Control of segmental asymmetry in *Drosophila* embryos

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SUMMARY

During *Drosophila* development, an important aspect of body patterning is the division of the embryo into repeating morphological units referred to as parasegments. The parasegmental domains are first defined at the blastoderm stage by alternating stripes of transcripts encoded by the pair-rule genes *fushi tarazu* (*ftz*) and *even-skipped* (*eve*) and later by stripes encoded by the segment polarity genes *engrailed* (*en*) and *wingless*. Here, we show that the *runt* gene (*run*) is required to generate asymmetries within these parasegmental domains. Using a heat-shock-inducible *run* transgene, we found that ectopic *run* expression leads to rapid repression of *eve* stripes and a somewhat delayed expansion of *ftz* stripes. Unexpectedly, we also found that ectopic *run* was a rapid and potent repressor of odd-numbered *en* stripes. Two remarkably different segmental phenotypes were generated as a consequence of these effects. In solving

the mechanisms underlying these phenotypes, we discovered that the positioning of *en* stripes is largely determined by the actions of negative regulators. Our data indicate that *run* is required to limit the domains of *en* expression in the odd-numbered parasegments, while the *odd-skipped* gene is required to limit the domains of *en* expression in the even-numbered parasegments. Activation of *en* at the anterior margins of both sets of parasegments requires the repression of *run* and *odd* by the product of the *eve* gene. The spatial restriction of gene expression via negative and double negative pathways such as these is likely to be a common theme during development.

Key words: *runt*, pair-rule genes, segment polarity, parasegments, *Drosophila*

INTRODUCTION

The earliest morphological signs of metamerization in the developing *Drosophila* embryo are in the form of parasegmental grooves (Martinez-Arias and Lawrence, 1985). The parasegmental domains defined by these grooves are equal in width to future segments, but are out of register. Odd-and even-numbered parasegments are first defined by the expression patterns of two pair-rule genes, *even-skipped* (*eve*) and *fushi tarazu* (*ftz*) (Lawrence et al., 1987). During the blastoderm stage of development, *eve* and *ftz* are expressed in a complementary set of seven, four-cell-wide stripes (Macdonald et al., 1986; Hafen et al., 1984). Two well-defined roles for both genes are to position stripes of expression of the segment polarity gene *engrailed* (*en*) at the anterior edge (DiNardo et al., 1985), and stripes of the segment polarity gene *wingless* (*wg*) at the posterior edge (Ingham et al., 1988) of each presumptive parasegment. In this way, *en* and *wg* stripes are juxtaposed at the parasegmental boundaries, and parasegmental grooves soon form in between.

In *ftz* and *eve* mutant embryos, stripes of *en* are lost, thereby assigning these genes as activators of *en*. However, stripes of *en* expression are somehow limited to the anterior margins of each *ftz* and *eve* stripe. Two contrasting models have been offered to explain this apparent restriction of *en*

expression. The combinatorial model (DiNardo and O'Farrell, 1987; Ingham et al., 1988) implicates overlaps with other pair-rule gene activators and repressors. Accordingly, overlaps between stripes of the *en* activators *eve* and *paired* (*prd*) stripes are necessary for initiation of the odd-numbered *en* stripes, while overlaps between the activators *ftz* and *odd-paired* (*opa*) are required for initiation of the even-numbered *en* stripes. This model, however, fails to explain the pattern of *en* stripe initiation: *en* stripes initiate during gastrulation in an anterior-to-posterior wave with even-numbered parasegment stripes initiating prior to odd-numbered parasegment stripes (DiNardo et al., 1985). Overlaps between the odd-numbered *en* stripe activators *eve* and *prd*, and the even-numbered *en* stripe activators, *ftz* and *opa*, occur long before gastrulation.

The second model, which we will refer to as the 'gradient/boundary' model, suggests that positioning of *en* stripes is functionally linked to the process of *ftz* and *eve* stripe resolution (Lawrence et al., 1987; Lawrence and Johnston, 1989). During gastrulation, *ftz* and *eve* stripes become narrower due to a graded loss of expression along their posterior edges. At the same time, the anterior edges of each stripe become more sharply defined. These observations suggested that both high levels of expression and sharp stripe boundaries are required for the activation of *en* in single-cell-wide stripes. Since the process of *ftz* and *eve*

stripe resolution occurs during gastrulation, it potentially explains the delayed onset of *en* expression. However, since stripe narrowing occurs at the same time for both *ftz* and *eve*, and does not exhibit any anterior-to-posterior variations, this also fails to explain the initiating pattern of *en* expression.

