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The repressor activity of Even-skipped is highly conserved, and is sufficient to activate *engrailed* and to regulate both the spacing and stability of parasegment boundaries

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SUMMARY

During segmentation of the *Drosophila* embryo, *even skipped* is required to activate *engrailed* stripes and to organize odd-numbered parasegments. A 16 kb transgene containing the *even skipped* coding region can rescue normal *engrailed* expression, as well as all other aspects of segmentation, in *even skipped* null mutants. To better understand its mechanism of action, we functionally dissected the Even-skipped protein in the context of this transgene. We found that Even-skipped utilizes two repressor domains to carry out its function. Each of these domains can function autonomously in embryos when fused with the Gal4 DNA-binding domain. A chimeric protein consisting only of the Engrailed repressor domain and the Even-skipped homeodomain, but not the homeodomain alone, was able to restore function, indicating that the repression of target genes is sufficient for *even skipped* function at the blastoderm stage, while the homeodomain is sufficient to recognize those target genes. When *Drosophila* Even skipped was replaced by its homologs from other species, including a mouse homolog, they could provide substantial function, indicating that these proteins can recognize similar target sites and also provide repressor activity. Using this rescue system, we show that broad, early *even skipped* stripes are sufficient for activation of both odd- and even-numbered *engrailed* stripes. Furthermore, these 'unrefined' stripes organize odd-numbered parasegments in a dose-dependent manner, while the refined, late stripes, which coincide cell-for-cell with parasegment boundaries, are required to ensure the stability of the boundaries.

Key words: *even skipped*, *engrailed*, Parasegment boundaries, *Evx*, Repressor, Segmentation, Homeodomain, Protein evolution

INTRODUCTION

The *even skipped* gene (*eve*) encodes a homeodomain (HD) transcription factor required during *Drosophila* segmentation for activation of *engrailed* (*en*) (Harding et al., 1986; Macdonald et al., 1986) and for proper organization of odd-numbered parasegments (DiNardo and O'Farrell, 1987; Frasch et al., 1988; Fujioka et al., 1995; Manoukian and Krause, 1992). It is activated in response to upstream gap genes in a striped pattern that is subsequently refined into narrow stripes that coincide cell-for-cell with the odd-numbered parasegment boundaries (Lawrence et al., 1987). This refinement involves auto-activation, in that early, broad stripes are needed to activate the refined, late stripe pattern (Goto et al., 1989; Harding et al., 1989). Somewhat paradoxically, transcription assays in cultured cells showed that Eve can act as a transcriptional repressor (Han and Manley, 1993; Jaynes and O'Farrell, 1988). This analysis identified an alanine/proline-rich repressor domain, similar in sequence composition to repressor domains in other proteins (reviewed by Hanna-Rose and Hansen, 1996). Further analysis indicated that this Eve repressor domain can function in vitro by interacting with TBP (Austin and Biggin, 1995; Um et al., 1995), and that the Eve N-terminal region can negatively regulate this activity (Li and Manley, 1999). In embryos, ubiquitous expression of Eve led to rapid repression of some target genes, indicating that Eve is a direct repressor of those genes (Manoukian and Krause, 1992). Subsequently, a second repressor domain that is active in embryos was identified, and was shown to interact with the corepressor Groucho (Gro) (Kobayashi et al., 2001). In contrast, the first repressor domain was shown to be Gro-independent (Jimenez et al., 1997). Recently, *Drosophila* Atrophin was identified as a corepressor that interacts functionally with Eve through the Gro-independent repressor domain (Zhang et al., 2002).

Detailed analysis of *eve* regulatory regions identified specific elements responsible for each aspect of its expression pattern, including individual elements for early stripes, as well as a

single element for the refined, late stripes (Fujioka et al., 1999; Goto et al., 1989; Harding et al., 1989; Sackerson et al., 1999). Null mutations for *eve* can be completely rescued by a 16 kb transgene, including the Eve coding region (Fujioka et al., 1999).

The initially identified *eve* allele was a hypomorph with a pair-rule phenotype for which the gene was named (Nüsslein-Volhard and Wieschaus, 1980). However, *eve* function is required for the expression of both odd- and even-numbered *en* stripes, which are activated by distinct mechanisms (DiNardo and O'Farrell, 1987; Howard and Ingham, 1986). The odd-numbered stripes require *paired* (*prd*) in addition to *eve*, while the even-numbered stripes require *eve*, *fushi tarazu* (*ftz*), and *odd paired*. How does Eve do this? Previous data suggested that the role of *eve* in the activation of *en* might be at least in part indirect. Early Eve stripes repress *prd* at a high concentration, and *sloppy paired (slp)*, a repressor of *en* (Cadigan et al., 1994; Grossniklaus et al., 1992), at a low concentration, producing one cell row that has an activator, but not a repressor of *en* (Fujioka et al., 1995). These cells activate the odd-numbered *en* stripes. For the even-numbered *en* stripes, Eve represses another repressor of *en*, *odd-skipped* (*odd*), at the anterior edges of *ftz* stripes to again create one cell row that has an activator, but not a repressor of *en* (Fujioka et al., 1995; Manoukian and Krause, 1992). In *eve* hypomorphic mutants, both sets of *en* stripes are expressed, but the spacing is abnormal. The odd-numbered parasegments are narrower than the even-numbered ones, and are deleted at late embryonic stages (Frasch et al., 1988), apparently through a combination of regulative processes (Pazdera et al., 1998; Hughes and Krause, 2001).

Eve is also expressed in *Drosophila* at later developmental stages. It is expressed (Frasch et al., 1987) and required in specific lineages within the dorsal mesoderm (Su et al., 1999) and the nervous system (Doe et al., 1988; Landgraf et al., 1999), and is expressed in the proctodeum and anal plate ring (Frasch et al., 1987). Eve homologs from several other species have also been shown to have important functions in development. In *Caenorhabditis elegans*, the *eve* orthologue (*vab-7*) is expressed in posterior epidermal cells, muscles and neuronal precursors, and was shown to be required for posterior patterning (Ahringer, 1996). In the beetle *Tribolium castaneum*, *eve* is expressed in a double-segmental pattern (Brown et al., 1997; Patel et al., 1994), and ablation of the protein (Tc-Eve) resulted in a pair-rule phenotype (Schroder et al., 1999). Mice and humans contain two *eve*-related genes. In the mouse, *Evx1* is activated in the primitive streak, and high levels of expression are localized to the region that will give rise to extraembryonic and ventral mesoderm, suggesting involvement of *Evx1* in dorsoventral specification of mesodermal cells (Bastian and Gruss, 1990; Dush and Martin, 1992). *Evx1* is also expressed in the tail bud and the central nervous system, where its function in specific neurons has been established (Moran-Rivard et al., 2001). At later stages, *Evx2* is expressed in the proctodeal region, as well as in the limbs, and has been shown to be required for digit formation (Herault et al., 1996).

