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Drawing lines in the sand: *even skipped* et al. and parasegment boundaries

James B. Jaynes[*] and **Miki Fujioka**
Department of Microbiology and Immunology, Thomas Jefferson University, Philadelphia, PA 19107, USA

Abstract

The pair-rule segmentation gene *even skipped* (*eve*) is required to activate *engrailed* stripes and to organize odd-numbered parasegments (PSs). The protein product Eve has been shown to be an active repressor of transcription, and recent models for Eve function suggest that activation of *engrailed* is indirect, but these models have not been fully tested. Here we identify the forkhead domain transcription factor Sloppy-paired as the key intermediate in the initial activation of *engrailed* by Eve in odd-numbered parasegments. We also analyze the roles of the transcription factors Runt and Odd-skipped in this process. Detailed analysis of *engrailed* and pair-rule gene expression in various mutant combinations shows how *eve* activates *engrailed* by repressing these *engrailed* repressors, and further indicates that mutual repression among pair-rule genes plays an important role in establishing parasegment boundaries. We present a new model of pair-rule gene function that explains the response of these boundaries to the relative levels of Eve and Fushi Tarazu.

Keywords

Segmentation; Parasegment boundary; Pair-rule gene; Eve; Homeodomain; Transcriptional repressor; Genetic network

Introduction

The *even skipped* gene (*eve*) encodes a homeodomain (HD) transcription factor (Macdonald et al., 1986) required during Drosophila segmentation for activation of *engrailed* (*en*) and for proper organization of odd-numbered parasegments (Fujioka et al., 1995; Harding et al., 1986). 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 (PS) 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 ala/pro-rich repressor domain similar in sequence composition to repressor domains in other proteins (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

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[*]Corresponding author. Kimmel Cancer Center, Department of Microbiology and Immunology, Thomas Jefferson University, 481A Jefferson Alumni Hall, 1020 Locust Street, JAH490, Philadelphia, PA 19107. Fax: 215-923-7144. *E-mail address:* E-mail: J_Jaynes@mail.jci.tju.edu (J.B. Jaynes).

some target genes, indicating that Eve is a direct repressor of those genes (Manoukian and Krause, 1992). Subsequently, a second repressor domain 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 (Jiménez et al., 1997). Recently, the corepressor Atrophin was identified through its ability to interact functionally with Eve through the Gro-independent repressor domain (Zhang et al., 2002).

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 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* (DiNardo and O'Farrell, 1987). How does Eve do this? Previous data suggested that *eve* might activate *en* indirectly. Early Eve stripes repress *prd* at a high concentration, and *sloppy paired* (*slp*), a repressor of *en*, at a low concentration, producing one cell row that has an activator, but not a repressor of *en*. These cells activate the odd-numbered *en* stripes (Fujioka et al., 1995). 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 PSs are narrower than the even-numbered ones and are deleted at late embryonic stages (Frasch et al., 1988), presumably because of the abnormal juxtaposition of cell types within them (Pazdera et al., 1998). Previous models invoking only the repression activity of *eve* do not explain, however, why odd-numbered *en* stripes do not expand in *slp* mutants until well after they are established (Cadigan et al., 1994b). It was suggested that secondary stripes of the primary pair-rule gene *runt* might serve a redundant function with *slp* to set the anterior borders of these *en* stripes (Fujioka et al., 1995), but this has not been tested.

In this paper, we analyze how the repressor activity of Eve, combined with repressive interactions among other pair-rule genes, allows it to carry out its complex series of functions in the subdivision of blastoderm embryos. In particular, we show that slp is the key intermediate between *eve* and *en*, so that repression of *slp* by *eve* activates the odd-numbered *en* stripes. We identify novel aspects of pair-rule gene interaction that lead to a more complete picture of how this group of genes resolves the broad patterns of gap genes into the narrow patterns of segment polarity genes in their proper relative positions. The resulting model can explain, among other things, how the relative concentrations of Eve and Ftz determine the subsequent widths of the odd- and even-numbered PSs.

Materials and methods

Drosophila strains and transgenic flies

The mutant strains used in this study were *Df(2R)eve*, *eve*^{R13}, *en*^{E}, *Df(2L)ed*^{sZ1} (mutant for both *slp1* and *slp2*), *odd*^{7L}, and *runt*^{LB5} (Gergen and Wieschaus, 1986), all null alleles. *Df(2R) eve* and either *Df(2L)ed*^{sZ1} or *odd*^{7L} were recombined onto the same chromosome for the double mutant analysis. These mutations were balanced over marked balancer chromosomes to allow identification of mutant embryos, as indicated in the figure legends.

Embryo analysis

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/CIPB 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 (Developmental Studies Hybridoma Bank) at 1:10 dilution visualized using appropriate secondary antibodies and the HRP-DAB reaction (Mullen and DiNardo, 1995).

Results

Repression of slp by Eve activates en

A key aspect of a previously proposed model for how *eve* organizes the odd-numbered PSs (Fujioka et al., 1995) was that Eve represses *slp* (and other repressors of *en*) at a low concentration, while at a higher concentration (Manoukian and Krause, 1992), it represses *paired* (*prd*, a crucial activator of *en*), thereby restricting *en* expression to a region at the edge of broad, early *eve* stripes. In *eve* mutants, both the *slp* and *prd* expression patterns expand throughout the *eve* domain (Baumgartner and Noll, 1990; Fujioka et al., 1995), and *en* is not activated. If repression of *slp* (and not *prd*) by low concentrations of *eve* is sufficient to activate *en*, then when both *eve* and *slp* are removed, *en* might be activated by *prd* in the *eve* domain. Activation of *en* in *eve*, *slp* double mutants was previously reported (Riechmann et al., 1997), but whether this occurs at the time of development when en is normally turned on or whether this is related to the ectopic activation of *en* that occurs at later stages in *slp* mutants remained unclear. We examined *eve, slp* double mutants and found that broad *en* stripes are expressed in the *eve* domains from the time when *en* expression is normally initiated (Fig. 1D, compare to Fig. 1C), showing that *eve* does, indeed, activate the odd-numbered *en* stripes through a double-negative mechanism. These abnormally broad en stripes persist to later stages (Fig. 1F and data not shown). Activation of the even-numbered *en* stripes also requires *eve* function, and it was previously shown that these *en* stripes require that *eve* repress another *en* repressor, *odd*, because they ‘‘reappear’’ in *eve, odd* double mutants (Coulter and Wieschaus, 1988; DiNardo and O’Farrell, 1987). In *slp* single mutants, *en* is expressed essentially normally until mid-germband extension (Fig. 1B), after which the odd-numbered *en* stripes begin to expand anteriorly, followed by a similar expansion of the even-numbered stripes (data not shown) (Cadigan et al., 1994b). This suggests that the positioning of the anterior borders of odd-numbered *en* stripes is not due solely to *slp*, but that another *en* repressor may act redundantly with *slp* until mid-germband extension (see below). We emphasize, however, that this putative repressor is not sufficient, in the absence of *eve* function, to prevent activation of *en* in the *eve* domain because *en* is activated there in *eve, slp* double mutants.

We also examined *odd* expression in *eve, slp* double mutants and found that *odd* stripes are extensively broadened, failing to retract from the posterior of the *ftz* domains as they normally do (Fig 1H and J, compare to Fig 1G and I) as well as from the anterior of the *ftz* domains due to the absence of *eve* (this is where even-numbered *en* stripes are normally activated). This lack of retraction from the posterior is due to the absence of *slp* (see below). As *en* expression becomes strong, it becomes clear that in *eve, slp* double mutants, *en* and *odd* are expressed in mutually exclusive patterns that together fill the trunk region of the embryo (Fig. 1L). We also examined *prd* expression and found that broad *prd* stripes are expressed during early gastrulation and germband extension (Fig. 1N and data not shown), relative to the much narrower stripes in the wild type (Fig. 1M), similar to the expanded *prd* expression seen in eve single mutants (Baumgartner and Noll, 1990). The broad *en* stripes are expressed within these *prd* stripes, suggesting that they are regulated similarly to the normal odd-numbered *en* stripes, which are activated by *prd* and repressed by *slp* (as well as by other *en* repressors, see below). That these *en* stripes are expressed within presumptive odd-numbered PSs is confirmed by double staining with *ftz* (Fig. 1P). Comparing the *ftz* pattern to that in the wild type (Fig. 1O), it is clear that *ftz* stripes fail to narrow properly in *eve, slp* double mutants, similar to the effect of *slp* mutants (Cadigan et al., 1994a).