Relatively little is known about the role of the *runt* gene (*run*) during these events. Stripes of *run* expression are out of phase with those of *ftz* and *eve*. Each *run* stripe initially overlaps with the posterior half of each *eve* stripe and the anterior half of each *ftz* stripe (Kania et al., 1990). In *run* mutant larvae, pattern deletions occur in regions corresponding approximately to these domains of expression, and the deleted regions are replaced by mirror-image duplications of the regions that remain (Gergen and Wieschaus, 1985). The basis for this phenotype has yet to be determined. An important clue, however, is that stripes of *hairy* (*h*) and *eve* are expanded in *run⁻* embryos, while *ftz* stripes initiate weakly and are prematurely lost (Ingham and Gergen, 1988). Due to its influential role on the initating expression patterns of *eve*, *h* and *ftz, run* has been subclassified as one of three 'primary' pair-rule genes.

It is surprising that, despite these effects on the expression of other pair-rule genes, and the dramatic nature of the *run⁻* phenotype, expression of the *en* gene appears to be essentially normal in *run⁻* embryos (DiNardo and O'Farrell, 1987; Martinez-Arias and White, 1988). As in wild-type embryos, 14 stripes of *en* are initiated, although their spacing and width are somewhat irregular. Since *en* is regulated by the *ftz* and *eve* genes, both of which are misexpressed in *run⁻* embryos (Carroll and Scott, 1986; Frasch and Levine, 1987; DiNardo and O'Farrell, 1987), it has not been possible to determine whether *run* has a direct role in *en* regulation.

The inability to distinguish between direct and indirect gene interactions via the analysis of mutant embryos has left a great deal of uncertainty regarding the actual circuitry of segmentation gene interactions. While it is possible to test for direct interactions in vitro, the significance of these interactions is often unknown. For example, the product of the *eve* gene (Eve) binds to sequences in the promoters of both the *eve* (Jiang et al., 1991) and *en* (Han et al., 1989) genes, consistent with genetic studies that suggested that *eve* is a positive regulator of both genes (Frasch et al., 1988; DiNardo and O'Farrell, 1987; Ingham et al., 1988). However, recent results with a heat-shock-inducible *eve* transgene indicate that both the *eve* and *en* genes are indirectly regulated by Eve through the regulation of intermediary genes (Manoukian and Krause, 1992). The use of inducible transgenes has also shown that the regulatory roles of a gene may change dramatically within very short periods of time (Manoukian and Krause, 1992), making the interpretation of expression patterns in mutant embryos even more complex. Hence, it is important to establish, whenever possible, whether gene interactions are direct or indirect in an in vivo setting prior to persuing the nature of these interactions in vitro.

We have used a heat-shock-inducible *run* transgene to learn more about the regulatory roles and properties of Run in vivo. By monitoring the response time of potential Run target genes following ectopic Run induction, we were able to address which genes were direct targets of Run and which
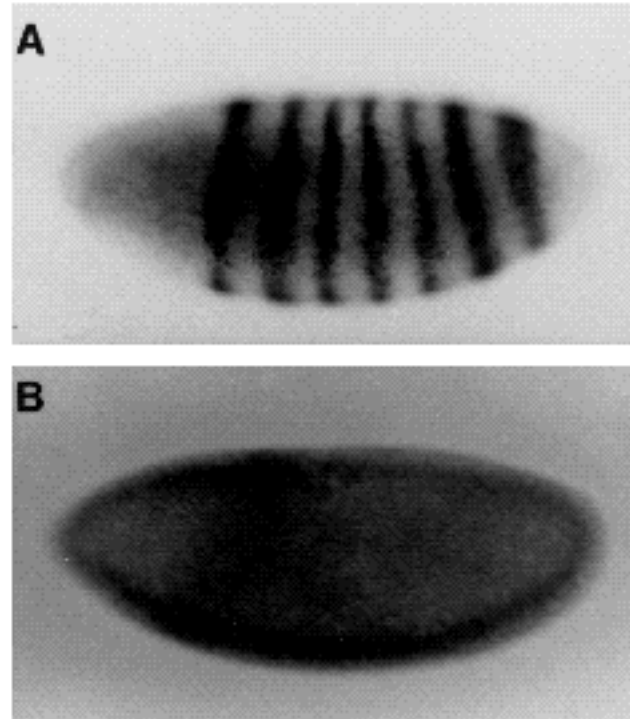