In some organisms where the functions of *eve* homologues have not been tested, expression patterns are suggestive of functions in segmentation (reviewed by Davis and Patel, 2002). For example, in the spider *Cupiennius salei* (Damen et al., 2000) and in the silk worm *Bombyx mori* (Xu et al., 1997), *eve* is expressed in stripes. In the short germ band insect *Schistocerca americana* (grasshopper) the *eve* homologue is expressed in a single domain of posterior mesoderm, and in identified neurons that are homologous to those expressing *eve* in *Drosophila* (Patel et al., 1992; Patel et al., 1994). Expression patterns have also been examined in *Xenopus laevis* (Ruiz i Altaba and Melton, 1989) and in the zebrafish *Danio rerio* (Joly et al., 1993; Sordino et al., 1996; Thaeron et al., 2000). These, along with recent studies of expression in amphioxus, *Branchiostoma floridae*, suggest that a role in specifying certain neuronal cell fates, and possibly an analogous role in other tissues, is retained throughout bilaterian animals (Ferrier et al., 2001).

In the work reported here, we utilized the ability to functionally replace the endogenous *eve* gene with a transgene to address three related issues. First, we analyzed the domains of Eve that are required for its function in early development, and found that repression of specific target genes is both necessary and sufficient during segmentation. Second, we replaced *Drosophila* Eve with its homologues from several species, and showed that both recognition of target sites and repression activity are conserved. Third, we showed that the broad, early stripes of Eve establish parasegment spacing and organization, while the late, refined stripes have a distinct role in the maintenance of parasegment boundaries.

MATERIALS AND METHODS

Construction of plasmids

Wild-type *eve* genomic DNA, from –6.4 kb to +9.2 kb (EGN92) (Fujioka et al., 1999), or from –6.4 kb to +8.6 kb (EGN86), was cloned into a modified pCaSpeR vector (Glass protein binding sites were inserted to intensify the eye color of transformants, as described previously) (Fujioka et al., 1999). The +8.6 kb end point was chosen because it gave stronger expression in RP2 and a/pCC neurons than the +8.4 kb end point that was used previously (EGN84) (Fujioka et al., 1999), the +8.6 kb end point showed a similar rescuing potency to the +9.2 kb end point, and, for ease of subcloning, the shorter construct was preferable. For deletion of the stripes 4+6 element, the region from +4.8 kb (*Xho*I site) to +5.7 kb (*Sph*I site) was removed from EGN92. For deletion of the late stripes, the region from –6.4 kb to –4.8 kb was removed from EGN84 and from EGN92.

In protein deletion constructs, a single copy of a FLAG tag (including an initiator ATG) followed by an *Nhe*I site was inserted in front of the normal initiator ATG. All modified protein coding regions except ΔC (Gro interaction domain deletion, see below) were inserted just downstream of the Flag tag using the *Nhe*I site. The N-terminal deleted protein (ΔN) starts at aa 61 (Gly). For the R domain deletion (ΔR), aa 167 (Ala) to 237 (His) were removed. At the new junction, two amino acids (Ser, Arg) were inserted because of the cloning site. The ΔC lines were described previously (Kobayashi et al., 2001), and do not include the FLAG tag. Clear homology among *Drosophila* Eve, Tc-Eve (flour beetle), and Sa-Eve (grasshopper) extends beyond the HD (which is aa 70-130), so that aa 61 (Gly) to 166 (Pro) were included in the H domain in this study. The ΔRC protein ends after aa 166. The En N terminus to aa 298 (Ser) was fused either upstream or downstream with the Eve H domain. Deletion of the Ala stretch, aa 167-177, generated the RΔA construct. All modified proteins were expressed from the EGN86 construct, except ΔC, which was expressed from EGN84.

For expression of Gal4 fusion proteins, the Gal4 DNA binding domain followed by an HA-tag was fused to either the Eve R+C

region (aa 140Y–C terminus), the R domain (aa 140Y–239H), or the C domain (aa 238M–C terminus) followed by the *eve* 3′ untranslated region, from nt +1306 (*Bst*UI) to +1521 (*Kpn*I). The *eve* 5′ promoter region from nt –275 (Sfi1) to +11 followed by a 38 bp multicloning sequence and *eve* DNA from nt +91 to +99 was inserted upstream of the ATG. The yeast *GAL4* translational initiation signal was changed to that of *eve* in order to boost expression. These constructs were driven by the elements for *eve* early stripes 1 and 5, from +6.6 kb (*Stu*I) to +8.2 kb (*Cla*I). For the reporter transgene, the elements 1 and 5 region, from +6.6 kb (*Stu*I) to +8.0 kb (*Ple*I) and the stripes 4+6 element, from +4.5 kb (*Bam*HI) to +5.2 kb (Csp45 1), were cloned upstream of the *lacZ* coding region and the *eve* promoter region from nt –275 (*Sfi*I) to +166, and the Gal4 UAS sequence from pUAST (Brand and Perrimon, 1993) was inserted between these elements (about 1 kb upstream of the promoter). Further details of constructions are available on request (also see diagram in Fig. 2).

Drosophila strains

The *Drosophila* mutants used in this study were *Df(2R)eve*, eve^{R13} (a.k.a. eve^{3}), and eve^{1} (a.k.a. eve^{ID19}). These mutations were balanced over marked balancer chromosomes to allow identification of mutant embryos. The production of transgenic flies was as previously described (Fujioka et al., 2000).

Analysis of embryos

In situ hybridization to whole-mount embryos was performed as described previously (Tautz and Pfeifle, 1989) using digoxigenin-labeled antisense mRNA, and visualized by the alkaline phosphatase-NBT/BCIP reaction (Roche). For double staining, in situ hybridization was followed by antibody staining (Mullen and DiNardo, 1995) with polyclonal α-Eve (Frasch et al., 1987) at 1:10,000 dilution, or with α-En monoclonal 4D9 (Patel et al., 1989) (obtained from the Developmental Studies Hybridoma Bank) at 1:10 dilution. The staining was visualized using the HRP-DAB reaction. Staining with α-FLAG monoclonal M5 (Sigma), used at 1:100, and α-Eve monoclonal 2B8 (Patel et al., 1994), used at 1:10, was visualized using HRP-DAB enhanced by nickel (Patel, 1994).

Survival rates of rescued transgenic lines were determined by counting the progeny from a cross of *Df(2R)eve* with eve^{R13}, each balanced over *SM6a*, *Cy* and either carrying the transgene on the *eve* mutant chromosome or homozygous for the transgene on the third chromosome. For transgenes that could not be recombined into a *Df(2R)eve* background, the corresponding eve^{R13} self-cross was used. Rescued adult flies, either *Df(2R)eve*/eve^{R13} or eve^{R13}/eve^{R13}, were identified by their wild-type (non-*Cy*) wing phenotype. All of the progeny from two vials were counted for each transgenic line, with the cross done in opposite directions. In order to assess single-copy rescue, homozygous transgenic lines in either a *Df(2R)eve* or an eve^{R13} background were crossed with either eve^{R13} or *Df(2R)eve* flies, respectively, without the transgene. Embryo staining of transgenic lines was done in a *Df(2R)eve* background unless otherwise indicated.