The roles of runt and odd in restricting the odd-numbered en stripes

According to the above results, *slp* would appear to be involved in setting the anterior border of each odd-numbered *en* stripe. However, as stated above, these *en* stripes do not expand anteriorly in *slp* mutants when they are first expressed, although they do so later. This suggests that *en* repressors other than *slp* also participate in setting these borders, which become the odd-numbered PS boundaries. At the time of germband extension, secondary *runt* stripes are expressed just anterior to the odd-numbered *en* stripes. In fact, previous studies suggested that Runt is a direct repressor of odd-numbered *en* stripes (Manoukian and Krause, 1993; Tracey et al., 2000; Tsai and Gergen, 1994). If the presence of Runt is part of the reason for the delay of *en* expansion in *slp* mutants, then in *slp*, runt double mutants, *en* stripes should expand earlier than they do in *slp* mutants alone. To test this prediction, we first analyzed *runt* null mutants (*LB5*) more thoroughly than has been done previously with respect to their patterns of expression of *slp* and *odd*. This was necessary because of the complex effects of *runt* on the expression of other pair-rule genes. In *runt* mutants, *hairy* expression is expanded, resulting in relatively narrow and weak *ftz* expression (in the even-numbered PSs) (Carroll and Scott, 1986). The early *odd* pattern is very similar to that of *ftz*, and it is probably similarly regulated by *hairy* (Jiménez et al., 1996). Also, early, broad *eve* stripes persist longer than normal (in the odd-numbered PSs) (Frasch and Levine, 1987; Ingham and Gergen, 1988) due to the role of *runt* in repressing these stripes (Manoukian and Krause, 1993; Tsai and Gergen, 1994). These effects have secondary consequences for both *slp* and *odd* expression. The situation is further complicated by the fact that the effects of *runt* vary in different parts of the embryo, particularly in the even-numbered PSs, so that *ftz* stripes 1, 4, and 5 remain relatively broad, while others are reduced (Lawrence and Johnston, 1989). The same is true for the primary *odd* stripes (Fig. 2B, compare to A) that are almost complementary to Eve stripes at this stage (and essentially coincident with *ftz* stripes). In wild-type embryos, the primary *odd* stripes narrow from the anterior due to repression by Eve, as well as from the posterior, and secondary *odd* stripes appear in the middle portion of each *eve* stripe (Fig. 2C,E) (Coulter et al., 1990). In contrast, in *runt* null mutants, the primary *odd* stripes disappear essentially completely (Fig. 2D,F), while the secondary odd stripes are broader than normal, again with variation among the different stripes: they are initially close to normal within *eve* stripes 1 and 5, but become much broader than normal within the other *eve* stripes (Fig. 2F). These stripe-specific differences are presumably the result of the ‘‘gap gene-like’’ effects of *runt* (Tsai and Gergen, 1994), which cause, among other things, early *eve* stripe 5 to be weaker than normal.

The net result in runt nulls is that, by early in gastrulation, *odd* is expressed within *eve* stripes (but only weakly in 1 and 5), but not in the *ftz* domains (Fig. 2H,J). In fact, this *odd* expression is sandwiched between pairs of late *eve* stripes, which are present at both the anterior and posterior edges of early *eve* stripes, rather than only at the anterior, as in the wild type, due to the ‘‘loss of polarity’’ that occurs in *runt* mutants (Carroll and Scott, 1986). This loss of polarity is reflected in ectopic late *eve* and *en* stripes in *runt* hypomorphs (data not shown), while in *runt* nulls, no ‘‘extra’’ *en* stripes are apparent (DiNardo and O’Farrell, 1987) (Fig. 2J). The process that ‘‘duplicates’’ odd-numbered *en* stripes in *runt* hypomorphs may be similar to that which occurs in the *eve* domains of *runt* nulls because none of the *en* stripes in *runt* nulls appear to be *ftz*-dependent (although a part of the *en* expression in *ftz* domain 4 may be; data not shown). Thus, in *runt* nulls, the *ftz* domains do not produce their normal *en* stripes, so that the *en* pattern appears to be more normal than in *runt* hypomorphs, where the presence of the *ftz*-dependent *en* stripes makes it apparent that there is an ectopic *en* stripe in the posterior of each *eve* domain (data not shown).

At the same time that *odd* expression is disappearing from the *ftz* domains in *runt* nulls, *slp* expression is induced. The timing of this *slp* activation is similar to that in the wild type (Grossniklaus et al., 1992), which occurs just before *en* activation. In the wild type, *slp* expression is confined to the posterior part of each PS, just anterior to each *en* stripe (Fig. 2K), but in *runt* nulls, it is expressed throughout each variably sized *ftz* domain (Fig. 2L). In fact, during germband extension, the patterns of *odd*, *en*, and *slp* appear to be mutually exclusive, and together they ‘‘fill up’’ the trunk region of the embryo (Fig. 2J,L; dots mark odd-numbered *en* stripes), as they do in the wild type, albeit in very different patterns. This complementarity is consistent with the observed interactions among these genes, in that en can be repressed by ectopic expression of either *slp* (Cadigan et al., 1994a) or *odd* (Saulier-Le Dréan et al., 1998), *slp* is repressed by ectopic *odd* (Saulier-Le Dréan et al., 1998), and ectopic En also represses *slp* (Kobayashi et al., 2003). Furthermore, the induction of *slp* within the *ftz* domains in *runt* nulls just precedes the loss of *odd* expression (Fig. 2M–P, arrows indicate *ftz* domain 4), suggesting that *slp* may be responsible for repression of *odd*. In the wild type, *odd* remains in the *ftz* domains anterior to each *slp* stripe (and posterior to each *en* stripe).

The complete loss of primary *odd* stripes in *runt* nulls may be a result of weaker than normal *ftz* expression. Even *ftz* stripes 1, 4, and 5 appear weaker than normal, although they remain broad (data not shown). As shown previously (Nasiadka and Krause, 1999) and in Fig. 2T, *ftz* is required to maintain these *odd* stripes. Furthermore, the activation of *slp* just precedes the fading of *odd* from the *ftz* domain, both in *ftz* mutants and in the wild type (Fig. 2Q–T, arrows indicate *ftz* domain 4). Thus, repression of *odd* by *slp* may restrict *odd* to the middle of each even-numbered PS in the wild type, where *ftz* expression is maintained at a high level (it has faded from the posterior half of the PS as part of the normal narrowing of *ftz* stripes). Thus, weakened *ftz* expression in runt nulls may be insufficient to maintain *odd* expression as *slp* is induced.

This proposed role of *slp* in the repression of primary *odd* stripes would predict that in *slp* null mutants, these *odd* stripes might fail to narrow from the posterior. (As stated above, narrowing from the anterior also occurs in the wild type due to repression by *eve*.) This is in fact the case as shown in Fig. 3B and D (compare with 3A and C; dots indicate regions of expansion). The primary *odd* stripes remain broader than normal, abutting the odd-numbered *en* stripes, which appear essentially normal at this stage. Because *odd* is probably sufficient to repress *en* (DiNardo and O’Farrell, 1987; Saulier-Le Dréan et al., 1998), this can explain why these *en* stripes fail to expand in *slp* mutants until later stages. The later expansion of *en* expression is roughly coincident with the fading of *odd* expression in this region (data not shown) and results in the repression of alternate *wingless* (*wg*) stripes (positions of dots in Fig. 3F). Later still, all the *en* stripes expand, and all the wg stripes are lost (Cadigan et al., 1994b).