Fig. 1. *run* expression in wild-type and HSR embryos. Wild-type (A) and HSR embryos (B) fixed at a similar developmental stage (2.5-3 hours AEL; cellular blastoderm) and hybridized with digoxigenin (DIG)-labeled *run* probe. HSR embryos were heat shocked for 6 minutes at 36°C and fixed 15 minutes after the initiation of the heat shock.

were indirect. Surprisingly, we found that Run regulates, not only other pair-rule genes, but the segment polarity gene *en* as well. Based upon these primary effects, we suggest how pattern rearrangements arise in ectopic *run* and *run⁻* embryos, and how Run functions in wild-type embryos. In unravelling these phenotypes, novel gene expression patterns and interactions were discovered that explain the complex pattern of *en* stripe initiation, much of which is apparently controlled by negative regulatory interactions.

MATERIALS AND METHODS

HSR flies were obtained from J. P. Gergen (Tsai and Gergen, personal communication). Two HSR lines, containing inserts at different chromosomal locations, were tested, both yielding similar results. Embryo collection, synchronization, heat shocks and fixation were performed as previously described (Manoukian and Krause, 1992). Briefly, embryos were collected in Plexiglas cylinders (8.5×15 cm) on apple juice/agar plates. For cuticle preparations, embryos were collected for 20 minutes and aged for the appropriate period at 25°C. For the precise timing of cuticle phenotypes, eight different 20 minute collections were made. Each represented a different 20 minute interval between 2 and 3.5 hours AEL, differing in age by 10 minutes (i.e. 2-2:20 AEL, 2:10-2:30 AEL, 2:20-2:50 AEL etc.). At the appropriate time, embryos were transferred to a glass coverslip, covered in a thin layer of halocarbon oil and heat shocked by floating the coverslip in a 36°C water bath within a closed container. Properly staged embryos were then selected under the microscope (50-60 embryos/collection) and
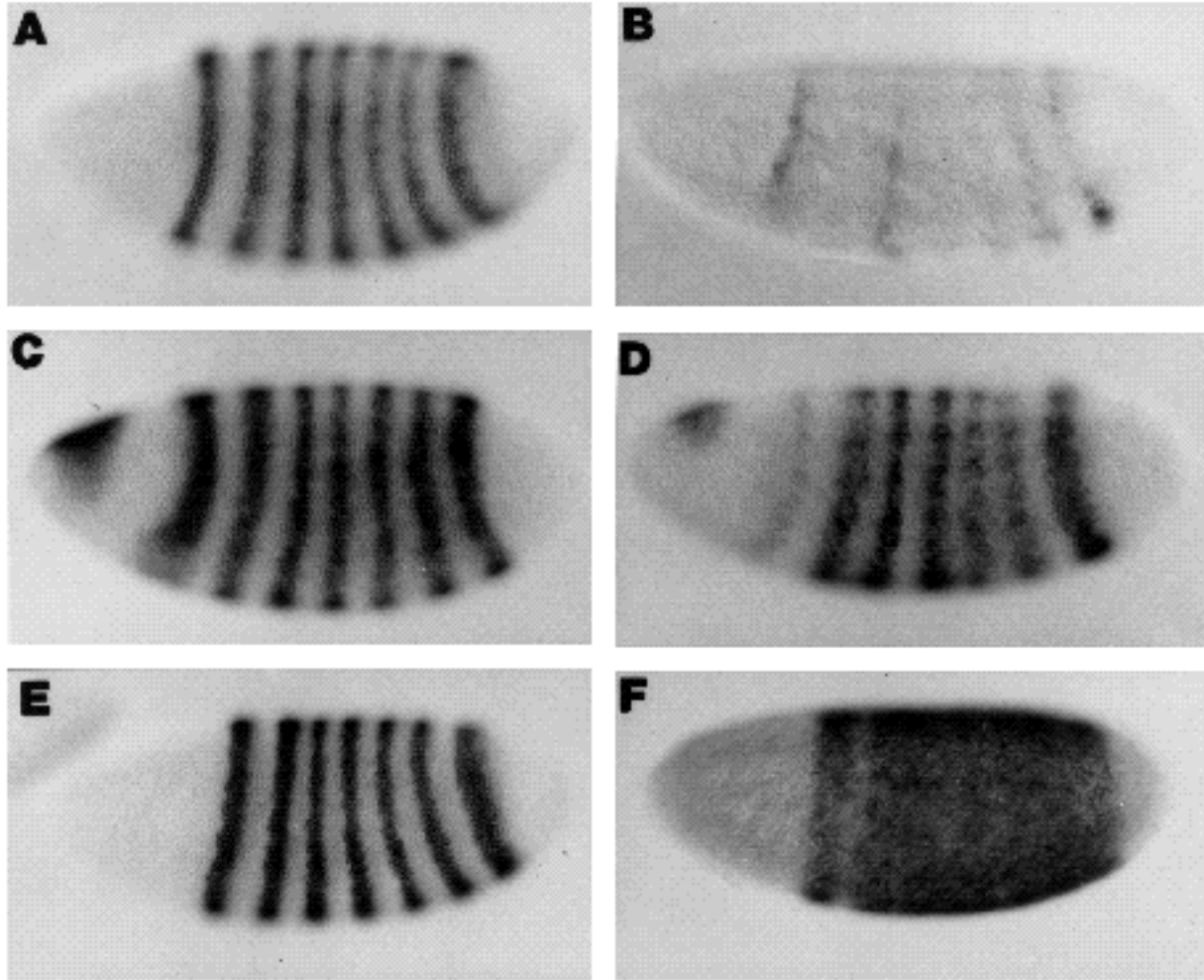
Fig. 2. Pair-rule gene expression in wild-type and HSR embryos. Whole-mount in situ hybridizations using DIG-labeled probes to detect wild-type (A, C, E) and HSR (B, D, F) pair-rule gene expression patterns: *eve* (A, B); *h* (C, D); *ftz* (E, F). The HSR embryos (right) were heat shocked collectively for 6 minutes at 36°C between 2.5 and 3 hours AEL. The embryos in B and D were fixed 30 minutes after the initiation of heat shock (AHS), while the embryo in F was fixed 40 minutes AHS.