RESULTS

Repression and targeting are sufficient for *eve* segmentation function

The ability of a transgene to completely rescue *eve* null mutants allowed us to analyze functional domains of the Eve protein in embryos. We established transgenic lines expressing variously modified Eve proteins, each driven by the complete *eve* regulatory region (–6.4 kb to +8.6 kb). In order to monitor the expression levels of modified proteins, a FLAG-tag was added to the N terminus of each protein. FLAG-tagged wild-type protein rescued the lethality of *eve* mutants effectively, although the percentage of rescued flies was slightly lower than with the normal protein (Table 1). Based on previous studies and sequence comparison with other species, for this study Eve was divided into 4 domains: an N-terminal region (N); the HD (H), which includes a conserved flanking region (see Materials and Methods); a repressor domain identified in transient assays in cultured *Drosophila* cells (R) (Han and Manley, 1993); and the remaining C terminus (C), which includes a Gro interaction domain (Kobayashi et al., 2001). Protein expression was monitored by staining embryos with α-FLAG and α-Eve antibodies.

Table 1. Efficiencies of adult rescue of *eve* null mutants by *eve* transgenes

	Lines rescued by 2 copies (%)	% rescued per line	Single copy rescue	% rescued per line
Wild type	14/20 (70%)	30 (18-39)	6/20	17 (11-33)
t-WT	4/4 (100%)	23 (14-30)	0/4	0
ΔN	12/13 (92%)	24 (11-30)	11/13	14 (5-25)
ΔR	0/5 (0%)	0		
RΔA	8/16 (50%)	17 (10-36)	ND	
ΔRC	0/4 (0%)	0		
H	0/8 (0%)	0		
H-En	3/8 (38%)	26 (8-48)	ND	
En-H	2/2 (100%)	35 (32-38)	ND	
Wild type 2	2/3 (67%)	32 (31-33)	ND	
Δlate	6/9 (67%)	11 (2-23)	ND	

The rescuing capabilities of *eve* transgenes listed in the first column were determined as described in Materials and Methods. The 'wild type' lines carry the EGN86 construct with the wild-type *eve* coding region. The 't-WT' lines carry the EGN86 construct with a FLAG-tagged *eve* coding region (see Materials and Methods). The 'wild type 2' lines carry the EGN92 construct with the wild-type *eve* coding region. The 'Δlate' lines carry the *eve* genomic region from –4.8 kb to either +8.4 kb or +9.2 kb with the wild-type *eve* coding region. All other lines are like t-WT except for the indicated alterations to the Eve coding region (see Materials and Methods and text for details). The second and fourth columns show the number of lines rescued (at least 1% of the total number of adults eclosing) over the total number analyzed, with the corresponding percentage in parentheses. The average percentage of rescued flies per line is shown in the third column (rescued by two copies of the transgene) and the fifth column (rescued by one copy of the transgene; ND means not done), with the range shown in parentheses. All adult flies eclosing over a period of 10 days were counted from at least two vials for each line (see Materials and Methods for additional details). The expected percentage for complete rescue is 33% (homozygous balancer flies do not survive).

At most insertion sites, the rescue transgene expressing either the wild-type protein or the tagged full-length protein (t-WT, Fig. 1A and Table 1) required two copies for efficient rescue (Fujioka et al., 1999) (Table 1). In contrast, when the N terminus was deleted (ΔN), most lines showed single-copy rescue (Table 1, 11/13 lines rescued, versus 6/20 lines rescued by a single wild-type copy), indicating that the activity of Eve was increased. Consistent with this conclusion, the spacing of En stripes showed that odd-numbered parasegments were slightly expanded relative to even-numbered ones (2-copy rescue, Fig. 1B), a phenotype similar to that caused by an extra copy of the wild-type rescue transgene (Kobayashi et al., 2001).

When the entire R domain was removed (ΔR, deletion of 70 aa), the transgene could no longer rescue adult flies (Table 1). The *en* pattern showed severely narrowed odd-numbered parasegments, with partial loss of odd-numbered *en* stripes (Fig. 1C). Thus, the R domain is necessary for function in the

embryo. The R domain contains an Ala/Pro-rich region that is similar to motifs found in other transcriptional repressors. We tested whether an Ala stretch within this region is required for function. Deletion of these 11 consecutive alanines (RΔA) decreased the percentage of lines that were rescued to adult viability (Table 1), and in most lines where rescue did occur, the efficiency of rescue was less than that of most wild-type rescued lines (Table 1). The lines that did not rescue gave equally narrowed odd-numbered parasegments throughout the embryo (data not shown), indicating reduced Eve function. These data suggest that the alanine stretch contributes to repression activity, but that considerable activity remains when it is removed.

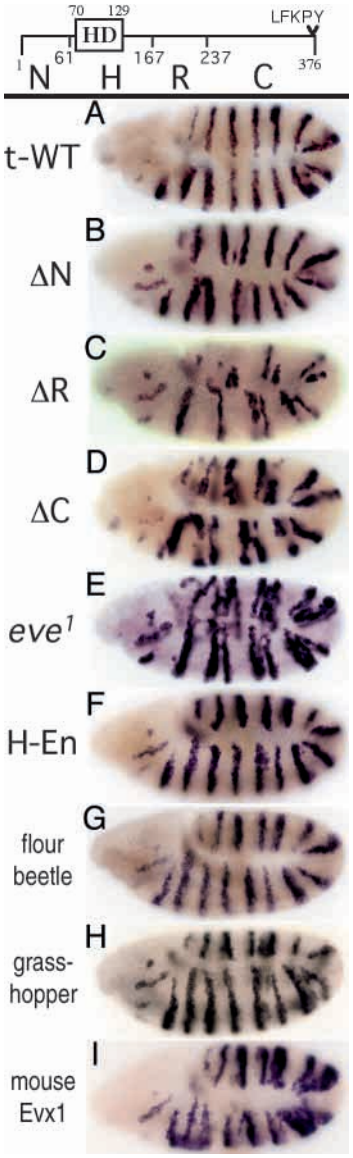

Fig. 1. Functional dissection of Eve, and functional rescue by Eve homologues. At the top is a diagram of *Drosophila* Eve protein domains (for details see Material and Methods). The HD and Gro interaction domain (LFKPY) are indicated. Flag-tagged modified Eve proteins were expressed using the *eve* rescue construct (from –6.4 kb to +8.6 kb) in a *Df(2R)eve* mutant background. Patterns of expression of *en* mRNA were monitored by in situ hybridization. (A) Rescue by tagged, wild-type Eve; note the equally spaced *en* stripes. (B) The same construct with the N domain removed (ΔN); note that the spacing of *en* stripes is similar to wild type, although odd-numbered parasegments are slightly wider than even-numbered ones, indicating an increased activity. (C) The same construct with the R domain removed (ΔR); odd-numbered parasegments are severely narrowed and some odd-numbered stripes are missing, indicating a severe, but not complete, loss of activity. (D) LFKPY-deleted Eve (ΔC) (Kobayashi et al., 2001); the odd-numbered parasegments are narrowed, as in *eve*^{1} mutants (E) at a semi-permissive temperature (18°C), indicating a partial loss of activity. (F) A chimera of the Eve HD and En repressor domains (H-En); both *en* stripes and parasegment spacing are rescued. (G) Tc-Eve (flour beetle); all *en* stripes are restored, but odd-numbered parasegments are slightly narrowed. (H) Sa-Eve (grasshopper); all *en* stripes are restored, but odd-numbered parasegments are narrower. (I) Mouse Evx1; *en* stripes are restored, but spacing is abnormal, due to a combination of increased protein stability and variations in expression among the early stripes (Evx1 stripes 4, 5, and 6 are weak, and the corresponding parasegments, 7, 9 and 11, are narrowed, see text).