As noted above, secondary *runt* stripes are present in the same portions of even-numbered PSs where *slp* is expressed in the wild type and where *odd* remains in *slp* mutants. Having characterized *runt* null mutants, we were in a position to test the effects of removing both *runt* and *slp* function. The most normal even-numbered PSs in *runt* nulls, at least in terms of their width, are PSs 4 and 5, where the broadest *slp* stripes are expressed (Fig. 2L; marked with red and green bars, respectively, in Fig 3G–J). As shown in Fig. 3I, *wg* is also expressed in this region (coincident with *slp* expression). In *runt*, *slp* double mutants, these regions express *en* (as do the other, narrower even-numbered parasegmental domains; Fig. 3H,J; compare with Fig. 3G,I). This result is consistent with the idea that both *runt* and *slp* contribute to setting the anterior boundaries of the odd-numbered PSs by repressing late *eve* and *en*. However, the observed expansion of *odd* in *slp* mutants suggests that *runt* may not be directly required for this function and that *slp* may be primarily responsible for setting this boundary in wild-type embryos. In the absence of *slp*, *odd* expands into the region, keeping *en* off until later (described above), while in *runt* nulls, *odd* is lost in this region. In *runt*, *slp* double mutants, *odd* is not present in the *ftz* domains, so that *en* can be activated.

Repression among the repressors of en contributes to segmentation

As shown above, in *runt* mutants, the trunk region is subdivided by the expression patterns of three genes (Fig. 2), *en* (essentially coincident with late *eve* expression), *odd*, and *slp*. Furthermore, in *runt, slp* double mutants (Fig. 3H), the embryo is subdivided by *odd* and *en* expression (which coincides with late *eve*), while in *eve, slp* double mutants, the trunk region is also subdivided by *odd* and *en*, but in a very different pattern (Fig. 1L). In *eve* null mutants, *en* expression is completely missing in the trunk region (Fig. 1A), while *odd* is present only in the *ftz* domains (Fig. 4A), and *slp* is expressed throughout the *eve* domains (Fujioka et al., 1995), so that again, *en*, *odd*, and *slp* subdivide the embryo. The cross-repressive interactions between *slp* and *odd*, and between late *eve* and *slp*, can account for the lack of overlap of their resolved patterns. Once established, mutual repression between *en* and *slp* (Alexandre and Vincent, 2003;Cadigan et al., 1994b;Kobayashi et al., 2003) can reinforce the odd-numbered PS boundary set up at the late *eve*– *slp* interface.

If the observed phenotypes are in fact due to the interactions described above, and if these interactions are sufficient to account for pair-rule gene cross-regulation, we can make several predictions of dramatic phenotypes that should occur in *runt*, *eve* double mutants. First, there should be no *odd* expression in the trunk region during gastrulation. This is indeed found to be the case (Fig. 4B). Second, we would expect *slp* to be expressed throughout the trunk region due to the absence of both *eve* and *odd*. This prediction is also borne out (Fig. 4C,D). We would also expect all periodicity in the pattern of *prd* expression to be lost in *eve, slp* double mutants. We found that this is indeed the case (Fig. 4E,F). Thus, repressive interactions between *slp* and *odd*, the repressors of *en* (and late *eve*), as well as repression of *slp* and *odd* by *eve*, appear to play an important role in organizing the pair-rule and *en* expression patterns. These interactions, while they can lead to complex effects, may be functionally rather simple in that *eve*, *odd*, and *slp* repress each others expression, while *en* (regulated similarly to late *eve*) is repressed by *odd* and *slp* (and possibly *runt*), and activated by *ftz* and *prd*. The upstream interactions that subdivide the embryo into two-segment-wide swaths of repeating pattern in the wild type can be thought of as setting up a repeating pattern of *odd*, *slp*, and late *eve* stripes (including the ‘‘minor’’ *eve* stripes in the anterior of the *ftz* domains, which are expressed at the same time as late *eve* stripes, overlapping the even-numbered *en* stripes). The segment polarity genes are then activated in a similar repeating pattern. The odd-numbered *en* stripes essentially follow the late *eve* pattern, replacing it at later stages. In fact, it has been shown that in the absence of *en*, late *eve* expression persists (Harding et al., 1986), consistent with a direct role for *en* in turning off *eve*. The *wg* pattern is a subset of the *slp* pattern (Grossniklaus et al., 1992). After the *wg* and *en* patterns are set up, they are maintained by positive feedback involving the secreted proteins Wg and Hedgehog, while *slp* and *en* continue to have roles in the maintenance of these patterns (Cadigan et al., 1994b;Fujioka et al., 2002).

Discussion

Repression of slp and odd by Eve to activate en

Previous genetic studies suggested that the activation of *en* by *eve* might be indirect because *en* stripes are restored in the *ftz* domains (even-numbered PSs) when both eve and the *en* repressor *odd* are simultaneously removed (DiNardo and O’Farrell, 1987), and *en* stripes are also restored when both *eve* and the *en* repressor *slp* are removed (Riechmann et al., 1997). The latter observation is consistent with a previous model of how *eve* organizes odd-numbered PSs by repressing different target genes at different concentrations (Fujioka et al., 1995), a model also consistent with the rescue of viable adult flies by expression of only the early, broad stripes of *eve* (in an *eve* null background), and the complete rescue of segmentation (in *eve* null mutants) by a chimeric repressor containing only the *eve* HD and repressor domains from En (Fujioka et al., 2002). We confirmed this model by showing that the *en* stripes expressed in *eve, slp* double mutants are present within odd-numbered parasegmental primordia (Fig. 1P), and that they are activated as early in development as are normal *en* stripes (Fig. 1D). The latter point also indicates that *slp* has a primary role in the initial activation of *en* stripes by *eve*, rather than acting only later to restrict *en* stripes, a possibility that was suggested by the fact that *en* stripes only expand at later stages of germband extension in *slp* mutants (Cadigan et al., 1994b). Two possible explanations for the delay in *en* expansion in *slp* mutants emerged from our studies. One is provided by the fact that *odd* stripes abut the anterior edges of odd-numbered *en* stripes in *slp* mutants due to the lack of retraction of these stripes (Fig. 3B,D). Thus, *slp* represses *odd*, and in the absence of *slp*, expanded odd expression may prevent *en* stripes from expanding until later stages, when *odd* expression fades. Another possibility is discussed below.

The complex role of runt in segmentation

A previous model of *eve* function suggested that *runt* acts redundantly with *slp* as a repressor of *en* to help set the anterior margins of odd-numbered PSs (Fujioka et al., 1995). Eve represses both *slp* and secondary *runt* stripes, both of which expand into the *eve* domains in *eve* mutants (Fujioka et al., 1995), and ectopic expression of either *runt* or *slp* is sufficient to repress *en* (Cadigan et al., 1994a; Manoukian and Krause, 1993). We tested this model by examining *runt, slp* double mutants. The interpretation of expression patterns in these mutants is complicated by the fact that *runt* is a primary pair-rule gene that drastically affects the patterns of expression of the other pair-rule genes. In *runt* null mutants, the other primary pair-rule genes *hairy* and *eve* are both overexpressed, with *hairy* stripes becoming variably wider, and *eve* stripes remaining broad well into germband extension, when they are normally restricted to the anterior edges of the odd-numbered PSs (Ingham and Gergen, 1988). Due possibly to indirect effects, *ftz, odd*, and *slp* all have drastically affected expression patterns as well. We catalogued these effects of removing *runt* function, and then looked at how they were altered by simultaneously removing *slp* function.

The straightforward prediction of the model that *runt* and *slp* act redundantly to set the anterior margins of odd-numbered *en* stripes is that these stripes will expand anteriorly in *runt, slp* double mutants. Such an anterior expansion means that they would extend into the *ftz* domains. In *runt* single mutants, the *ftz* domains are abnormally narrow, with the exception of *ftz* domains 1, 4, and 5, which are close to normal width (Lawrence and Johnston, 1989) (Fig. 2J,L; *ftz* domain 7 is broad, but the effects there appear to be different, and we have not dealt with them here). Consistent with the model, in *runt, slp* double mutants, *en* is expressed throughout the *ftz* domains (Fig. 3H,J).

