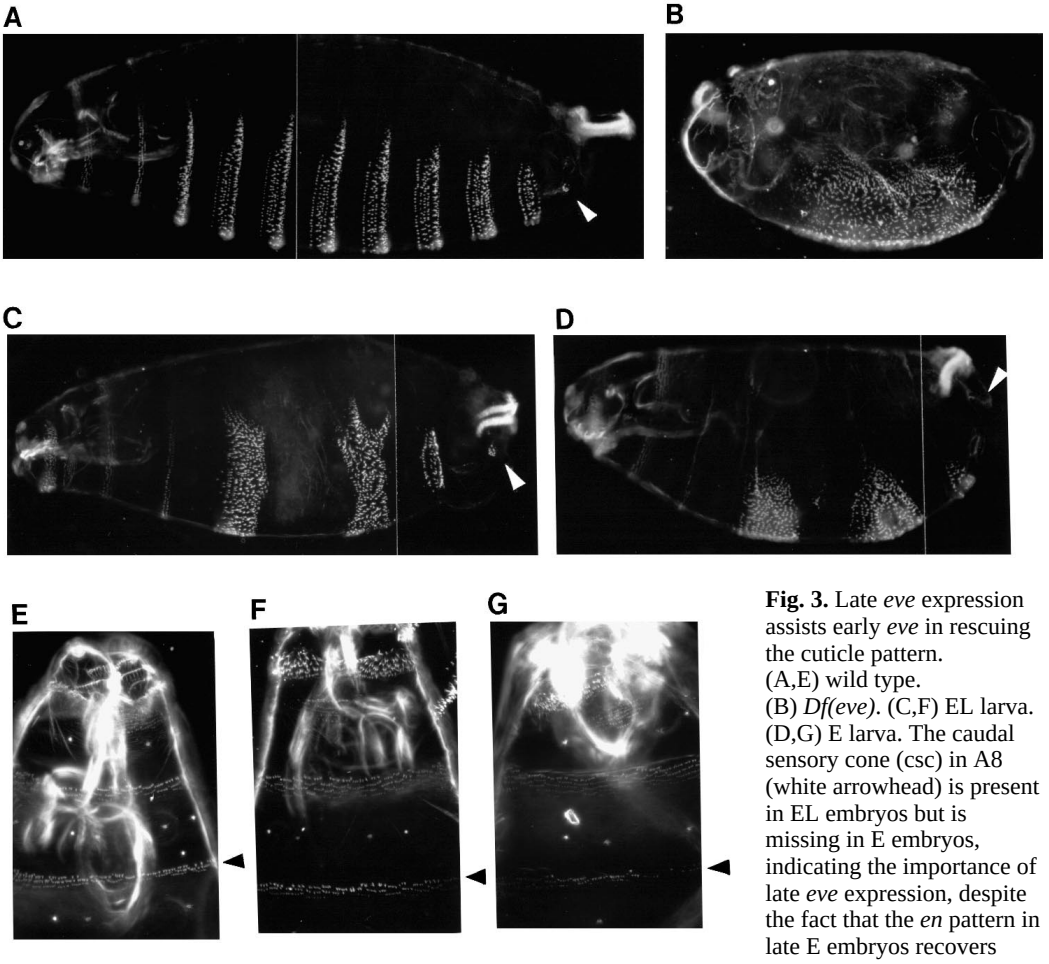

Fig. 3. Late *eve* expression assists early *eve* in rescuing the cuticle pattern. (A,E) wild type. (B) *Df(eve)*. (C,F) EL larva. (D,G) E larva. The caudal sensory cone (csc) in A8 (white arrowhead) is present in EL embryos but is missing in E embryos, indicating the importance of late *eve* expression, despite the fact that the *en* pattern in late E embryos recovers significantly and resembles that in EL embryos. (E,F,G) The head/thoracic region, with the black arrowhead indicating the T3 denticle band, which derives from the region of *eve* stripe 3. Rescue of the T3 band in EL embryos (F) is more complete than in E embryos (G).