The C terminus interacts with Gro, and was previously shown to be required for full Eve function (Kobayashi et al., 2001). When the Gro interaction domain was removed, a hypomorphic phenotype resulted that was somewhat less severe than when the R domain was removed (Fig. 1D compare with C). When either R or the Gro interaction domain was removed, although there was no rescue of viable adults (Table 1) (Kobayashi et al., 2001), the protein retained some function, because the rescued embryonic phenotypes (Fig. 1C,D) were similar to those of hypomorphic (pair-rule) *eve* alleles (Fig. 1E: most or all *en* stripes present, but odd-numbered parasegments severely narrowed) rather than null mutants (loss of all *en* stripes in the trunk region). In order to test whether Eve activity was entirely dependent on these two repressor domains, we deleted both the R and C domains. No expression of *en* was restored, showing that little, if any, Eve activity remains without these repressor domains (Table 1 and data not shown). These data also suggest that the Eve HD by itself has no detectable rescuing activity. Although we established a number of transgenic lines expressing only the Eve HD (and flanking region, Table 1), α-FLAG antibody staining showed that they expressed only low levels of protein at the blastoderm stage, in contrast to each of the other rescue transgenes, and, as expected, there was no rescue of *en* stripes (data not shown).

Engrailed (En) is a well-characterized transcriptional repressor, and the N terminus of the protein contains two repressor domains, one Gro-dependent and the other Gro-independent (Tolkunova et al., 1998). Although the En domains function similarly to those of Eve, they have little sequence similarity. In particular, the Gro interaction domains appear to have evolved independently, since they show similarity to two distinct families of Gro interacting proteins (Kobayashi et al., 2001; Smith and Jaynes, 1996). In order to test whether a generic repressor function, acting in conjunction with the targeting activity provided by the Eve HD, can substitute for normal Eve function during segmentation, we established transgenic lines expressing En-Eve fusion proteins in a normal *eve* pattern. We tested both the En N terminus fused upstream of the Eve HD (En-H), and the same two regions in reverse order (H-En). Three out of 8 H-En and 2 out of 2 En-H lines rescued *eve*-null flies to adulthood (Table 1), and restored normally spaced *en* stripes (Fig. 1F and data not shown), while the other lines showed the hypomorphic *eve* phenotype of narrowed odd-numbered parasegments (data not shown). The range of phenotypes, which is also seen with wild-type Eve protein (Table 1), is presumably due to variations in expression caused by chromosomal position effects. These data suggest that the Eve HD and conserved flanking region is sufficient for targeting the protein to the correct set of target genes during segmentation, and that in addition to this targeting activity, repressor function is both necessary and sufficient to carry out the early blastoderm functions of Eve.

Eve homologues can function in segmentation

Eve homologues have been isolated from several species. The sequences show a high degree of conservation within the HD, and also have recognizable similarity at the C terminus. We tested whether the homologues retain repressor activity and the ability to recognize similar target sites by expressing Flag-

tagged versions of them in *Drosophila* embryos mutant for endogenous *eve* function. The homologues from *Caenorhabditis* (Vab-7), *Schistocerca* (Sa-Eve), *Tribolium* (Tc-Eve), and mouse (Evx1) were expressed in an *eve* null background in the normal pattern, using the rescue construct. Protein expression was monitored using α-Flag antibodies. Vab-7 showed almost no expression at early blastoderm, although it was clearly expressed later in the nervous system, proctodeum, and anal plate ring. Furthermore, only 1 out of 4 lines showed detectable expression in the mesoderm. These data suggest that Vab-7 is unstable at blastoderm and probably also in the mesoderm. Reflecting the lack of accumulation in early stripes, Vab-7 did not rescue *en* stripes (data not shown). Both Sa-Eve and Tc-Eve were expressed at apparently normal levels, and in 4 out of 4 lines each, both orthologues restored all *en* stripes (Fig. 1G,H). However, no rescue to adulthood was observed in either case. Consistent with the relative evolutionary distance, Tc-Eve showed better rescue of odd-numbered parasegment spacing (as well as rescuing even-numbered parasegments). Obtaining transgenic lines expressing Evx1 was difficult, and the lines established had reduced viability, indicating that expression of Evx1 has dominant effects. Although 2 out of 4 lines showed relatively weak expression in early stripes 4, 5 and 6, all lines were able to restore the normal 14 *en* stripes (Fig. 1I and data not shown). (The slightly abnormal patterns of Evx1 expression are probably due to chromosomal position effects at the site of transgene insertion, which are likely due to the selection of sites that reduce overall expression levels during the establishment of the transgenic lines.) Evx1 levels were maintained much longer than normal (data not shown), suggesting that the protein is abnormally stable. Where Evx1 was expressed at approximately normal levels (or possibly higher due to the increased protein stability), odd-numbered parasegments were as wide or wider than even-numbered ones, whereas where Evx1 was expressed at low levels, odd-numbered parasegments were narrower, and the corresponding *en* stripes were broadened (e.g. in the regions of *eve* stripes 4, 5 and 6 in Fig. 1I). These data indicate that these Eve homologues provide normal *eve* function in segmentation, a function that requires not only that they recognize appropriate target genes, but also that they act as transcriptional repressors. Evx1 rescuing activity may be relatively low, with the increased protein stability able to partially compensate for a reduced activity.