Functional redundancy between runt and slp?

Due to the crucial functions of *runt* as a primary pair-rule gene, in *runt* mutants, the *ftz* domains are incompletely organized. Rather than *slp* replacing *odd* in the posterior half of each even-numbered PS, and *odd* persisting just anterior to *slp*, as in the wild type, *slp* completely replaces *odd* (Fig. 2). Similarly, in *runt, slp* double mutants, *odd* is lost throughout the *ftz* domains (Fig. 3H). This differs from *slp* single mutants, where *odd* persists in the posterior part of the *ftz* domains and may therefore be responsible for preventing expansion of *en* until mid-germband extension, when *odd* expression fades. Thus, the expansion of *en* in *runt, slp* double mutants is consistent either with secondary *runt* stripes providing a redundant function with *slp* in setting the anterior borders of odd-numbered *en* stripes, or with *odd* delimiting these borders when *slp* is mutated. A third possibility is that both are true, that is, *runt* and *slp* may each be required for this function in the wild type, while in the absence of *slp*, expanded *odd* expression substitutes for *slp*, until it fades during germband extension. A way to distinguish between these possibilities might be to examine *en* expression in *odd, slp* double (null) mutants to determine whether secondary *runt* expression is sufficient to prevent *en* expansion.

Unfortunately, these are not available because *odd* and *slp* are very close together on the 2nd chromosome.

Either *runt*, *slp*, or *odd* appears to be sufficient to repress the odd-numbered *en* stripes when ectopically expressed (Cadigan et al., 1994a; Manoukian and Krause, 1993; Saulier-Le Dréan et al., 1998), supporting the possibility of redundancy. However, in *eve* nulls, both the *slp* and secondary *runt* expression patterns expand throughout the *eve* domains (Fujioka et al., 1995), and in *eve, slp* double mutants, low level *runt* expression also occurs throughout the *eve* domain (data not shown). Despite this, in the absence of *slp*, *en* is activated (Fig. 1), perhaps because secondary *runt* expression is not activated soon enough (it normally becomes detectable just as *en* is activated) and is relatively weak. This suggests a stringent requirement for *slp*. Thus, *runt* may serve only an auxiliary role at this stage of segmentation.

A remaining question is why *odd* is not present in the *ftz* domains of *runt*, *slp* double mutants (Fig. 3H). The explanation presented here for the loss of *odd* from the *ftz* domains of *runt* nulls is that *slp* represses it (Fig. 2), so why is *odd* not maintained there in *runt, slp* double mutants? As *odd* is known to be very sensitive to repression by Eve (Manoukian and Krause, 1992), this may be explained by the fact that late *eve* stripes expand into this region (data not shown), which presumably occurs because *slp* and *runt* are absent while *prd* is present (Fujioka et al., 1995). In wild-type embryos, *prd* fades from the middle of each *ftz* domain before *en* is activated, but as in *runt* nulls (Baumgartner and Noll, 1990), this does not occur in *runt, slp* mutants (data not shown). This is consistent with the fact that *odd* is not there, because *prd* persists throughout the *ftz* domain in *odd* mutants (Baumgartner and Noll, 1990). Apparently, the combination of the persistence of early *eve* (due to the absence of *runt*) and the reduced level of *ftz* (due to expanded *hairy* expression, which can also be ascribed to the absence of *runt*), in the absence of *slp*, results in the activation of late *eve* and the repression of *odd* throughout the *ftz* domain. Thus, in *runt, slp* mutants, *odd* is not maintained at a sufficient level to prevent late *eve* expression, and Eve therefore represses *odd*. However, the regulation of *prd* in the *ftz* domain is complex and may also involve *odd-paired* (Baumgartner and Noll, 1990;DiNardo and O'Farrell, 1987) or *naked cuticle* (Mullen and DiNardo, 1995;Zeng et al., 2000). These genes are known to be involved in regulating *odd* and *en* in this region (DiNardo and O'Farrell, 1987;Mullen and DiNardo, 1995), but we have not examined their roles.

A model of pair-rule gene interactions

Key observations emerging from these studies are the repressive interactions between *slp* and *odd*, which help to explain the phenotype of *runt* mutants, and the identification of *slp* as a primary intermediary in the activation of *en* by *eve*. Several other important interactions were also revealed by these studies, and are described above and in Fig. 5A and Table 1. When combined with the previously characterized interactions among pair-rule genes, they can account for such complex effects as those seen in *runt* mutants. They also allow us to understand the process of segmentation more succinctly as a series of interactions that subdivide the trunk region into repeating patterns of *en* (coincident with late *eve* stripes), *odd*, and *slp*. These patterns come to be mutually exclusive due at least in part to corepressive interactions among the three genes (see Fig. 5), and serve as a template for the expression of other segment polarity genes, such as *wg* (which is activated within each *slp* stripe, adjacent to *en*). The experiments of Fig. 4 constitute a test of the model. The model shows how most periodicity is lost in the *ftz* domains of *runt* null mutants, as well as accounting for the loss of periodicity in the *eve* domains of *eve* nulls. In neither case is all periodicity lost because *hairy*, *odd*, and *ftz* are still expressed in striped patterns. However, the model predicts that all periodicity in the *odd*, *slp*, and *prd* patterns will be lost in *runt, eve* double mutants, and this prediction is borne out: both *slp* and *prd* are expressed throughout the trunk region in the double mutants, and *odd* expression is completely lost there. This suggests that the model does, indeed, account for the key interactions between spatially localized factors that act to establish the periodic pattern in *Drosophila* embryos.

It has been well documented that the relative widths of odd- and even-numbered PSs are regulated by the relative levels of Eve and Ftz at the syncytial blastoderm stage (Frasch et al., 1988; Fujioka et al., 1995). However, only a partial understanding of the mechanisms involved has previously been achieved (Hughes and Krause, 2001). It has been shown that the widths of odd-numbered PSs contract when Eve function is reduced (Frasch et al., 1988; Fujioka et al., 1995), and expand when the dose of *eve* is increased (Fujioka et al., 1999; Hughes and Krause, 2001), or when Eve's repressor activity is increased (Fujioka et al., 2002; Kobayashi et al., 2001). Reciprocally, when Ftz is overexpressed (Hughes and Krause, 2001), or when its level is increased by a mutation that increases its stability (Kellerman et al., 1990), the even-numbered PSs expand. In each case, expansion of one set of PSs is at the expense of the other. The primary determinant of these relative widths appears to be the positioning of the PS boundary, which coincides with the anterior borders of the *en* stripes. The interactions described here are sufficient to account for these effects, as illustrated in Fig. 5B, and explained in the figure legend.

In addition to the interactions shown in the model, which can account for the major effects that occur in the mutant combinations described here, a number of other interactions have been documented. Some of these are described above, but for clarity were not included in Fig. 5, while a number of others have been observed in overexpression experiments and may serve an auxiliary role in sculpting expression patterns. Further, the transitions from gap gene to pair-rule gene control of pattern formation, and that from pair-rule to segment polarity gene control, are not discrete. As a result, gap gene effects persist as primary pair-rule genes exert their effects on each other. Segment polarity gene interactions probably have a role in parallel with that of the later cross-regulatory interactions among pair-rule genes because they can begin almost as soon as their expression patterns become discernable, very soon after cellularization of the blastoderm is complete. Additionally, quite a number of genes known to be involved in early pattern formation along the anterior–posterior axis have not been included here. Most of these probably exert their effects through the genes shown in Fig. 5 (e.g., *partner of paired*) (Raj et al., 2000), which are usually considered to be the major players that have spatially restricted expression patterns at this stage, but some may not. Nonetheless, this model can serve as a backbone for understanding this complex system of interactions, which represent a crucial step in what is probably the best studied regulatory network guiding the development of multicellular organisms.