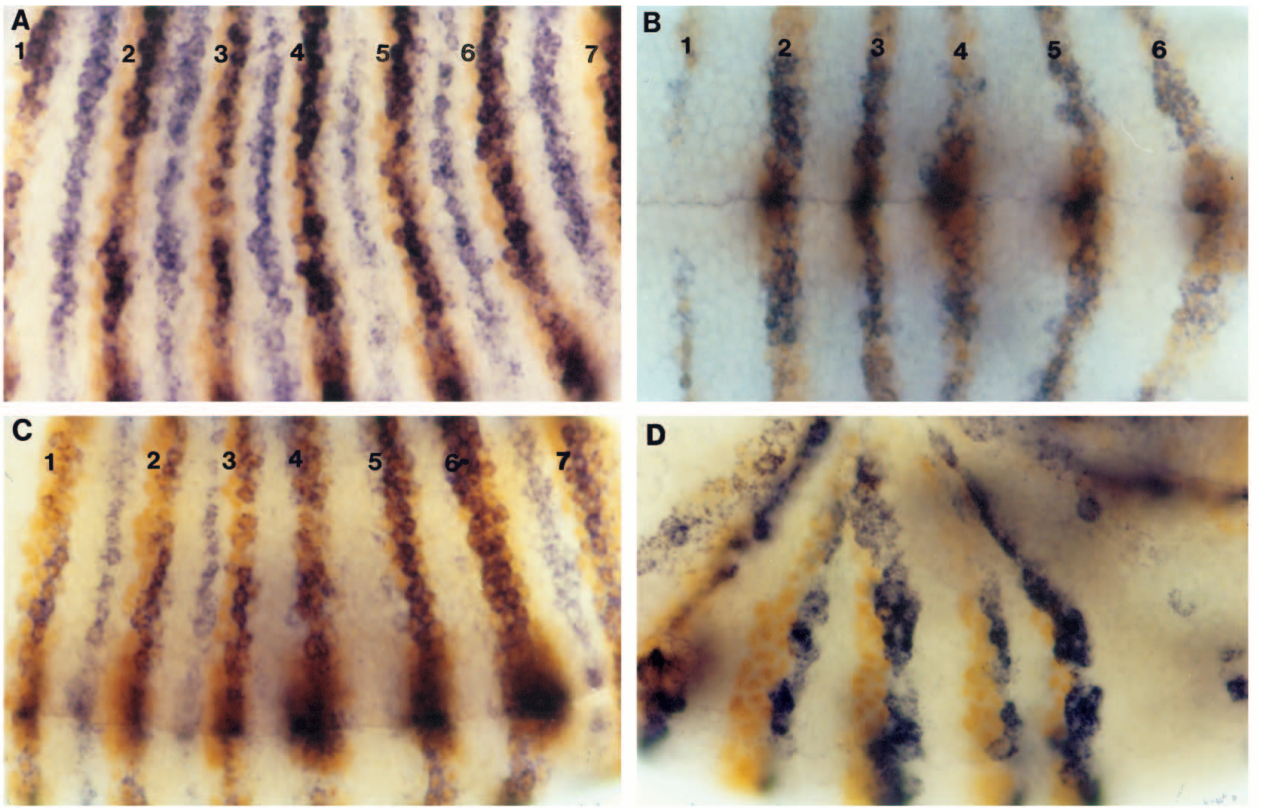

Fig. 4. Early *eve* stripes mediate the clearing of *odd* expression from the anterior-most cells of posteriorly adjacent Ftz stripes. Embryos were hybridized with *odd* probe (blue), followed by staining with a Ftz monoclonal antibody (orange, A,B,C; Ftz stripes are numbered) or an *en* monoclonal antibody (orange, D). (A) Wild type; *odd* mRNA is cleared from the anterior-most cells of Ftz stripes. (B) *Df(eve)*; *odd* expression is coincident with Ftz expression; both Ftz stripe 1 and *odd* stripe 1 are weaker than in wild type. (C) E embryo; *odd* expression is cleared from the anterior cells of Ftz stripes 1, 2, 3, and 7. (D) E embryo; even-numbered *en* stripes (2, 4, and 6 are shown) are induced just anterior to *odd* stripes (where Ftz is expressed). (*odd* is also expressed, more weakly, just posterior to odd-numbered *en* stripes.)

the two that suggest the functional importance of late *eve* stripes. For example the T3 denticle band, which derives from the domain of *eve* stripe 3, is completely recovered in EL embryos but is incomplete in E embryos (Fig. 3F,G). In addition, the portion of the A8 segment that derives from the domain of *eve* stripe 7 is normal in EL embryos, whereas in E embryos it is missing several structures, including the caudal sensory cone (arrowhead in Fig. 3C,D). Interestingly, in both E and EL embryos an abnormally wide band of naked cuticle is formed in the middle trunk region. This may be a consequence of the presence of stripe 5/6, which results in prolonged expression of *wg* in this region relative to that in *Df(eve)*, presumably by an indirect mechanism. This aspect of the phenotype is dosage sensitive and is not seen in most embryos containing only one copy of the transgene (data not shown). Overall, the cuticle patterns confirm the effectiveness of early *eve* expression both in establishing stable parasegmental borders and in organizing intervening odd-numbered parasegments. However, late expression is additionally required for complete functional rescue.

odd-skipped mediates the regulation of even-numbered *en* stripes by *eve*

Although genetic experiments have shown that *eve* plays a role in activating both even- and odd-numbered *en* stripes, they do not tell us whether the interaction is direct. In fact, evidence suggests that it is not. In studying the consequences of ectopic *eve* expression from a heat-shock promoter, Manoukian and Krause (1992) concluded that the activation of *en* expression by *eve* is likely to be an indirect effect. A possible mediator of the activation of *ftz*-dependent (even-numbered) *en* stripes by *eve* is *odd-skipped* (*odd*), since even-numbered *en* stripes, which fail to form in *eve* hypomorphic mutants, 're-appear' in *eve–/odd–* double mutants (DiNardo and O'Farrell, 1987). Primary *odd* stripes overlap the posterior portion of *ftz* stripes. Thus *en*, which is expressed in the anterior part of *ftz* stripes, may be activated by *ftz* and repressed by *odd* (Coulter et al., 1990). Furthermore, Manoukian and Krause (1992) concluded, from their studies on ectopic Eve expression, that Eve represses *odd* and *ftz* directly, but *odd* is sensitive to lower levels of Eve than is *ftz*. Based on this observation, they postulated that the role of *eve* in regulating *en* in the *ftz* domain is to repress *odd* in the anterior row of *ftz*-expressing cells. We tested this model by double staining wild-type, *Df(eve)*, and E embryos with probes for *odd* mRNA and Ftz protein (Fig. 4). Staining of wild-type embryos shows that the Ftz and *odd* stripes are completely coincident at the precellular blastoderm stage, but *odd* expression disappears from the anterior-most cells of Ftz stripes during gastrulation (A), as was shown previously (Manoukian and Krause, 1992). Moreover, this 'clearing' begins in anterior stripes and progresses toward the posterior in a manner similar to the progressive appearance of *en* stripes (Manoukian and Krause, 1992). In contrast, in *Df(eve)* embryos, *ftz* stripes and

odd stripes are completely coincident throughout gastrulation (B). In E embryos, the anterior-most cells of *ftz* stripes 1, 2, 3, and 7 are devoid of *odd* expression, correlating with the appearance of *en* in these locations, whereas stripes 4, 5, and 6 of *ftz* and *odd* overlap even at late gastrulation (C). Double staining for En protein and *odd* mRNA shows that *odd* message is indeed absent from *en* expressing cells (D). These results strongly support the *eve/odd* double negative model of *en* activation discussed above, and further show that early *eve* expression alone is sufficient for this effect in the *ftz* domain.