transferred back to apple juice plates for aging overnight. Embryos for in situ hybridization and immunochemical detection of proteins were collected for 20 or 30 minute intervals, as specified, and were mounted under glycerol instead of oil for heat shock and recovery. Subsequent steps have all been previously described (Manoukian and Krause, 1992). All experiments have been repeated at least two times.

RESULTS

Fly lines containing P-element-mediated insertions of an *hsp*70 promoter-*run* cDNA fusion gene were obtained from C. Tsai and J. P. Gergen. Initial experiments showed that short heat shocks of 5-7 minutes at 36°C,administered at the cellular blastoderm stage of development, were sufficient to cause lethal segmentation defects in approximately 90% of heat-shock *run* (HSR) embryos. Under these conditions, *run* transcripts were distributed throughout the embryo within 10-15 minutes of the initiation of heat shock (Fig. 1B). The distribution of induced *run* protein (Run) correlates well with that of *run* transcripts (Tsai and Gergen, personal communication).

Effects of ectopic Run on *eve, h* and *ftz*

Transcripts encoded by the pair-rule genes *eve*, *h* and *ftz* were monitored in HSR embryos by whole-mount *in situ* hybridization to test the regulatory circuitry between these four genes. Embryos were heat shocked for 6 minutes, 2.5-3 hours after egg laying (AEL) and fixed at various times after heat shock (AHS). At the age of 2.5-3 hours AEL, embryos are in transition between the syncitial blastoderm and cellular blastoderm stages of development (stages 5(1)-5(3)). Patterns of pair-rule gene expression, at this time, are being resolved into well-defined stripes.