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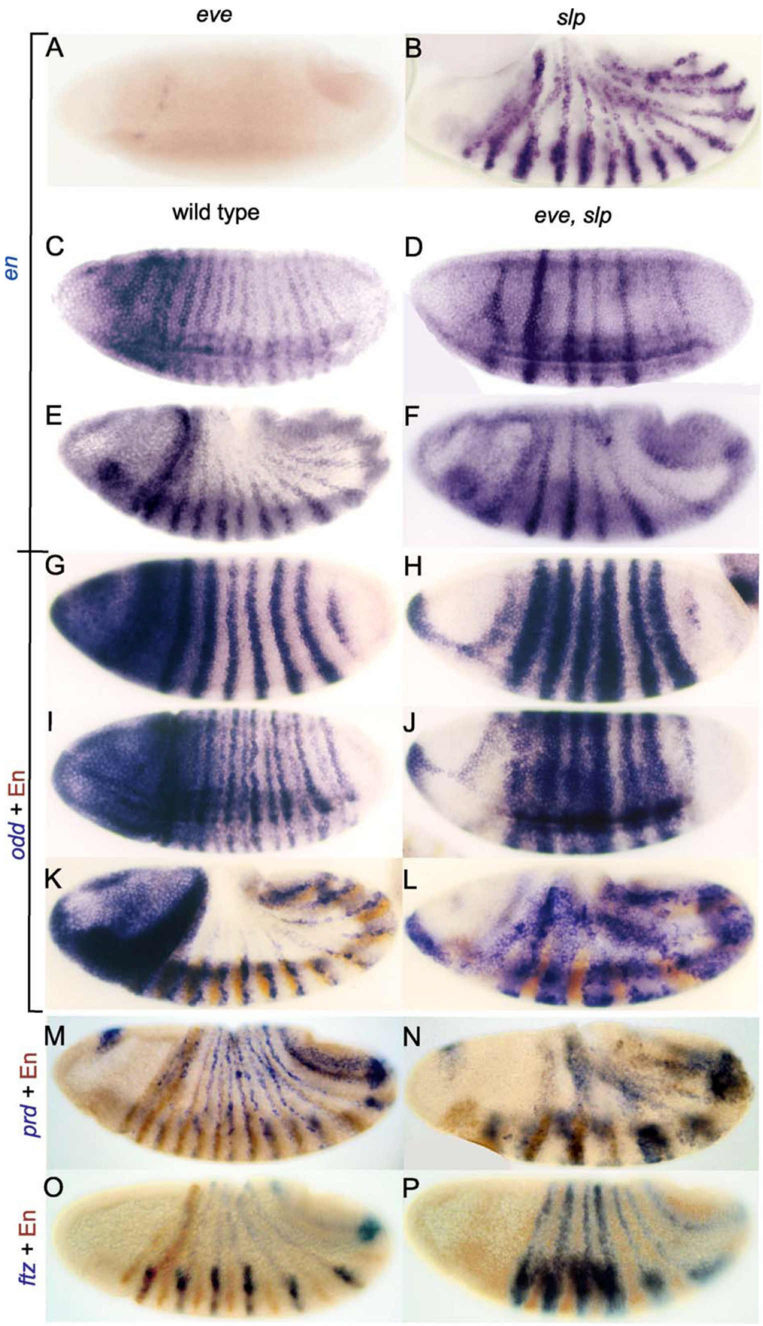

Figure 1.

Eve activates en by repressing *slp*. Expression of *en*, absent in *eve* mutants, is restored in odd-numbered PSs in *eve*, *slp* double mutants. Wild-type embryos (C–P, left side) or embryos null mutant for *eve* (A), *slp* (B), or both *eve* and *slp* (C–P, right side) were stained (blue) by in situ hybridization to RNA from either *en*(A–F), *odd* (G–L), *prd* (M, N), or *ftz* (O, P); those in C–L were simultaneously stained for *lac*Z RNA from the *hb-lacZ* transgene on the balancer chromosome to identify the mutants (wild-type embryos have light blue staining throughout the head region). Those in G–P were then antibody-stained (brown) for En protein. Note that *en* expressionis absent inthe trunk region of *eve* mutants (A) butis restored (in an abnormal pattern) when *slp* function is simultaneously removed (D, F). Note also that *odd* expression is expanded in the double mutants (H, J), that *odd* and En expression are essentially complementary in these mutants (L), and that the En expression overlaps with that of *prd* (N), but not *ftz* (P), indicating that it is in odd-numbered PSs.

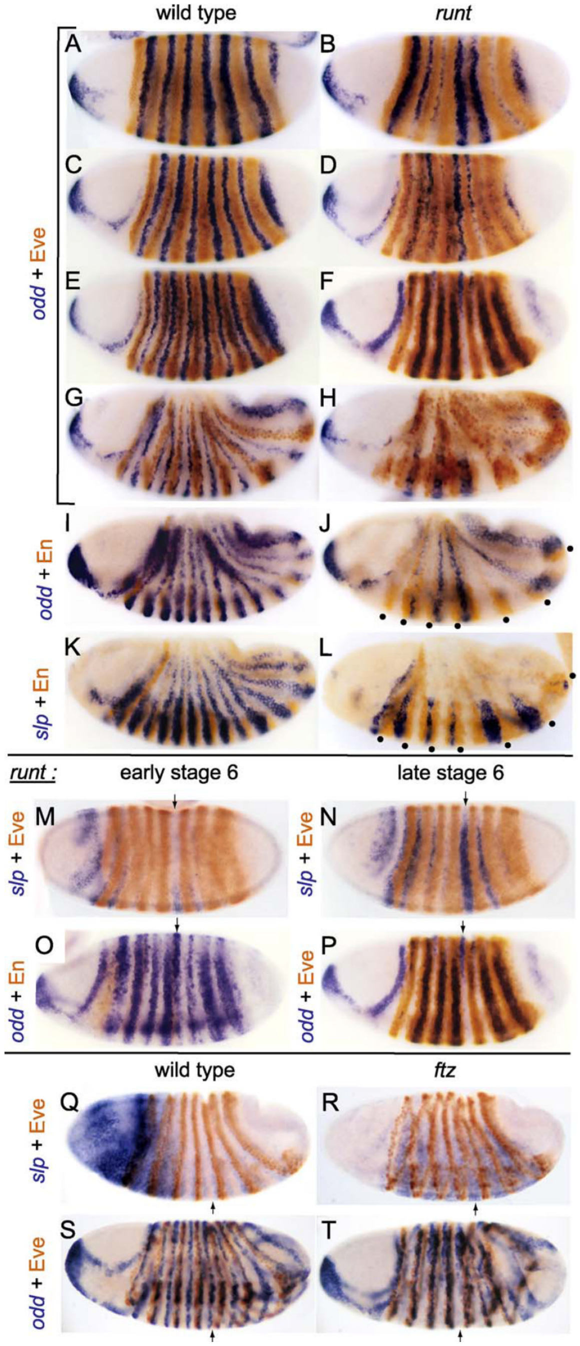

Figure 2.

Pair-rule and *en* expression patterns in *runt* null mutants, and maintenance of *odd* expression by *ftz*. Wild-type embryos (A–K, left side, and Q, S) or embryos null mutant for *runt* (all others except R and T, which are null for *ftz*) were stained (blue) by in situ hybridization to RNA for either *odd* (A–J, O,P, S, T) or *slp* (K–N, Q, R; those in Q and R were simultaneously stained for *lac*Z RNA from the *hb-lacZ* transgene on the balancer chromosome to identify the mutants, as in Fig. 1), and then antibody-stained (brown) for either Eve (A–H, M, N, P–T) or En (I–L, O). Note the stripe-specific effects of *runt* on *odd* expression at the blastoderm stage (B), with the 1st, 4th, and 5th stripes being the most normal (corresponding to PSs 2, 8, and 10). The subsequent evolution of these incipient PSs diverges from that of PSs 4, 6, and 12, in that they remain of approximately normal width (seen as gaps between Eve stripes in F, where *odd* expression is “replaced” by that of *slp*, as shown in M–P), while 4, 6, and 12 are abnormally narrow. Note also that in *runt* mutants, abnormally wide secondary *odd* stripes come up within Eve stripes 2–4, 6, and 7 (seen in F; these are in PSs 3, 5, 7, 11, and 13). By stage 8 (G–L), *odd* RNA is seen clearly only in odd-numbered PSs in *runt* mutants, while *slp* is expressedin even-numbered PSs: the positions of the originally strong, wide stripes of *odd* are now those of the wide stripes of *slp*, that is, PSs 2, 8,and 10. The odd-numberedEn stripes are marked with dots in J and L (stripes 1–2and 9–10 are fused), while *ftz* domain 4is marked with arrows in M–T. Note that in *runt* mutants (M–P) and in *ftz* mutants (R, T) *odd* expression is fading ventrally as *slp* is activated, and that in *ftz* mutants, *slp* is expressed throughout the *ftz* domains, while *odd* is lost there (although still present dorsally in T, *odd* is also lost there by stage 9, well before it fades in the wild type; not shown).