eve minor stripes are not required for *en* regulation in the *ftz* domain

The results thus far leave an unanswered question as to the source of Eve in the *ftz* domain. As suggested previously (Manoukian and Krause, 1992), a logical candidate has been seven *eve* minor stripes, which appear during gastrulation and alternate with the strong late stripes, apparently coinciding with late, narrowed *ftz* stripes (Harding et al., 1986; Macdonald et al., 1986; see Fig. 5A). According to this idea, the level of Eve in these minor stripes, which is very low, is sufficient to repress *odd* but not sufficient to repress *ftz*, effectively dividing the *ftz* stripes into *odd* expressing and non-expressing cells (Manoukian and Krause, 1992). However, some of our observations are not consistent with this model. For example, the clearing of *odd* expression from the anterior of *ftz* stripes 1, 2, and 3 begins during the cellular blastoderm stage, prior to the appearance of the minor stripes (not shown). To clarify the matter further, we examined E and EL embryos for expression of minor stripes (Fig. 5). Gastrulating E embryos do not express minor stripes at detectable levels. However, during germ-band elongation, they do express seven faint stripes near where minor stripes are expected (data not shown). It is not clear whether these late appearing stripes are belated minor stripes. In fact, there is a *cis*-acting element downstream of the *eve* transcription unit that can drive reporter gene expression in seven late *ftz*-like stripes (Charles Sackerson and T.G., unpublished data), and the E-*eve* construct may contain part of this *cis*-acting element. However, irrespective of how these stripes relate to normal *eve* expression, they cannot be the source of *odd* repression, since these stripes still appear in the regions that lack early *eve* stripes, and yet they do not rescue the corresponding *en* stripes (8 and 12). In contrast to E embryos, EL embryos do detectably express some minor stripes, although at lower levels than in wild type (this expression is not clearly reproduced in Fig. 5B). We find that expression of minor stripes is boosted by certain deletions within L in the locations of rescued even-numbered *en* stripes, suggesting that L is at least partly responsible for the minor stripes; one such embryo (a transformant of RVΔSac in Fig. 1) is shown in Fig. 5C (compare C with the EL embryo shown in B). These observations indicate that E embryos, lacking L, do not have proper minor stripe expression, yet there is rescue of even-numbered *en* stripes. Therefore, we conclude that the minor stripes are not required for the regulatory effects of *eve* in the *ftz* domain, and that it is probably the posterior trailing edge of early stripes that mediate this aspect of *eve* function.

Other pair-rule genes mediate early *eve* regulation of *en*

Odd-numbered *en* stripes fail to appear in loss-of-function

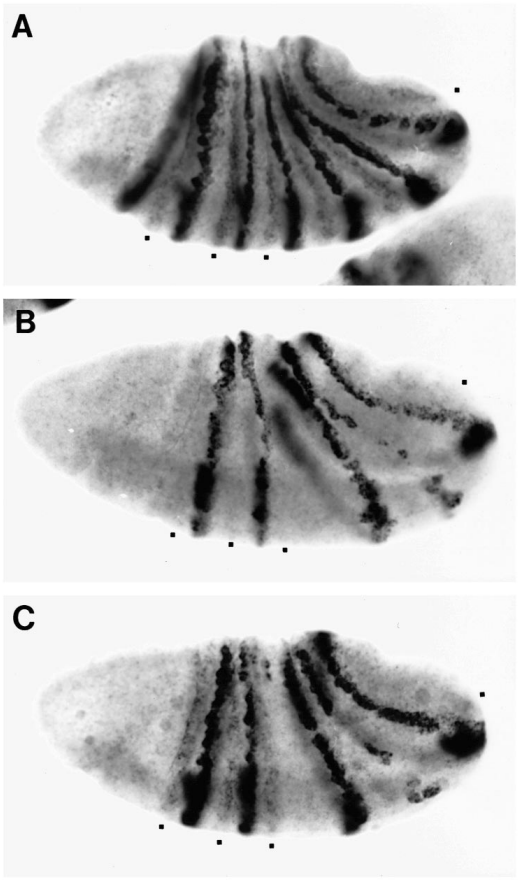

Fig. 5. *eve* minor stripes are derepressed by a deletion within the late element (L). Gastrulating embryos were hybridized with an *eve* probe. (A) wild type; minor stripe expression is centered between *eve* major stripes. Minor stripes in the domains of *ftz* stripes 1, 2, 3, and 7 are marked with square dots. (B) EL embryo; weak minor stripes are just detectable in the domains of *ftz* stripes 1, 2, 3, and 7 (square dot; more clearly visible under the microscope). Embryos in A and B were from the same staining population. (C) RVΔSac-*eve*; the transgene contains a small deletion within a shortened, but fully active L (see Fig. 1A). Minor stripes apparently corresponding to those seen in EL embryos (C) are strongly enhanced, indicating that L plays a major role in driving minor stripe expression.