We have provided previous evidence that it is possible to distinguish between direct and indirect target genes of ectopically expressed proteins based upon the timing of their response (Manoukian and Krause, 1992). In general, transcripts encoded by genes that appear to be direct targets of ectopically expressed proteins are altered in their patterns of expression within 15-30 minutes of the beginning of a heat-shock pulse. Genes that do not appear to be direct targets of the ectopically expressed protein require 30 minutes or longer to respond. In HSR embryos, transcripts encoded by
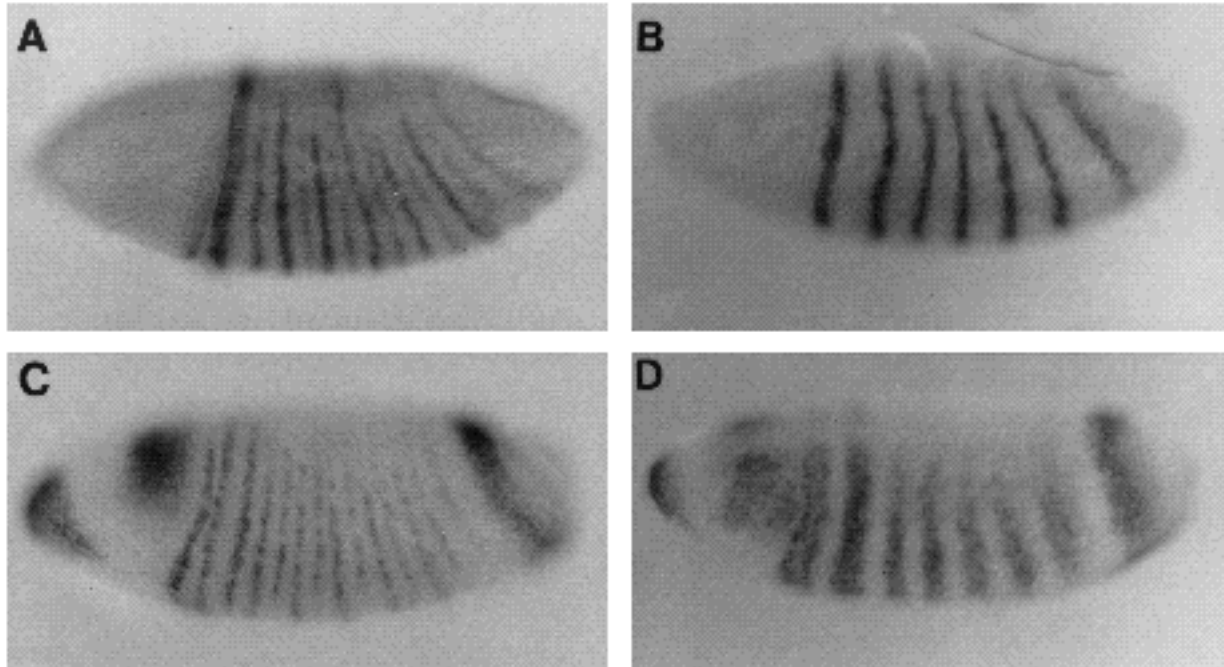
Fig. 3. Expression of the segment polarity genes *en* and *wg* in HSR embryos. Similar stage wild-type (A, C) and HSR (B, D) embryos hybridized with DIG-labeled *en* (A, B) or *wg* (C, D) probes. HSR embryos (right) were heat shocked collectively for 6 minutes at 36°C (between 2.5 and 3 hours AEL) and then fixed 30 minutes AHS in the case of *en* (B) and 45 minutes AHS in the case of *wg* (D).

the *eve* gene showed significant effects 15-30 minutes after the initiation of heat shock (AHS). Fig. 2B shows an HSR embryo at the cellular blastoderm stage of development (around 3 hours AEL), fixed 25 minutes AHS. Significant repression of *eve* was observed relative to the wild-type embryo shown in Fig. 2A. Interestingly, all stripes were not equally affected. Stripe 2 was the most efficiently repressed while stripes 3 and 7 were relatively resistant to repression.