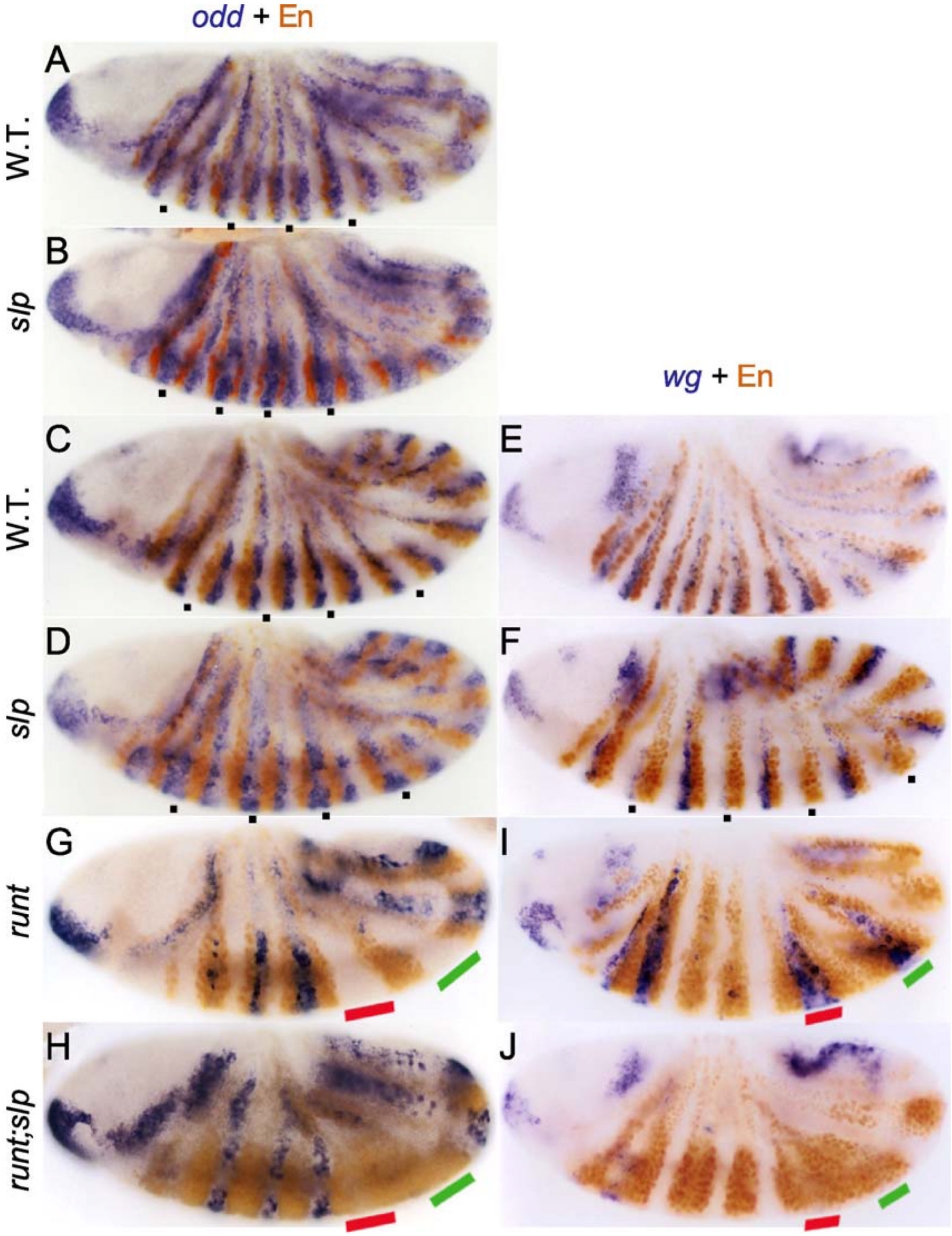

Figure 3.

Contributions of *odd* and *runt* to the odd-numbered parasegment boundaries. Wild-type embryos or those null mutant for *slp* (B, D, F), *runt* (G, I), or double mutant for *runt* and *slp* (H, J) were stained (blue) by in situ hybridization to RNA for either *odd* (left column) or *wg* (right column), followed by anti-En staining (brown). Note, in *slp* mutants at stages 7 and 8 (B, D), the expanded *odd* expression just anterior to odd-numbered En stripes (the posterior parts of PSs 2, 4, 6, and 8 are marked in A–D and F with small squares). At these stages, En expression is approximately normal; however, at stage 9 (F), odd-numbered En stripes have expanded anteriorly, and *wg* expression in the same regions has been lost. Note also that the regions where neither *odd* nor En is expressed in *runt* mutants (G, I) express En in *runt*, *slp* double mutants (H, J); PSs 8 and 10, where *slp* (Fig. 2) and *wg* (I) are expressed between En stripes in *runt* single mutants, are marked with red and green bars, respectively.

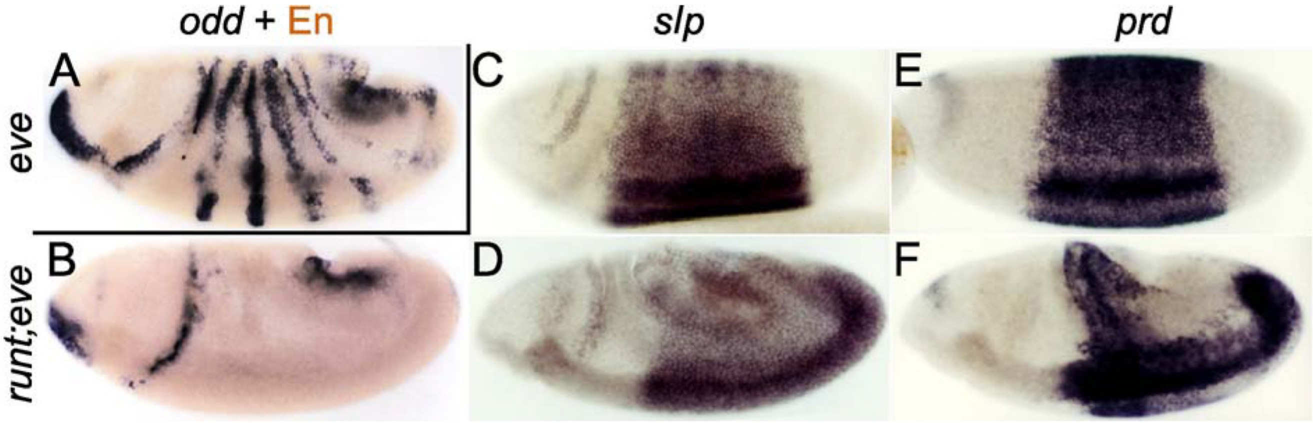

Figure 4.

The role of *eve*, *runt*, and *odd* in limiting *slp* and *prd* expression. Embryos either null mutant for *eve* (A) or double mutant for *runt* and *eve* (B–F) were stained (black) by in situ hybridization to RNA for either *odd* (A, B), *slp* (C, D), or *prd* (E, F), followed by either anti-En staining (A, B; brown) or anti-Eve staining (C–F; the embryos shown did not stain, indicating that they are mutant for *eve*). Note that *odd* expression, which is present in *eve* nulls (A), is absent from the trunk region in *runt*, *eve* double mutants (B), and that these same double mutants express both *slp* and *prd* throughout the trunk (C–F; *prd* has faded from most dorsal and lateral regions by late stage 8, seen in F).