mutants of two pair-rule genes, *eve* and *prd*, and the posterior of 'early' *prd* stripes overlap the anterior of early *eve* stripes where *en* is activated (DiNardo and O'Farrell, 1987; Ingham et al., 1988). From these observations, it was suggested that the combination of *eve* and *prd* may directly activate odd-numbered *en* stripes. For the simplest version of this model to be correct, both Eve and Paired must be transcriptional activators. While Paired does act as a transcriptional activator in cell culture (Han et al., 1989), a growing body of evidence suggests that Eve is a transcriptional repressor (Jaynes and O'Farrell, 1988; Han et al., 1989; Biggin and Tjian, 1989; Johnson and Krasnow, 1992; Manoukian and Krause, 1992; Han and Manley, 1993). If this is its molecular activity in activating odd-numbered *en* stripes, we might be able to identify intermediates among the known pair-rule genes. Therefore, we examined the expression patterns of all known pair-rule genes (i.e. *hairy, runt, prd, odd-paired* and *sloppy-paired (slp)*, in addition to *ftz* and *odd*) in E and EL embryos. We found that for each of these genes, altered

expression patterns are similar in E and EL embryos during blastoderm and early gastrulation (data not shown). This finding suggests that late *eve* expression does not strongly affect the patterning of other pair-rule genes during the period critical to the activation of *en*, just as it is not crucial to the proper localization of odd-numbered *en* stripes. However, late *eve* does enhance expression of these *en* stripes. Intermediates thus may share a border of expression with late *eve*. Therefore, we double-stained EL embryos for expression of *eve* and each of three genes, *prd*, *runt*, and *slp*, which previous work had shown might be involved in *en* regulation (see below).

As shown in Fig. 6A, early *prd* stripes 2 and 3 are sharply defined, while stripes 4-6, in the absence of strong early *eve* stripes 4-6, are less clearly defined, due to ectopic *prd* expression throughout the region. This is consistent with the previous observation that *eve* maintains *prd* repression between early *prd* stripes (Gutjahr et al., 1993). (Stripe 1 is regulated differently from the rest (Gutjahr et al., 1993) while the appearance and refinement of *prd* stripe 7 is delayed relative to the others.) In addition, the relative sharpness of *prd* stripes 2 and 3 also indicates that peak concentrations of early *eve* restrict the posterior borders of *prd* stripes. That *prd* is not sensitive to lower concentrations of *eve* is seen in the inability of the weak stripe 5/6 to repress *prd* (Fig. 6A). In embryos after cellularization (B,C), *prd* apparently becomes resistant to repression by *eve*, as stripes 2 and 3 continue to overlap with the high concentrations of Eve in the anterior portions of narrowing *eve* stripes, while *prd* stripes 4-6 remain broad despite strong *eve* late expression in the region. Thus, early *eve* appears to restrict the posterior borders of early *prd* stripes, and this border is maintained as *eve* stripes are refined.

runt has been shown to be a potent and apparently direct repressor of expression of both late *eve* and odd-numbered *en* stripes (Frasch and Levine, 1987; Goto et al., 1989; Manoukian and Krause, 1993). Conversely, ectopic *eve* can repress *runt*, perhaps directly (Manoukian and Krause, 1992). The seven early *runt* stripes, which do not appear to be sensitive to *eve* repression, overlap the posterior half of early *eve* stripes (Fig. 6D), consistent with a contribution from *runt* to the localization of late *eve* stripes and odd-numbered *en* stripes. After cellularization, the *runt* pattern evolves into 14 stripes that eventually cover all the trunk region, except where late *eve* (and odd-numbered *en*) and *ftz* (and even-numbered *en*) are activated (E,F). An antagonistic relationship between late *runt* and late *eve* is suggested by the mutual exclusivity of their expression, even in the region of late *eve* stripes 5 and 6 in EL embryos, which are not entirely normal, as described earlier. In contrast to its effect on late *runt*, early *eve* expression appears to reinforce early *runt* expression posterior to *prd* (Fig. 6D,E; Klingler and Gergen, 1993). The sensitivity of late *eve* and late (14-stripe) *runt* to repression by Runt and Eve, respectively, may generate the observed mutually exclusive patterns, with early expression providing an initial spatial bias, and late expression sharpening the boundaries. By repressing *runt* in cells that normally express *en*, late *eve* may thus boost the level of odd-numbered *en* stripes relative to that seen in E embryos.

Previous studies with both *slp* mutants and ectopic Slp expression indicated that *slp* is a repressor of *eve* and *en*, and may be involved in restricting the anterior borders of late *eve* and odd-numbered *en* stripes (Cadigan et al., 1994a,b). *slp* expression starts relatively late, and the initial *slp* pattern is complementary to that of *eve*, as shown in Fig. 6G, suggesting that *slp* is negatively regulated by *eve*. In E embryos at this stage as well, while *slp* expression is normal in the stripe 2, 3, and 7 region, it is derepressed in the stripe 4 region (data not shown), showing that bell-shaped early stripes are responsible for this effect. It appears that *slp* is more sensitive to repression by *eve* than is *prd*, since the low level of Eve in stripe 5/6 is sufficient to repress *slp*, but not *prd*. This is also consistent with the fact that *slp* stripes are normally localized further from the center of early *eve* stripes than are the corresponding *prd* stripes. This apparent sensitivity to repression by *eve* continues as the *slp* pattern evolves into 14 stripes, as *slp* is absent in cells expressing high levels of *eve* (Fig. 6H,I). (Cells in the region of *eve* stripe 1 do express both *eve* and *slp* (not shown), but regulation of several genes in this region appears to be different from that in the rest of the trunk.) Similar to the cross-regulation by late *eve* and *runt*, the mutual exclusivity of the *eve* and *slp* patterns is consistent with the notion that mutual repression by *slp* and late *eve* helps to determine the anterior borders of both late *eve* and odd-numbered *en* stripes.