Transcripts encoded by the *h* gene were not strongly affected when fixed 25-30 minutes AHS. Stripe 1 showed the highest sensitivity to ectopic Run, exhibiting mild repression by 30 minutes AHS (Fig. 2D). Repression approaching the efficiency of *eve* repression was only seen when embryos were fixed between 30 and 45 minutes AHS (not shown). Expression of the *ftz* gene also did not appear to be affected prior to 30 minutes AHS. However, starting at 30 minutes AHS, *ftz* stripes widened dramatically. In embryos that had been heat shocked prior to *ftz* stripe resolution (2:20-2:50 AEL), and fixed 30-45 minutes later, *ftz* was often expressed in a single solid stripe that filled the entire trunk of the embryo (Fig. 2F). When these embryos were permitted to develop for another 15-30 minutes prior to fixation, this solid pattern of *ftz* expression began to split into a pattern of seven, segment-wide stripes (see below).

In embryos where *ftz* had already resolved into stripes prior to the time of *run* induction (around 2:50-3:10 AEL), the seven stripe pattern could no longer be consolidated into a solid band of expression. Nevertheless, at 30-45 minutes AHS, *ftz* stripes were abnormally wide (shown below). During this period (gastrulation and germ band extension), *ftz* stripes normally resolve from four cells in width to one or two cells in width. In embryos heat shocked at 2:50-3:10

AEL, this narrowing process failed to occur. Thus, both early and late Run inductions resulted in wider than normal *ftz* stripes in germ-band-extending embryos.

Expression patterns of *ftz* and *eve* promoter-*lacZ* fusion genes have also been examined in HSR embryos (A. S. M. and H. M. K., unpublished observations; Tsai and Gergen, personal communication). These responded similarly to ectopic Run, indicating that these effects are mediated at the level of transcript initiation.

Expression of segment polarity genes in HSR embryos

As with the pair-rule genes *eve*, *h* and *ftz*, we monitored expression of the segment polarity genes *en* and *wg* for altered patterns in HSR embryos (Fig. 3). When Run was induced shortly before or after the onset of *en* expression (around 2:50-3:20 AEL), odd-numbered *en* stripes were repressed within 15-30 minutes AHS (Fig. 3B). Expression of the *wg* gene, on the other hand, did not change prior to 30 minutes AHS. However, subsequent to this time, the initiating pattern of *wg* changed from 14 narrow stripes to 7 wide stripes (Fig. 3D). These wide stripes were positioned between the broadened stripes of *ftz* (see below).