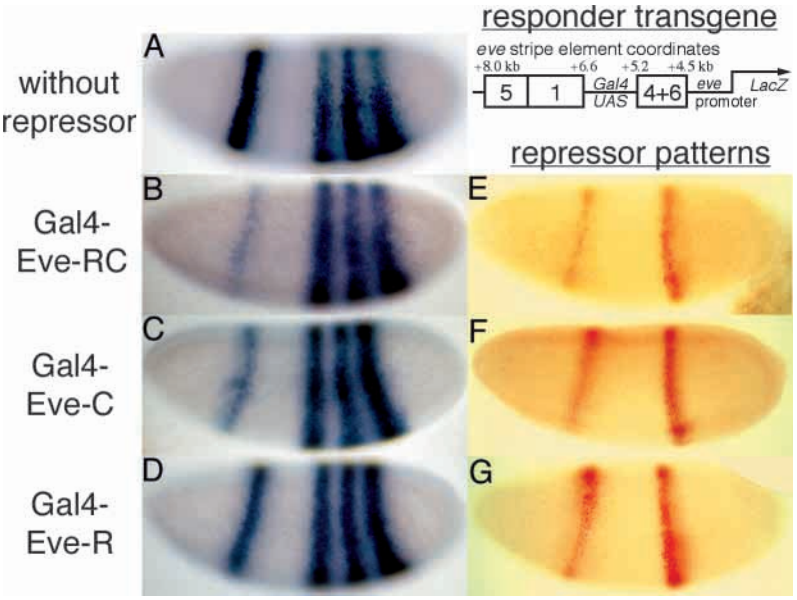

Fig. 2. Eve repressor domains function autonomously in embryos. (A-D) Expression patterns of a responder transgene (diagrammed at the top right) driven by the stripes 4+6, 1 and 5 enhancers and containing a Gal4-UAS sequence, visualized by in situ hybridization (to *lacZ* mRNA). (E-G) Expression patterns of the Gal4 fusion proteins indicated on the left, which contain Eve repressor regions, driven by the stripe 1 and 5 enhancers. The patterns in E and G were visualized by staining with polyclonal α-Eve antiserum, and that in F with monoclonal antibody 2B8, which specifically recognizes the Eve C terminus (see text), all in *Df(2R)eve* embryos. (A) The responder transgene alone, expressed in the pattern of *eve* stripes 1, 4, 5 and 6. Note that stripe 5 is slightly stronger than stripes 4 and 6. (B) The responder repressed by Gal4-Eve-RC, present in the stripe 1 and 5 regions, as shown in E (one copy of each transgene). Note that stripe 1 is dramatically reduced, while stripe 5 is slightly reduced relative to A. (C) The responder repressed by Gal4-Eve-C. Note that stripe 1 is strongly reduced, although not as much as in B, while stripe 5 is again slightly reduced. (D) The responder repressed by Gal4-Eve-R. Note that stripe 1 is clearly reduced, while stripe 5 may also be reduced.

Eve repressor domains are functionally autonomous

In order to test whether individual Eve repressor domains have the ability to repress target genes in embryos, we constructed Gal4 fusion protein-expressing transgenes. The Gal4 DNA binding protein was fused with either the R repressor domain, or the C domain, or both together, downstream of the *eve* promoter driven by the enhancer elements for stripes 1 and 5. Lines carrying these transgenes were each crossed with lines carrying an artificial target transgene, which contained a Gal4 UAS target site and a *lacZ* reporter gene along with the stripes 4+6, 1 and 5 enhancer elements (see Materials and Methods for details). Thus the reporter is expressed in stripes 1, 4, 5 and 6, while the repressors are expressed only in stripes 1 and 5. Each of the Gal4-repressor domain fusion proteins was expressed as expected (Fig. 2E-G), as determined by staining with polyclonal α-Eve antiserum, or with monoclonal α-Eve antibody 2B8 (for those containing the C terminus of Eve), in a *Df(2R)eve* background. This antibody recognizes the Eve homologues from grasshopper and *Tribolium*, as well as crustacean species (Duman-Scheel and Patel, 1999), but fails to recognize the Gro interaction domain-deleted *Drosophila* protein, or proteins without the C terminus (data not shown), suggesting that 2B8 recognizes an epitope within the conserved C terminus (LFKPYK in *Drosophila* and *Tribolium*, and LFQPYK in *Schistocerca*). Target gene expression was monitored by in situ hybridization.

When homozygous Gal4-Eve-RC-expressing lines were crossed to homozygous responder lines, so that all progeny contained one copy of each transgene, stripe 1 expression was strongly repressed, while stripe 5 was more weakly repressed,