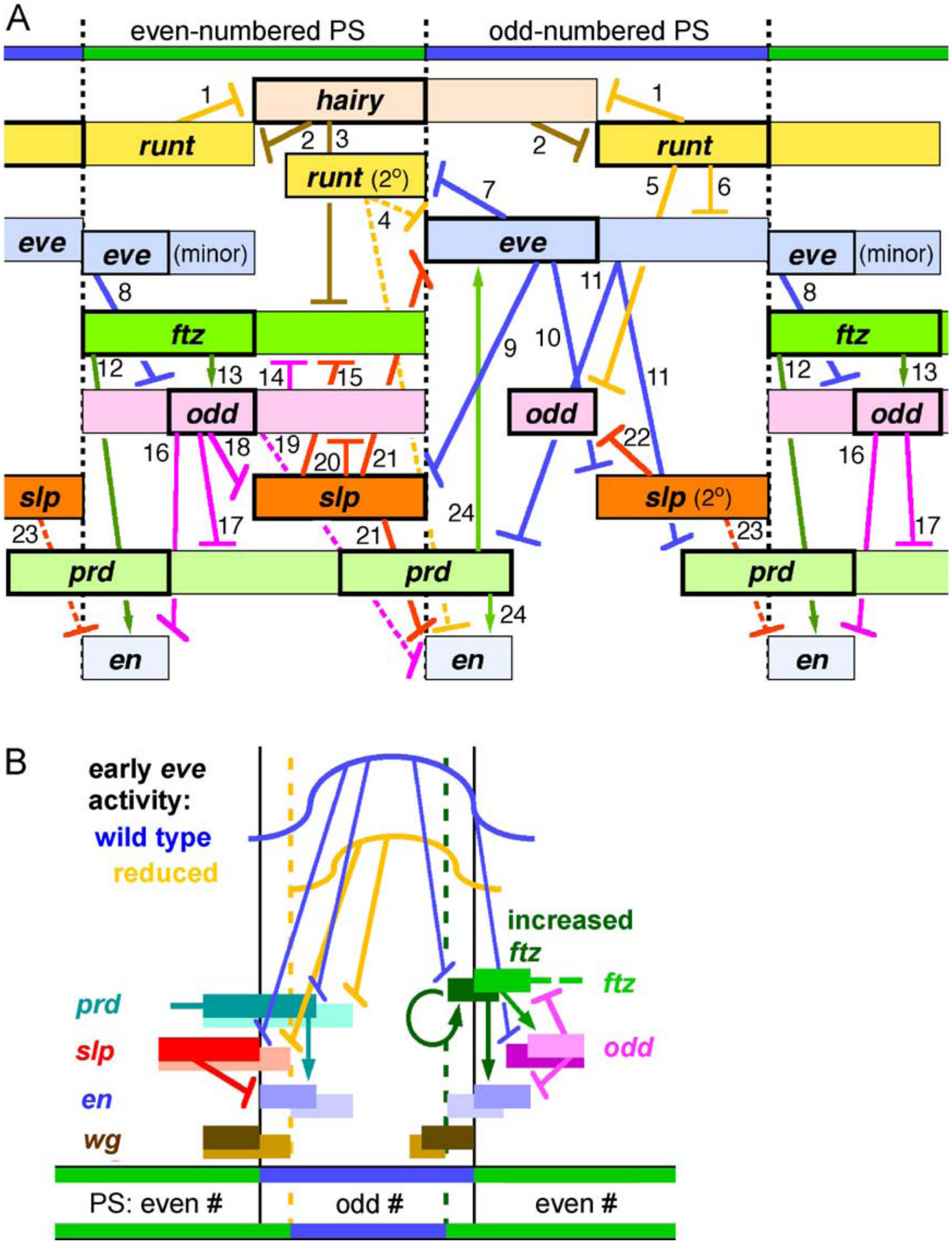

Figure 5.

A model of pair-rule gene interactions that position *en* stripes. Regulatory interactions are represented by arrows (activation) or T-shapes (repression). Anterior is to the left. (A) The anterior and posterior extents of initial expression patterns are represented by colored boxes. Refined patterns that are either activated later or result from subsequent interactions among pair-rule genes are shown either as a heavily outlined box or (in the cases of *runt* and *eve*) as a box at a slightly lower position in the diagram (for *eve*, these "minor" stripes remain weak relative to the thick-outlined late *eve* stripes). The initial patterns of the primary pair-rule genes *hairy*, *runt*, and *eve* are largely determined by the concentrations of the gap proteins, and *ftz* also has a strong input from gap proteins. Mutually repressive interactions between *hairy* and *runt* further contribute to the formation of their complementary patterns, which are diagrammed here. Regulatory interactions that point downward in the diagram contribute to the initial patterns of expression of downstream genes, while those that point upward generally produce a subsequent refinement of the initial pattern or regulate a part of the pattern that appears later. The experimental justification for each interaction is listed in Table 1, with the exception of number 10, which is as follows: in *runt* nulls, where *eve* expression persists abnormally, *odd* comes on in broad stripes in the *eve* domain in place of *slp* (Fig. 2); coupled with the fact that in *eve* nulls, *slp* is expressed throughout the *eve* domain (Fujioka et al., 1995), this suggests that late *eve* expression is normally responsible for setting the anterior border of secondary *slp* expression; this function may be taken over by *odd* as *odd* represses *eve* (see 6[b],Table 1), and *odd* may also help to limit the posterior border of odd-numbered *en* stripes at this stage (Saulier-Le Dréan et al., 1998). Interaction number 4 is shown as a dotted line because, as described more fully in the text, *odd* expression is lost from the *ftz* domains in *runt* nulls as well as in *runt*, *slp* double mutants, so that *odd* may be primarily responsible for keeping *en* and late *eve* from expanding anteriorly in slp mutants, while slp may be solely responsible for setting this border in the wild type. Implicit in a number of these regulatory relationships is the fact that an effect of one gene on another may change during the course of refinement of pair-rule gene patterns, and similarly, that an effect in odd-numbered PSs does not imply the corresponding effect in even-numbered PSs, and vice versa. Such complexities can be explained by the existence of distinct regulatory elements in a gene, which drive expression of different aspects of its pattern and respond to distinct regulatory inputs. This is the case for the regulation of *eve*. In other cases, such as for *runt*, it may not be possible to dissect the cis-acting sequences into distinct elements (Klingler et al., 1996), and such complexities may be due to multiple factors acting combinatorially through common or overlapping elements. (B) Regulatory interactions can account for the well-established concentration-dependent positioning of PS boundaries by Eve and Ftz (see text for appropriate references). In the anterior half of each odd-numbered PS, the early eve stripe provides a concentration gradient of Eve protein just before and during cellularization of the blastoderm (shown as a blue curve at the top). Reduced Eve activity is represented by the yellow curve; other expression patterns are represented by colored boxes, with altered patterns that result from either decreased eve activity, manifested primarily at the left PS boundary in the diagram, or increased *ftz* activity, manifested primarily at the right PS boundary, represented by boxes offset below the wild-type patterns. The odd-numbered *en* stripe is activated by Prd and repressed by Slp, and both *prd* and *slp* are repressed by Eve. A high level of Eve is required to repress *prd* (Fujioka et al., 1995;Manoukian and Krause, 1992), which is activated earlier than *slp* in the trunk region, while a low level of Eve suffices to repress *slp*. The anterior border of this *en* stripe is positioned by the posterior edge of the *slp* stripe, while its posterior border is positioned by the posterior edge of *prd* expression, both of which are sensitive to the genetic dose of *eve* (Fujioka et al., 1999) and to the level of Eve's repressor activity (Fujioka et al., 2002;Kobayashi et al., 2001). The net result of these interactions is that the positions of both the anterior and posterior borders of the odd-numbered en stripe respond to changes in the level of Eve, with lower Eve levels resulting in reduced-width odd-numbered PSs (and a boundary at the dotted yellow line), and therefore expanded even-numbered PSs. The positions of the even-numbered *en* stripes are particularly sensitive to the concentration of Ftz. Ftz activates these *en* stripes, while Odd represses them, setting the anterior and posterior borders, respectively. Higher levels of Ftz (or increased stability of Ftz) result in auto-activation over a wider region (Hiromi and Gehring, 1987;Ish-Horowicz et al., 1989), but also cause an anterior expansion of *odd* stripes. Eve represses *odd* at low concentrations, and is also capable of repressing *ftz* at higher concentrations (DiNardo and O'Farrell, 1987;Fujioka et al., 1995;Manoukian and Krause, 1992), while Odd causes subsequent downregulation of *ftz* expression. This combination of interactions allows the positions of the even-numbered *en* stripes to move anteriorly in response to higher Ftz activity, expanding the even-numbered PSs (with a boundary at the dotted green line) at the expense of the odd-numbered ones. Reduced levels of Ftz function or increased levels of Eve function produce the opposite effects of those described above, and mechanisms that are the converse of those described can account for these effects. The positions of the wg stripes move in conjunction with those of the *en* stripes. At the anterior boundaries of the odd-numbered PSs, *wg* is repressed by both En (Heemskerk et al., 1991) and late Eve expression (Ingham et al., 1988;Manoukian and Krause, 1992), which shares an anterior border with *en* (Lawrence et al., 1987). At the anterior boundaries of the even-numbered PSs, *wg* is repressed by *ftz* and *en* (Heemskerk et al., 1991;Ingham et al., 1988;Ish-Horowicz et al., 1989), so that in each case, *wg* expression is activated just anterior to that of en.