DISCUSSION

The expression patterns of most pair-rule genes are seemingly in a state of flux. In the case of *eve*, the course of these changes can be divided into an early establishment phase and a later refinement phase based both on the shape of the stripes and on the *cis*-regulatory elements involved. The refinement process has been described as a progressive loss of expression from the posterior of early, broad stripes. This process, as we have shown here, can be effectively reproduced as a simple combination of two static patterns. Broad, early stripes are initiated in response to combinations of earlier acting genes, particularly the gap genes, acting on stripe-specific regulatory elements within the *eve* gene. These early stripes simply fade, probably uniformly, giving way to late expression driven by a separate regulatory element. This element responds both to early *eve* expression and to regulatory inputs from other pair-rule genes to give narrow, late expression in the anterior portion of early stripes. The temporal overlap of these discrete early and late stripes gives the observed, apparently progressive refinement. In this work we have also shown that Eve protein expression driven by a combination of early and late elements can functionally substitute for the endogenous *eve* gene during embryonic pattern formation. We used these separable regulatory elements to express Eve in either an early-only pattern (E embryos) or an early-plus-late pattern (EL embryos) in portions of otherwise *eve* deficient embryos. This allowed us to separately determine the contributions of the early and late *eve* patterns to the regulation of its target genes. We found that there are important functional distinctions between early and late expression, as discussed below. This approach provides a number of new insights into how the domains of pair-rule gene expression are divided into multiple functional subregions, providing for correct activation and restriction of expression of downstream genes. Our results suggest that the early *eve* domain is subdivided by interactions that depend on both concentration-dependent and combinatorial mechanisms.

Activation of *en* is an important function of *eve* in both even- and odd-numbered parasegments. We find that early

expression alone is sufficient for normal activation of *en* in even-numbered parasegments. This has implications for the mechanism of *eve* action in the *ftz* domain, as discussed below. In contrast, we find that the odd-numbered *en* stripes, which form in the domain of late *eve* expression, require both early and late expression for complete activation. While this dual requirement is partially attributable to the fact that the early *eve* pattern serves to enhance and maintain late expression, there is activation of functional levels of *en* by early expression alone. Strikingly, this *en* expression is properly restricted to the anterior portion of the normal early stripes in E embryos. This, along with other results discussed below, suggests that the early bell-shaped *eve* stripes function as morphogenetic gradients over the distance of just a few cell diameters, regulating downstream genes in a concentration-dependent manner to specify the fates of individual cell rows.

Activation of even-numbered *en* stripes

Activation of even-numbered *en* stripes in the *ftz* domain is apparently attributable to the low level of *eve* expression at the posterior edges of early stripes. A previously proposed model of *en* activation suggested that *en* stripes are initiated in nuclei that express *ftz* but not *odd*, with *eve* being responsible for the 'clearing' of *odd* from the anterior-most cells of each *ftz* stripe (Manoukian and Krause, 1992). Our data confirm these features of the model, showing that *odd* disappears from the anterior row of *ftz*-expressing cells immediately posterior to each early *eve* stripe, where *en* is activated. However, there is a discrepancy between our data and the previous proposal that the minor stripes of *eve* that appear relatively late in the anterior cells of *ftz* stripes might be the source of *eve* function there (Manoukian and Krause, 1992). Our data point to early expression as the primary source of *eve* function in the *ftz* domain. Since early stripes are bell-shaped, the source of Eve in the *ftz* domain is presumably the posterior trailing edge of the gradient, although the functional level of Eve there may be below the detection limits of conventional staining procedures. A similar conclusion has been drawn about functional levels of Hairy protein in apparent interstripe regions (Landelli and Ish-Horowicz, 1993).

In the model by Manoukian and Krause (1992) cited above, it was postulated that the differential sensitivity of *odd* and *ftz* to repression by *eve* was sufficient to subdivide the *ftz* domain into *odd*-expressing and non-expressing cells. Based on the observation that several other potential target genes of *eve* also show differential sensitivities to ectopic Eve induction, these researchers further concluded that Eve acts as a concentration-dependent morphogen. Our examination of pair-rule gene expression in E and EL embryos shows that late stripes contribute relatively little to the regulation of downstream pair-rule genes by early stripes. Thus, the morphogenetic activity of Eve arises from the bell-shaped early stripes, rather than from the saw-toothed gradient that results from the sum of early and late expression. However, our data also call for caution in invoking a morphogenetic activity for Eve in the *ftz* domain. For example, the *eve* expression within *ftz* stripe 5 of E embryos, part of *eve* stripe 5/6, is apparently uniform, and the level is higher than that normally found within the *ftz* domain (as well as more persistent). Nonetheless, there is some clearing of *odd* in the anterior portion of *ftz* stripe 5, and this leads to a variable, weak rescue of *en* stripe 10. This suggests that something other than a difference of early *eve* concentration contributes to the distinction between anterior and posterior portions of *ftz* stripes; this additional regulation may involve a combinatorial mechanism. This notion is also suggested by the observation that *en* stripe 2 is rescued by E-*eve* within the anterior-most cells of *ftz* stripe 1 (Fig. 2E,G), apparently by the abnormally extended anterior edge of *eve* stripe 2, which increases in expression from anterior to posterior throughout *ftz* stripe 1 (Fig. 1D,G). However, it should be pointed out that this *en* stripe is partially *eve* independent (a semblance of this stripe still forms in *eve* null mutants), and thus may be regulated differently from other *en* stripes. Thus, while differential sensitivity of *ftz* and *odd* to a gradient of *eve* may contribute to *odd* clearing and *en* activation in the *ftz* domain, additional mechanisms are apparently also involved.