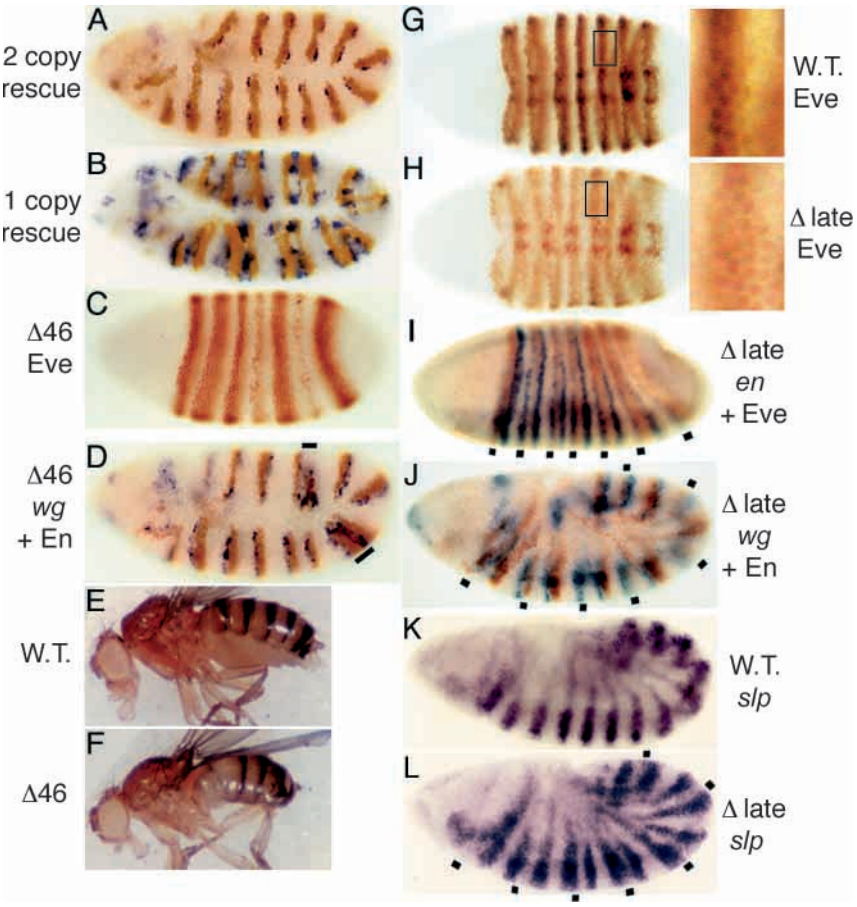

Fig. 3. Early *eve* stripes set parasegment spacing and activate *en*, while the late stripes ensure maintenance of *en* expression and repression of *slp*. (A,B,D,J) Expression patterns of *wg* (blue) and En (orange), visualized by in situ hybridization and monoclonal (4D9) α-En staining. (C,G,H) Expression pattern of Eve, visualized by polyclonal α-Eve staining. (I) Expression pattern of *en* (blue) and Eve (orange), visualized by in situ hybridization and polyclonal α-Eve staining. (K,L) Expression pattern of *slp*, visualized by in situ hybridization. (A) An *eve* null mutant rescued by two copies of the wild-type rescue transgene (from –6.4 kb to +9.2 kb); both *wg* and En are expressed normally, and parasegments are equally spaced. (B) An *eve* null mutant rescued by a single copy of the same wild-type rescue transgene; note that the odd-numbered parasegments are severely narrowed (all *wg* and En stripes are expressed, but there are few if any non-*en*/*wg*-expressing cells in odd-numbered parasegments). (C) Early Eve stripe expression from a transgene that lacks the early stripe 4+6 enhancer (Δ46). (D) The embryonic phenotype of an *eve* null mutant rescued by Δ46; note that parasegments 7 and 11 (marked by bars) are severely narrowed, although *en* stripes 7 and 11 are expressed, at least at early stages (by this stage in this embryo, stripe 11 has almost faded). (E) Wild-type adult fly with normal segmentation. (F) The adult phenotype of an *eve* null mutant rescued by Δ46; note that there are two fewer abdominal segments. (G) Normal Eve expression, in *Df(2R)eve* rescued by two copies of the wild-type rescue transgene. On the right is a magnified view of the boxed region; note that the anterior (left) edge is sharply defined, with the anterior-most cell usually expressing the highest level. (H) Eve expression from two copies of a transgene that lacks the late element (Δlate), in a *Df(2R)eve* background; note that there is residual expression from early stripes, but that the high level expression at the anterior edge of each early stripe is missing. On the right is a magnified view of the boxed region; note that the anterior edge is less sharply defined than in G, that the stripe appears broader, and that the anterior-most cell row is not usually the highest expressing. The embryo in H is actually over-stained relative to that in G, as suggested by the fact that the stripes appear narrower in G, owing to a lack of detection of the low level expression in the posterior of each stripe. (I) The Δlate rescued phenotype early in gastrulation: odd-numbered *en* stripes are activated normally (marked by dots); note the regular parasegment spacing (except for parasegment 3, which is slightly narrower due to weaker than normal expression of early Eve stripe 3 in this line). (J) The Δlate rescued phenotype during germ band extension: odd-numbered *en* stripes are either narrowed or lost (marked by dots), and some *wg* stripes are expanded posteriorly. (K) Expression pattern of *slp* (indistinguishable from wild type) in an *eve* null mutant rescued by the wild-type rescue transgene. (L) Expression pattern of *slp* in a Δlate-rescued *eve* null; note that in even-numbered parasegments, *slp* is expanded posteriorly (into the regions of odd-numbered *en* stripes, marked by dots).

relative to lines carrying one copy of the target gene alone (compare Fig. 2B with A). The Gal4-Eve-C fusion protein was able to repress the target gene effectively (particularly stripe 1, Fig. 2C), while Gal4-Eve-R showed somewhat weaker repression activity (Fig. 2D). Two copies of Gal4-Eve-R were able to repress the target gene to a similar degree as one copy of Gal4-Eve-C (data not shown), indicating that the R domain also has autonomous repressor activity in vivo, but is perhaps less potent than the C-terminal Gro interaction domain.

Early *eve* stripes establish parasegment spacing in a dose-dependent manner

Rescue of *eve* null mutants to adulthood requires two copies of the transgene at most insertion sites, while a pair-rule hypomorphic phenotype results with a single copy, showing that *eve* function in early embryogenesis is strongly dose-dependent (Fujioka et al., 1999; Fujioka et al., 1995; Nüsslein-Volhard et al., 1984). We analyzed embryos that carried a single copy of the rescue transgene in an *eve* null background, and found that while all *en* stripes are expressed, the odd-numbered parasegments are narrowed, as shown by Engrailed (En) and *wingless* (*wg*) expression (compare Fig. 3B with A). This indicates that a low concentration of Eve is sufficient for establishing all *en* stripes, while proper spacing of parasegments requires a higher concentration (see also Hughes and Krause, 2001). Eve expression normally occurs in two phases, regulated by different *cis*-acting elements (Goto et al., 1989; Harding et al., 1989). In the first (early) phase, each stripe is broad, and protein concentration appears to be highest in the middle of each stripe. In the second (late) phase, each stripe is narrow, with sharply defined anterior edges that correspond to the anterior borders of subsequent *en* expression (Lawrence et al., 1987). We asked whether these two phases of *eve* stripe expression have distinguishable functions. In order

to distinguish their functions, we first removed the early stripe 4+6 element from the entire rescue construct, which weakens expression specifically in early stripes 4 and 6 (Fig. 3C). The low concentration of Eve in these early stripes resulted in narrowed parasegments 7 and 11 (Fig. 3D). Surprisingly, this transgene was able to partially rescue *eve* null mutants, producing viable adults with fewer segments in the abdomen (compare Fig. 3F and E). Because the late stripes are under (indirect) control of the early stripes (Fujioka et al., 1995), late stripes 4 and 6 were often weaker than normal, and sometimes patchy and incomplete (Fig. 3C and data not shown). However, the abnormal spacing of parasegments is unlikely to be caused primarily by the changes in late expression, since the late stripes also showed the same shifts in position as the corresponding *en* stripes, suggesting that the shifted positions of both late *eve* and *en* are due to reduced levels of early Eve expression (see also Fujioka et al., 1995). Thus, these data suggest that odd-numbered parasegment spacing reflects primarily the activity of Eve in early stripes.

Late *eve* stripes are required to stabilize parasegment boundaries

To test the function of late Eve stripes, the late element was deleted from the wild-type rescue construct. Six out of 9 lines carrying this transgene (Δlate) rescued *eve* null mutants to adulthood, a similar percentage as with the wild-type rescue transgene. However, the percentage of individuals rescued for each line was on average lower than with the wild-type rescue construct (Table 1). As expected, the normally strong Eve expression in the anterior-most cell rows of odd-numbered parasegments never appeared in these rescued lines (compare Fig. 3H with G). Analysis of *en* expression showed that even-numbered *en* stripes were rescued effectively, while odd-numbered *en* stripes were expressed initially (Fig. 3I), but then became weak and incomplete (Fig. 3J). The initial *en* spacing was not significantly affected, although odd-numbered parasegments became slightly narrower at later stages, suggesting that *en* stripes were being repressed from the anterior. These lines were analyzed further to identify the cause of the fading of *en* stripes. To activate *en*, early Eve stripes must repress repressors of *en*, including *slp*, in odd-numbered parasegments (Fujioka et al., 1995) (see also Raj et al., 2000). In the Δlate lines, *slp* was repressed at the blastoderm stage (data not shown), allowing odd-numbered *en* stripes to come on. However, *slp* expression expanded posteriorly as gastrulation proceeded (compare Fig. 3L with K), into the cells where late Eve is normally expressed. Therefore, the function of the late Eve stripes is primarily to maintain repression of *slp* (and possibly other *en* repressors) in order to keep odd-numbered *en* stripes from being repressed shortly after their initial activation.

DISCUSSION

The transcriptional activity of Eve in segmentation

Previous studies showed that Eve has two distinct repressor domains, one dependent on the corepressor Gro and the other Gro-independent. Paradoxically, a primary function of Eve in this process is to allow activation of *en* stripes in both even- and odd-numbered parasegments. We used our ability to functionally replace the endogenous *eve* gene with a transgenic copy to evaluate the relative contribution of these and other domains to the function of Eve in this process. We found that neither repressor domain is sufficient to properly organize the odd-numbered parasegments, although all (or most) *en* stripes can be restored by either one alone (Fig. 1C,D). However, the relative width of the odd-numbered parasegments is reduced, so that they are unstable, and are deleted at later developmental stages. This gives rise to the pair-rule phenotype that earned *even skipped* its name (the even-numbered abdominal denticle bands are in odd-numbered parasegments) (Nüsslein-Volhard and Wieschaus, 1980).