Table 1

Summary of pair-rule interactions.

Interaction # (Fig. 5A)	Experimental basis for interaction	Genetic background	Reference
1	*hairy* expression expands	*runt*$^-$	Ingham and Gergen, 1988; Hartmann et al., 1994
2	*runt* expression expands	*hairy*$^-$	Ingham and Gergen, 1988
	runt is activated by a Hairy-AD[a] fusion	*hs-HairyAct*	Jiménez et al., 1996
3	*ftz* stripes expand, fail to narrow properly	*hairy*$^-$	Ingham and Gergen, 1988
	ftz is activated by a Hairy-AD[a] fusion	*hs-HairyAct*	Jiménez et al., 1996
4	both late *eve* and odd-numbered *en* stripes expand anteriorly (they do so only later in *slp* single mutants; however, see legend to Fig. 5A)	*runt*$^-$; *slp*$^-$	this work
	odd-numbered *en* stripes are very sensitive to repression by Runt	ectopic *runt*	Aronson et al., 1997; Manoukian and Krause, 1993
5	secondary *odd* stripes expand posteriorly (they do not in *slp*$^-$)	*runt*$^-$	this work (Fig. 2)
6[b]	early *eve* stripes persist	*runt*$^-$	Ingham and Gergen, 1988
	eve is rapidly repressed	*hs-runt*	Manoukian and Krause, 1993
	eve is activated by a Runt-AD[a] fusion	*hs-RunAct*	Jiménez et al., 1996
7	secondary (late) *runt* expression expands throughout the *eve* domain	*eve*$^-$	Fujioka et al., 1995
	runt is rapidly repressed	*hs-eve*	Manoukian and Krause, 1992
8	*odd* fails to retract from the anterior of *ftz* domains, preventing activation of *en*[c]	*eve*$^-$	Coulter and Wieschaus, 1988; DiNardo and O'Farrell, 1987; Fujioka et al., 1995
	odd is rapidly repressed	*hs-eve*	Manoukian and Krause, 1992
9	*slp* expression expands throughout the odd-numbered parasegments, preventing activation of *en*	*eve*$^-$	Fujioka et al., 1995 (this work, Fig. 1)
10	(see legend to Fig. 5A)		this work, Fig. 2
11	*prd* expression expands throughout the *eve* domain	*eve*$^-$	Baumgartner and Noll, 1990; Fujioka et al., 1995
	prd is rapidly repressed	*hs-eve*	Manoukian and Krause, 1992
12	even-numbered *en* stripes are not activated	*ftz*$^-$	DiNardo and O'Farrell, 1987; Howard and Ingham, 1986
	ectopic *ftz* activates *en*	*hs-ftz*	Ish-Horowicz et al., 1989
13	*ftz* is required to maintain *odd* expression[d] (and thereby to limit *slp* expression, as seen in *runt* nulls)	*ftz*$^-$(*runt*$^-$)	Nasiadka and Krause, 1999 (this work, Fig. 2)
	odd is rapidly activated	*hs-ftz*	Nasiadka and Krause, 1999
14	*ftz* stripes fail to narrow properly[e]	*odd*$^-$	Mullen and DiNardo, 1995
15	*ftz* stripes fail to narrow properly[f]	*slp*$^-$	Cadigan et al., 1994b
16	*odd* sets the posterior border of even-numbered *en* stripes	*odd*$^-$	DiNardo and O'Farrell, 1987
		eve$^-$ *rescue*	Fujioka et al., 1995
		hs-eve	Manoukian and Krause, 1992
17	*prd* persists throughout the *ftz* domain	*odd*$^-$	Baumgartner and Noll, 1990; Saulier-Le Dréan et al., 1998
18[g]	*slp* is expressed throughout *ftz* domains in *runt* nulls due to failure of *ftz* to maintain *odd* expression[h]	*runt*$^-$ (*ftz*$^-$)	this work (Fig. 2) (Nasiadka and Krause, 1999)
19,20	*odd* expands, preventing odd-numbered *en* stripes from expanding anteriorly (until *odd* fades during germband extension)[i]	*slp*$^-$	this work (Fig. 3)

Interaction # (Fig. 5A)	Experimental basis for interaction	Genetic background	Reference
21	odd-numbered *en* stripes “come back” when *slp* is removed from *eve*$^{-}$	*eve*$^{-}$, *slp*$^{-}$	this work (Fig. 1)
	ectopic *slp* expression represses *en*	*hs-slp*	Cadigan et al., 1994a
22	2° *odd* stripes missing in *eve*$^{-}$, due to expansion of *slp*	*eve*$^{-}$	this work (Fig. 4); (Fujioka et al., 1995)
23	even-numbered *en* stripes expand anteriorly late in germband extension	*slp*$^{-}$	Cadigan et al., 1994b
	ectopic *slp* expression represses *en*	*hs-slp*	Cadigan et al., 1994a
24	*prd* activates both late *eve* and odd-numbered *en* stripes	*eve*$^{-}$	Fujioka et al., 1996; DiNardo and O'Farrell, 1987

[a] Activation domain.

[b] *eve* is also repressed by *odd* (Saulier-Le Dréan et al., 1998), which may contribute to further narrowing not diagrammed in Fig. 5A (*odd* stripes in the odd-numbered PSs appear later than those in the even-numbered PSs); in addition, further narrowing occurs as early *eve* expression fades and late *eve* is activated by Prd, so that final late *eve* stripes are essentially coincident with odd-numbered *en* stripes.

[c] Although shown in Fig. 5A as emanating from the later “minor stripe” aspect of *eve* expression in the diagram, early *eve* stripes are probably sufficient for this function (Fujioka et al., 1995).

[d] This regulation is likely to be direct (Nasiadka and Krause, 1999).

[e] This effect may be indirect (Saulier-Le Dréan et al., 1998).

[f] This regulation is likely to be direct (Yu et al., 1999).

[g] *ftz* is also required for the *eve* minor stripes (data not shown), which may also limit *slp* expression.

[h] Ectopic *odd* was shown to repress *slp* (Saulier-Le Dréan et al., 1998), but in *odd* mutants, *en* is expressed here rather than *slp* (DiNardo and O'Farrell, 1987), due to its activation at higher levels of *ftz* (*en* can then repress *slp*).

[i] This effect of *odd* is likely to be direct (Saulier-Le Dréan et al., 1998).