The rapid repression of odd-numbered *en* stripes by ectopic Run suggests that Run may be a direct repressor of *en*, although only in odd-numbered parasegments. Alternatively, Run could act indirectly on these *en* stripes by repressing *eve* or *paired* (*prd*) gene expression, since both genes are required for the initiation of the odd-numbered *en* stripes (DiNardo and O'Farrell, 1987). In Fig. 2, we showed that *eve* was indeed repressed by ectopic Run, such that *eve* transcripts were greatly diminished by 30 minutes AHS.
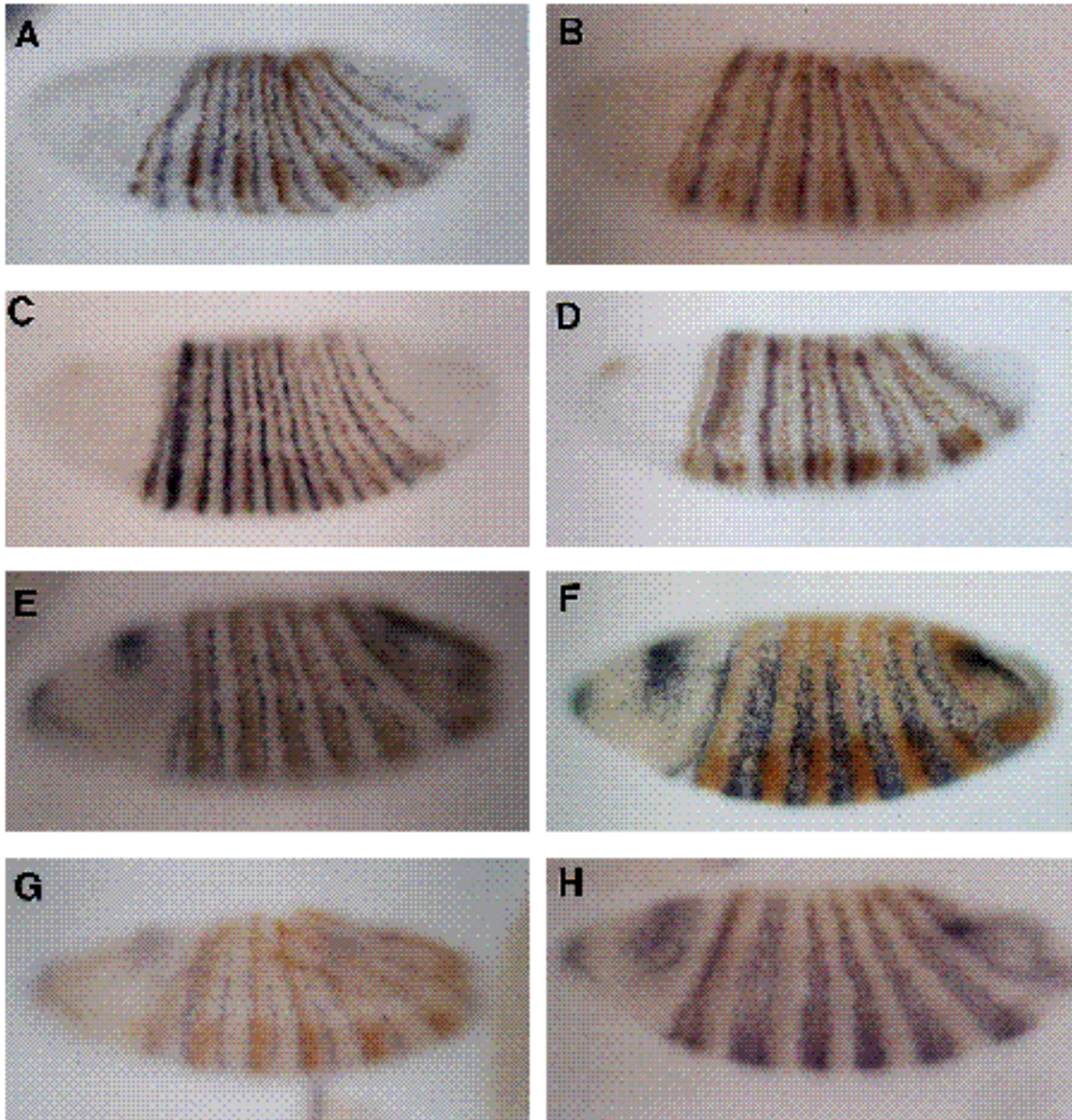
Fig. 4. Double labeling of wild-type and HSR embryos for pair-rule proteins and segment polarity gene transcripts. Wild-type embryos (A, C, E, G) are on the right and HSR embryos (B, D, F, H) are on the left. HSR embryos in the top half (B, D) were fixed 30 minutes after the initiation of a 6 minute heat shock (AHS) at 36°C, whereas the HSR embryos in the bottom half (F, H) were fixed 45 minutes AHS. All embryos shown are approximately 3.5 hours AEL in developmental age. Embryos were double labeled, either for *eve* protein (brown) and *en* transcripts (blue; A, B); *prd* protein (brown) and *en* transcripts (blue; C, D); *ftz* protein (brown) and *wg* transcripts (blue; E, F); or *eve* protein (brown) and *wg* transcripts (blue; G, H).

However, it seems unlikely that this is fast enough to account for the repression of *en* transcripts within the same period of time.

In order to differentiate between direct and indirect actions of Run upon *en*, we colocalized *en* transcripts (blue) together with either *eve* protein (Eve) or *prd* protein (Prd) in HSR embryos (Fig. 4). Panels B and D show that odd-numbered *en* stripes were repressed 25 minutes AHS despite the presence of both Eve and Prd (brown). Thus, Run is capable of overiding the ability of Eve and Prd to activate

en in the odd-numbered parasegments, while not affecting expression of *en* in the even-numbered parasegments.