Activation of odd-numbered *en* stripes by *eve*

Odd-numbered *en* stripes in E embryos, which express only early *eve* stripes, are weak but properly placed. The proper localization of these stripes without late *eve* expression supports the notion that the bell-shaped gradient of early *eve* provides cues that these *en* stripes normally respond to. *eve* probably acts indirectly in activating *en*, since Eve is a repressor in cultured cells and in in vitro transcription assays (Han et al., 1989; Biggin and Tjian, 1989; Johnson and Krasnow, 1992; Han and Manley, 1993), and abnormal *en* activation by ectopically expressed Eve is delayed (Manoukian and Krause, 1992). It is therefore likely that this regulation involves secondary factors that are normally under the control of early *eve* stripes. One of these genes is *prd*. It encodes a transcriptional activator (Prd), based on cell culture assays (Han et al., 1989), and is required for the activation of odd-numbered *en* stripes. The posterior of 'early' *prd* stripes and the anterior of early *eve* stripes overlap where *en* is activated, and these *en* stripes fail to appear in loss-of-function mutants of either gene (DiNardo and O'Farrell, 1987; Ingham et al., 1988). It was suggested therefore that the combination of *eve* and *prd* specifies odd-numbered *en* stripes. Consistent with this model, ectopic expression of Prd causes posterior expansion of these *en* stripes (Morrissey et al., 1991). Thus, in addition to activating these stripes, Prd is apparently involved in determining the posterior borders of odd-numbered *en* stripes. However, rather than simply acting combinatorially with *prd*, early *eve* regulates the posterior border of *prd* expression, as *eve* mutations cause ectopic activation of *prd* in this region (Fig. 6A; Gutjahr et al., 1993). Thus, this determinant of the posterior border of odd-numbered *en* stripes is itself restricted by high concentrations of early *eve*.

The restriction of the anterior borders of odd-numbered *en* stripes may be provided, at least in part, by secondary (late) *runt* stripes. These stripes appear just prior to *en* activation and abut the *en* anterior borders, occupying the anterior portion of late *prd* stripes (Fig. 6; Manoukian and Krause, 1993; Klingler and Gergen, 1993). Eve is probably a direct repressor of *runt* (Manoukian and Krause, 1992), and we observe an expansion of late *runt* expression in the *Df(eve)* portion of locally rescued embryos (Fig. 6; see also Frasch and Levine, 1987). Thus, late *runt* expression is apparently repressed at a lower concentration of Eve than is *prd*, thereby allowing *en* activation in cells that express *prd* but not *runt*. However, *runt* is unlikely to be the only repressor at this border, because these *en* stripes do not obviously expand in *runt* mutants. Moreover, odd-numbered *en* stripes do not re-appear in *eve/runt* double mutants (our unpublished observation). It is likely that stripes of *slp*, which also abut the anterior