The Gro-independent repressor domain was defined previously in repression assays in cultured cells (Han and Manley, 1993), based on transient transfections with artificial reporter genes. Later, this region was shown to interact physically with the TATA-box binding protein TBP, and to repress transcription in vitro (Um et al., 1995). Recently, a similar region was shown to interact physically with the human Atrophin homologue, which acts as a corepressor (Zhang et al., 2002). A phosphorylation-dependent function of down-regulating the repressor activity of this region in vitro was ascribed to the N-terminal domain of Eve (Li and Manley, 1999). Consistent with this result, we found that deletion of the N terminus caused an increase in Eve activity in vivo (Table 1, and Fig. 1B). One possible explanation for this effect is that the deleted protein is more stable than wild-type Eve, since PEST sequences are deleted, although antibody staining against the Flag-tagged proteins indicated only a minor, if any, increase in protein levels. When both repressor domains were removed, neither the Eve HD alone (with conserved flanking regions) nor the HD with the N terminus were able to provide any significant functional activity in segmentation.

The histone deacetylase Rpd3 was previously shown to affect *eve* function (Mannervik and Levine, 1999). In *Rpd3* mutant embryos, although the expression pattern of *eve* is not changed, even-numbered *en* stripes are very weak or missing owing to a lack of repression of *odd*. However, odd-numbered *en* stripes are expressed with only minor alterations. This is in contrast to the relative effects on odd- versus even-numbered *en* stripes when the *eve* dose is reduced (Fig. 3B compared with A), or in hypomorphic mutants (Fig. 1E), suggesting that *Rpd3* may affect the repression of *odd* more than that of *slp* and *prd*. The *Rpd3* effect similarly contrasts with the effects of removing either of the Eve repressor domains (Fig. 1C,D), suggesting that *Rpd3* specificity cannot be explained by a selective effect on one of the Eve corepressors (see also Kobayashi et al., 2001). This is true despite the fact that Rpd3 has been shown to mediate Gro repressor activity (Chen et al., 1999). Therefore, the apparent specificity of action of *Rpd3* during segmentation is not easily explained solely through an effect on Eve activity. Conceivably, Rpd3 might affect the target specificity of the Eve HD, perhaps through selective effects on chromatin structure at different target sites. Another possibility is that it might affect the activities of other pair-rule gene products in addition to Eve. For example, it has been shown that Slp interacts with Gro in vitro (Kobayashi et al., 2001). If *Rpd3* reduces *slp* activity, then the effect of *Rpd3* on Eve repressor function might be partially antagonized at the odd-numbered parasegment boundaries by its effect on *slp*.

Both of the repressor domains of Eve have autonomous

activity, since they can repress an artificial target gene in vivo when fused with the Gal4 DNA-binding domain. Transgenes expressing such fusion proteins with either domain alone are capable of repressing transgenes containing a UAS target site for binding by Gal4 (Fig. 2). However, maximal repression activity requires both repressor domains, consistent with the fact that Eve requires both domains for full function in segmentation.

Repression of our Gal4 binding site-containing transgene by the Eve-Gal4 fusion proteins showed a consistently stronger effect on stripe 1 than on stripe 5. Although the stripe 5 element in our reporter is further away from the Gal4 binding sites and is also less well repressed than the stripe 1 element, the apparent specificity of repression is probably not due to a distance effect. We infer this from the fact that a similar stripe preference was seen when Gal4 binding sites were inserted upstream of the same stripe elements, this time closer to the stripe 5 region (M. F., G. L. Y. and J. B. J., unpublished observations). The stronger repression activity on stripe 1 expression may be due to the earlier activity of the stripe 1 enhancer, relative to that of stripe 5. Since these elements are also used to drive the expression of the repressors, the earlier activity of the stripe 1 element causes earlier accumulation of the repressors in the stripe 1 region, which may result in more effective repression. Alternatively, the Eve repressor domains may have some functional specificity that allows them to work more effectively on the stripe 1 enhancer.

A chimeric protein consisting of the Eve HD (including the conserved flanking regions) and a heterologous repressor domain from the En protein is able to fully rescue segmentation (Table 1 and Fig. 1F), while the HD region alone shows no activity. This suggests that repression of its direct target genes is sufficient for the function of Eve as a segmentation gene, and that the HD region is sufficient to recognize those target genes.

Functional similarities of Eve homologues

Eve homologues have been studied in several species. We were interested to know whether there is functional conservation in the recognition of specific target sites as well as in transcriptional activity, and if so, whether these aspects of conservation extend to mammals. The strongest conservation is found in the HD and the immediate flanking sequences, with recognizable homology also in the C-terminal region. We analyzed the ability of several homologues to function in early *Drosophila* development. Expression of each protein was driven by the complete *Drosophila* regulatory region, and their ability to rescue the phenotype of *eve* null mutants was assessed. This provides a sensitive assay for function, since proteins with reduced activities give a range of distinctive hypomorphic phenotypes (Fig. 1 and Fig. 3) (see also Fujioka et al., 1999; Kobayashi et al., 2001). The *Caenorhabditis* orthologue, Vab-7 (Ahringer, 1996), was expressed at very low levels at the blastoderm stage, presumably due to protein instability, so that its activity could not be determined. Homologues from the flour beetle (Tc-Eve) (Brown et al., 1997), grasshopper (Sa-Eve) (Patel et al., 1992), and mouse (Evx1) (Bastian and Gruss, 1990; Dush and Martin, 1992) did, however, provide varying degrees of rescuing activity, paralleling their evolutionary relatedness to *Drosophila*. Tc-Eve rescued all of the *en* stripes, and parasegments were well organized (Fig. 1G), reflecting the relatively close evolutionary distance. Tc-Eve is expressed in stripes in the beetle, and has been shown to have a role in segmentation in that organism. In contrast, Sa-Eve is not normally expressed in stripes, and, correspondingly, provides a less complete rescue than does Tc-Eve. Nonetheless, Sa-Eve is capable of rescuing all of the *en* stripes, with the parasegments being better organized than in *eve* hypomorphic mutants (Fig. 1H).

Evx1, the mouse homologue that is expressed in early development, was able to provide a very significant rescuing activity in *Drosophila* (Fig. 1I). This suggests that it not only recognizes endogenous Eve target sites, but that it also has transcriptional repressor function, since we have shown that this function is required for any such rescue. Although Evx1 apparently acts as a repressor in *Drosophila* embryos, it may also exhibit other activities in other contexts (Jones et al., 1992).

Interestingly, the Gro interaction motif of Eve (LFKPY), located at the C terminus, is conserved in the flour beetle (*Tribolium*) and the grasshopper (*Schistocerca*), and appears to be recognized by the monoclonal antibody 2B8 (Patel et al., 1994). Without this motif, Eve is no longer recognized by the antibody, which recognizes the Eve homologues in other arthropods, including crustaceans (Duman-Scheel and Patel, 1999). This suggests that the motif is functionally conserved and that interaction with Gro homologues is thus likely to be a conserved feature of Eve function. The repressor activity of Evx1 may also reflect, at least in part, a conserved interaction, since the C terminus also shows sequence similarity to the Gro interaction domain of Eve.