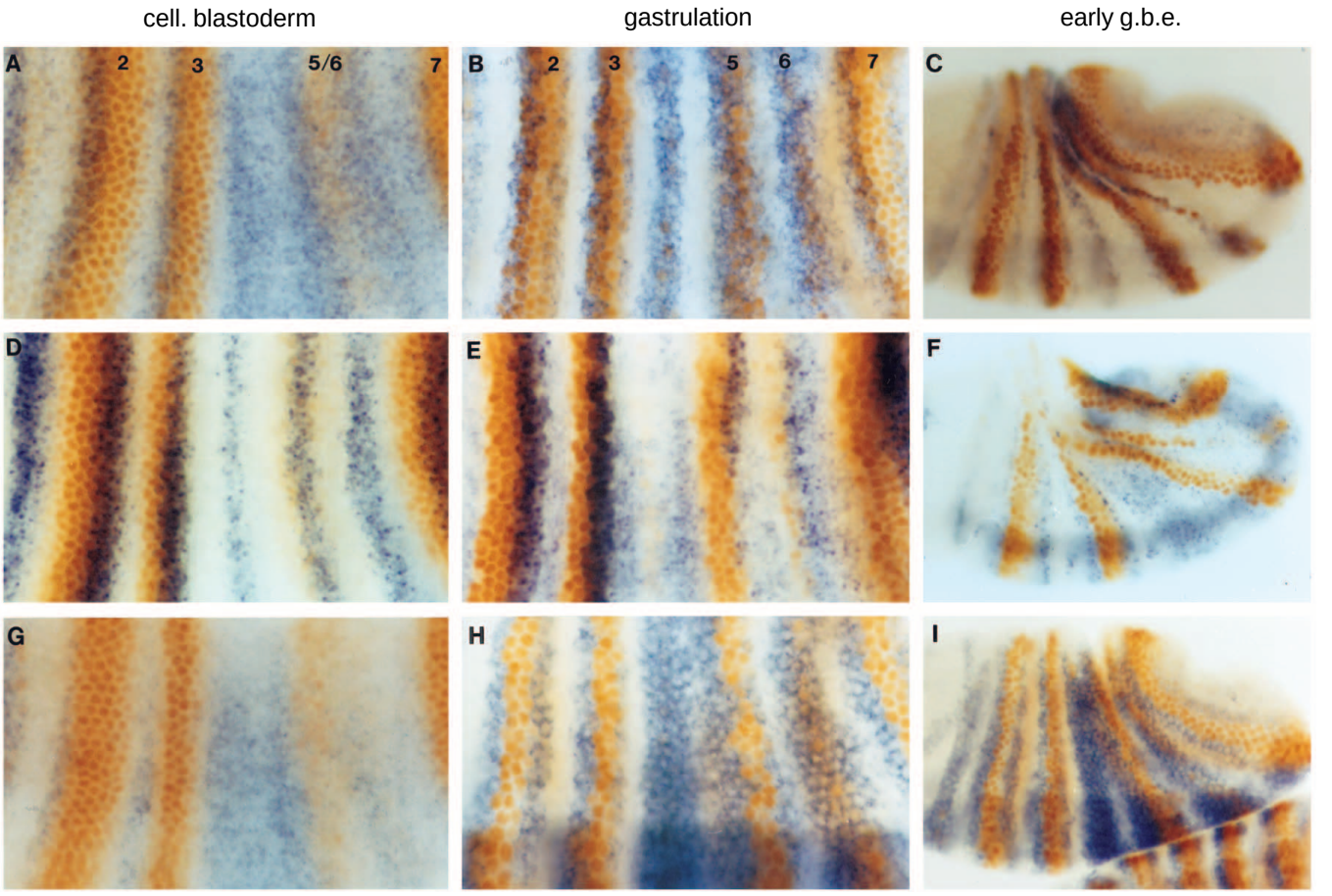

Fig. 6. Eve represses *prd*, *runt*, and *slp*, regulators of late *eve* and *en*. EL embryos were stained for Eve protein (orange) and either *prd*, *runt*, or *slp* RNA (blue) at three developmental stages. (A,B,C) *prd* and Eve. Eve stripes are numbered. At cellular blastoderm (A), *prd* stripes 2 and 3 overlap the anterior portions of early Eve stripes (this is less clear with *prd* stripe 7, which develops later than the others). Early *prd* expression is posteriorly restricted by Eve, as suggested by the ectopic *prd* expression observed in the region of stripes 4-6, which also shows that the level of Eve in early stripe 5/6 is not sufficient to repress *prd*. *prd* and Eve continue to overlap at gastrulation (B) and early germ band extension (C), indicating that late *prd* expression is insensitive to Eve repression. (D,E,F) *runt* and Eve (same developmental stages as A,B,C above). Early *runt* stripes overlap the posterior portion of early *eve* stripes and do not expand in the Eve-deficient region (D), suggesting that they are not sensitive to Eve repression. However, late *runt* expression, which appears at the anterior of *eve* stripes, seems to be sensitive to repression by Eve (see text), and possibly vice versa, as their patterns show mutual exclusivity with sharp boundaries, particularly at the anterior border of late *eve* (E,F; see also Manoukian and Krause 1993). (G,H,I) *slp* and Eve (same stages as above). *slp* expression, appearing later than *eve*, is ectopically expressed in the Eve-deficient regions (G), suggesting that it is repressed by early Eve. This apparent sensitivity continues to later stages (H,I).

of odd-numbered *en* stripes (Fig. 6; Grossniklaus et al., 1992), provide a redundant repression function here, since odd-numbered *en* stripes expand in *slp* mutants and are repressed by ectopic *slp* expression (Cadigan et al., 1994a,b). Our data show that *slp* is repressed by Eve in a concentration dependent manner, and by a lower concentration of Eve than that required to repress *prd*, since the level of Eve in the stripe 5/6 region of E embryos represses *slp* but not *prd* (Fig. 6). Thus, the anterior border of odd-numbered *en* stripes is abutted by two redundant repressors of these stripes (based on the fact that ectopic expression of either Runt or Slp causes *en* repression), both of which are repressed by early *eve* expression.

A model of *en* activation by a morphogenetic gradient of early *eve* expression

Previous models for activation of *en* invoked a combinatorial interaction between *prd* and late *eve* to explain the localization of odd-numbered *en* stripes. Our results show that the effects of *eve* are more complex. We have found that late *eve* expression is not required for proper localization of these *en* stripes. Moreover, both late *eve* and *prd* are regulated by early *eve*, and late *eve* can be activated by a low level of early *eve* that cannot repress *prd*. Moreover, two other pair-rule genes, *slp* and *runt*, that negatively regulate *en* and share stripe borders with *en* are also regulated by early *eve*. It was previously shown that ectopically expressed Eve represses both *prd* and *runt*, but that a higher concentration of Eve is required to repress *prd* (Manoukian and Krause, 1993); we have found that repression of *prd* requires the high concentration of *eve* present in the middle of early stripes. Further, we have found that *slp* is repressed by early *eve* in a concentration dependent manner, and that *slp* is repressed at a lower concentration of *eve* than is *prd*. Based on these observations, we propose that *eve* acts as a concentration-dependent morphogen in its activation of odd-numbered *en* stripes, as illustrated in Fig. 7.

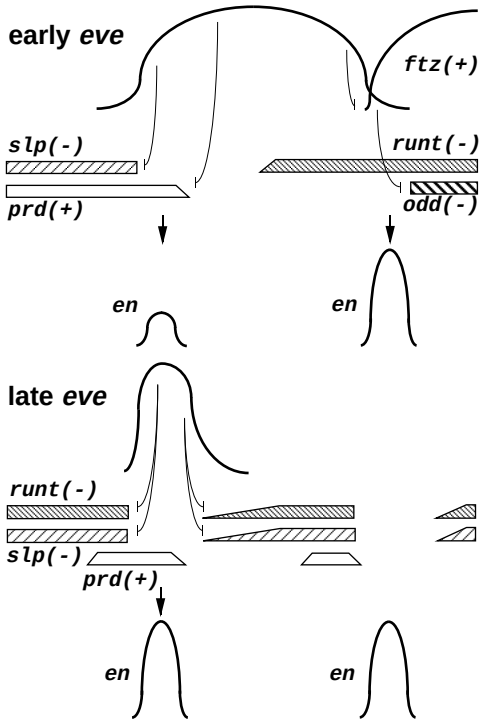

Fig. 7. A model of *en* activation by the Eve morphogen. Target genes are repressed by different concentrations of Eve in early stripes, leading to specific patterning of *en* activators (*prd* and *ftz*) and repressors (*slp*, *runt*, and *odd*). Combinations of these gene activities lead to activation of both odd-numbered *en* stripes and late *eve* stripes at the anterior edge of early *eve* stripes, and even-numbered *en* stripes at the posterior edge of early *eve* stripes. Late *eve* expression reinforces the repression of *en* repressors, allowing strong expression of odd-numbered *en* stripes. See the text for details.