In *Drosophila*, the concentration of Eve within each early stripe forms a gradient, and this graded distribution has morphogenic activity, crucial to the repression of different target genes in different cell rows (Fujioka et al., 1995). A graded pattern of mouse Evx1 expression is also seen in the primitive streak, and has been suggested to play a role in specifying cell fates (Dush and Martin, 1992). Thus, the action of Eve as a morphogen to subdivide embryonic domains may be a conserved aspect of function.

Eve homologues share common features in their expression patterns, which include the posterior region of embryos and specific cells during neurogenesis. In *Drosophila*, posterior expression is seen in the proctodeum, and later in the anal plate ring. However, the function of this posterior expression has not been established. In the nervous system, Sa-Eve is expressed in identified neurons that are homologous to those expressing Eve in *Drosophila* (Patel et al., 1992), and this conserved expression pattern is also seen in crustaceans (Duman-Scheel and Patel, 1999). In both *Drosophila* (Doe et al., 1988; Landgraf et al., 1999) and *Caenorhabditis* (Esmaeili et al., 2002), Eve has been shown to be important for correct neuronal fate specification, particularly in terms of axonal path finding. The functional importance of mouse Evx1 in the developing central nervous system has recently been established genetically by showing that in *Evx1* mutant embryos, a majority of V0 interneurons fail to extend commissural axons (Moran-Rivard et al., 2001). It will be interesting to determine whether the mechanisms connecting Eve function to axonal guidance are analogous between vertebrates and invertebrates.

The importance of *eve* stripe refinement

In the complete absence of *eve* function, *en* is not expressed in the trunk region, and there is little evidence of segmentation at the end of embryogenesis. In hypomorphic *eve* mutants, the odd-numbered *en* stripes are expressed at posteriorly shifted positions, so that the odd-numbered parasegments are too narrow, and are deleted at later embryonic stages. [A contribution to the narrowing of odd-numbered parasegments may also come from an anterior shift of even-numbered *en* stripes (see Hughes and Krause, 2001).] The positions of the odd-numbered parasegment boundaries, which are the anterior edges of odd-numbered *en* stripes, are foreshadowed by the anterior borders of refined, late *eve* stripe expression, prompting the suggestion that the late stripes are the more important functional aspects of expression (Lawrence et al., 1987), with the early, broad stripes serving only to help activate the late stripes. However, a previous model of *eve* function suggested that the early stripes, acting as morphogenic gradients, set independently the anterior margins of both late *eve* and odd-numbered *en* stripes, which coincide because of their similar regulation by repressors (including *slp*) and the activator *prd* (Fujioka et al., 1995). We tested these models by removing late *eve* stripe expression while retaining normal early stripes. In *eve* null embryos rescued by a transgene deleted for the late expression element, although there is variable partial refinement under the influence of *runt*, which represses each early stripe from the posterior, the well-refined, late stripes never appear (Fig. 3H). In these embryos, odd-numbered *en* stripes form normally (Fig. 3I). However, they are variably lost during germband extension, coincident with an expansion of *slp* expression (Fig. 3J,L). Nonetheless, without refined, late *eve* stripes, many embryos are able to survive to fertile adulthood (Table 1). Thus, it appears that the initial expression pattern of *en* and the overall organization of parasegments are determined primarily by the broad, early stripes. The late, refined stripes are required to maintain the pattern of *slp*, and to prevent partial repression of *en* shortly after it is activated. The expansion of *slp* is probably sufficient to explain the loss of *en*, since ectopic *slp* expression causes repression of these *en* stripes (Cadigan et al., 1994). The hypothesis that early stripes position odd-numbered *en* stripes in a concentration-dependent manner is also supported by the phenotype of embryos rescued by a transgene missing the stripe 4+6 element, which have severely reduced levels of early stripes 4 and 6, and activate odd-numbered *en* stripes in those regions at posteriorly shifted positions (Fig. 3D). A model of these functions of early and late *eve* expression is presented in Fig. 4.

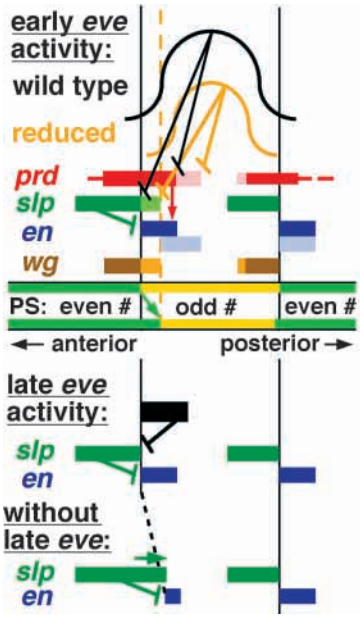

Fig. 4. Model of Eve repressor function in segmentation. Early Eve activity establishes *en* expression in the proper positions. Reduction of Eve concentration or activity in the syncytial blastoderm (top half of figure; a gradient of both wild-type and reduced *eve* activity is diagrammed) reduces repression of at least two key target genes, *slp* and *prd*. Concentration-dependent effects at the anterior edge of each Eve stripe include expanded *prd* and *slp* expression (lighter colored bars) (see also Fujioka et al., 1995; Kobayashi et al., 2001). Prd activates both *en* and *wg*, while *slp* represses *en*. Thus *slp* (and possibly other *en* repressors that are repressed by Eve) can effectively subdivide the *prd* domain into *wg*- and *en*-expressing cells (*en* and *eve* repress *wg*). The border between *slp* and *en* becomes the parasegment boundary, and the overall width of the parasegment is largely determined by the location of this border. A dotted line indicates the shifted position of the parasegment boundary when early *eve* activity is reduced. The net effect of reducing early *eve* activity is to reduce the width of the odd-numbered parasegments, and to sometimes expand the odd-numbered *en* stripes, since *prd* sometimes expands more than does *slp*. There may also be effects at the posterior border of each early *eve* stripe, but these appear to be relatively minor. For example, *ftz* stripe 4 expression does not appreciably expand or shift in the absence of *eve* stripe 4 (Fujioka et al., 1995), although there may be an anterior shift of some *ftz* stripes in the absence of *eve* (see also Hughes and Krause, 2001). PS, parasegments; ⊥ indicates repression of target genes. Late Eve expression is required to maintain *en* expression. The absence of late *eve* activity (bottom half of figure) results in the expansion of *slp* expression, and the concomitant loss of *en* expression, beginning with the anterior of each *en* stripe. The continued presence of Eve just posterior to *slp* is thus necessary to prevent 'encroachment' of *slp* into the *en* stripe, and disruption of the incipient parasegment boundary.

The prevalence of repression as a mechanism of early developmental regulation among pair-rule and gap genes is striking. In the case of Eve, this activity provides not only for the activation of *en* with the appropriate spacing between cell rows, but also for the maintenance of *en* expression in the face of opposing repressive activities. One of these opposing activities is that of *slp*, which apparently helps to set the anterior boundary of both late Eve and *en* expression (Fujioka et al., 1995). Thus, spatially localized repressors may have advantages over activators in making and maintaining cell fate decisions, where mutually exclusive patterns of transcription factor expression help to establish and reinforce those decisions. Such mutually exclusive patterns can be directly established and reinforced by repressors acting to repress each others expression in adjacent domains, while activators can do this only indirectly.

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