In this model, high levels of early *eve* repress *prd*, restricting its posterior border, while lower levels allow *prd* expression. In *eve* mutants, *prd* is ectopically expressed, but *en* is not activated. This is accounted for in the model by the proposed negative regulators of the *en* anterior border, *slp* and *runt*, which are also ectopically activated in *eve* mutants. These repressors set the anterior border of odd-numbered *en* stripes by being repressed at lower levels of early *eve* than is *prd*. These two concentration thresholds for repression by Eve provide a narrow region where *prd* is activated, but not *runt* and *slp*, resulting in activation of *en*. *en* is activated only toward the anterior, and not the posterior, of the early *eve* stripe due to the expression of early *runt* in the posterior.

wg is also repressed by early *eve*, since its expression expands throughout the *eve*-deficient portion of E embryos. *prd* is required for *wg* activation adjacent to odd-numbered *en* stripes (Ingham and Hidalgo, 1993), and *wg* is very sensitive to repression by both ectopically expressed Eve (Manoukian and Krause, 1993) and En (Heemskerk et al., 1991). Activation of *en* in the posterior row of *prd*-expressing cells is therefore capable of restricting *wg* expression to the anterior portion of late *prd* stripes, adjacent to the odd-numbered *en* stripes. Consistent with this model for subdivision of the *prd* stripe in E embryos, weaker than normal odd-numbered *en* stripes induced in E embryos are flanked by slightly expanded *wg* stripes (Fig. 2H). Thus, multiple target genes are repressed at different concentrations of Eve at the anterior border of early stripes to provide for the two adjacent cell identities (*wg*- and *en*-expressing cells) that establish the parasegmental border.

There is a strong correlation between the locations of stable late *eve* stripes and odd-numbered *en* stripes in all known circumstances, suggesting that they respond to a very similar set of regulatory interactions. Thus, like *en*, the anterior border of late *eve* is probably determined by the combined repressive action of secondary *runt* and *slp* stripes, whose sharp posterior borders abut late *eve* stripes (Fig. 6). Consistently, *eve*, like odd-numbered *en* stripes, is sensitive to repression by ectopic expression of either Slp or Runt (Cadigan et al., 1994a). Furthermore, *eve* stripes expand anteriorly in *slp* mutants during germ-band elongation (Cadigan et al., 1994b), after *runt* has faded. The reinforcing effect of late *eve* expression on odd-numbered *en* stripes is most likely due to its preventing late *runt* and *slp* expression from encroaching into the posterior-most row of *prd*-expressing cells, where *en* is activated (Fig. 7). Late *eve* can also restrict *wg* to the anterior-most row of *prd*-expressing cells, adjacent to the incipient *en* stripes. Thus a presumptive parasegment border may initially result from mutual repression between *eve* on one side and *runt* and *slp* on the other. Interestingly, *prd*, which is completely repressed by high levels of early *eve* expression, is not repressed by the high levels of late *eve*. This may be the result of *prd* having distinct early and late regulatory programs, analogous to those of *eve* (Gutjahr et al., 1993).

It is interesting to note that a number of pair-rule genes, including *eve*, *prd*, *runt*, and *slp*, have both pair-rule expression patterns and, later, narrower expression patterns in every segmental primordium. This suggests an evolutionary relationship between molecular interactions that occur at the pair-rule and segment polarity stages of pattern refinement. However, assuming such an evolutionary relationship, our results suggest that the molecular interactions at these two stages of development have been modified during evolution, such that, as with the effects of *eve* on *prd*, a strong interaction at one stage may be completely absent at the other. These modifications are apparently important to pattern refinement, in this case allowing the early Eve gradient to first restrict and then subdivide a domain of *prd* expression to establish the parasegment border. Early *prd* expression appears to activate late *prd*, so that the posterior restriction of early *prd* by *eve* is reflected in the late *prd* pattern.

A satisfying aspect of the model that *eve* acts as a bell-shaped gradient morphogen is that it can explain the relative shift of late *eve* and *ftz* stripes that has been observed in *eve* hypomorphs. Earlier work described this as an anterior shift of *ftz* expression relative to that of *eve* (Frasch et al., 1988). However, one could also account for this data as a posterior shift of late *eve* expression (DiNardo and O'Farrell, 1987). The latter interpretation is supported by the fact that we see little or no *ftz* shift between the eve^{+} and *eve*-deficient portions of locally rescued embryos, while there is an apparent posterior shift of late *eve* expression in the stripe 5/6 region, where the early *eve* expression level is lower than normal. In *eve* hypomorphs, the level of *eve* activity is reduced, presumably resulting in a similar posterior shift of late *eve* stripes. Since late *eve* stripes of approximately normal width still form, the determinants of both their anterior and posterior borders apparently shift toward higher concentrations of early *eve*. Therefore, this coordinated shift in response to reduced *eve* activity further supports the model that, in addition to acting

combinatorially with other pair-rule gene products, Eve behaves as a concentration-dependent morphogen.

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