Initiation of *wg* expression in wild-type and mutant embryos has indicated that *wg* expression is subject to negative regulation by *ftz* and *eve* (Ingham et al., 1988). Fig. 4E shows that, in wild-type embryos, the odd-numbered *wg* stripes (blue) initiate as Ftz stripes (brown) begin to narrow at their posterior edges. In HSR embryos, heat shocked between 2:20 and 2:50 AEL and fixed 45 minutes later, Ftz was expressed in seven wide stripes in which no *wg* was

expressed (Fig. 4F). However seven wide stripes of *wg* filled the regions between each Ftz stripe.

Double staining of Eve (brown) and *wg* transcripts (blue) suggests that these *wg* stripes expanded due to repression of Eve. In wild-type embryos, these *wg* stripes are normally restricted to the posterior edges of the odd-numbered parasegments where Eve stripes first begin to diminish (Fig. 4G). In gastrulating HSR embryos, expansion of *wg* into the anterior regions of the odd-numbered parasegments was coincident with Eve repression (Fig. 4H).

Ectopic Run generates two segmental phenotypes

HSR embryos were subjected to 5-10 minute heat shocks during different stages of early development to determine when the effects of ectopic Run were lethal. Inductions were lethal (96% with a 7 minute heat shock), primarily when induced during a 50-60 minute window of sensitivity (around 2:20-3:20 AEL). In contrast, wild-type embryos, subjected to 15 minute heat shocks at these stages of development, exhibited only a minor increase in lethality (around 5%). Double staining embryos for *en* protein (En) and *wg* transcripts, several hours after Run induction (6 hours AEL), showed that two distinct pattern rearrangements could be generated (Fig. 5B, C). Cuticle preparations showing the external features of unhatched larvae show the two corresponding segmental phenotypes that evolved from these patterns (Fig. 5E, F).

The first En/*wg* pattern (Fig. 5B) and the corresponding cuticle pattern (Fig. 5E) were induced by heat shocks administered between 2:20 and 2:50 AEL. Both patterns exhibited reversals of polarity within each segment. This phenotype will be referred to as the class 1 phenotype. The second En/*wg* pattern (Fig. 5C), and the corresponding cuticle pattern (Fig. 5F), were generated by heat shocks administered between 2:50 and 3:20 AEL. These exhibited characteristics of a pair-rule phenotype, with pattern deletions in alternate segments. This will be referred to as the class 2 phenotype.

In wild-type embryos, En (brown) is expressed at the anterior edge, and *wg* (blue) is expressed at the posterior edge, of each parasegment. Hence En and *wg* stripes are juxtaposed at parasegmental boundaries, with each En stripe preceded by a stripe of *wg* (Fig. 5A). In class 1 embryos, odd-numbered En stripes, and the *wg* stripes immediately in front, were somehow replaced by sets of En and *wg* stripes of inverted polarity: these En stripes were in front of the *wg* stripes rather than behind (Fig. 5B). Below, we show that these ectopic En stripes are Ftz-dependent. The induction of these inverted En/*wg* stripes generated a pattern of mirror-image symmetries, where lines of symmetry passed between adjacent stripes of En and between adjacent stripes of *wg*. These mirror-image duplications were reflected in the cuticle preparation shown in Fig. 5E. Focusing upon the abdominal denticle belts in this cuticle, one can see that the posterior regions of odd-numbered denticle belts have been replaced by mirror-image duplications of the anteriormost denticles, and that the even-numbered denticle belts have been replaced by mirror-image duplications of the posteriormost denticles. When the En/*wg* and cuticle patterns are considered together, we can interpret these rearrangements as the replacement of the posterior regions of even-numbered parasegments by duplications of the remaining anterior regions, and the replacement of the anterior regions of odd-numbered parasegments by duplications of the remaining posterior regions (diagrammed in Fig. 5H). These rearrangements have been confirmed by examination of intermediate cuticle phenotypes (not shown) and by double staining embryos fixed at various time points following Run induction (see below).

In the class 2 pattern, odd-numbered En stripes were repressed or failed to initiate and were not replaced by ectopic En stripes (Fig. 5C). Loss of these stripes led to an eventual loss of *wg* expression and deletions centered about the cells that failed to express En and *wg*. In the resulting cuticle (Fig. 5C), these deletions were reflected by fusions between adjacent denticle belts (ie. anterior A3/posterior A4) (Fig. 5F, I). Again, this interpretation was supported by intermediate cuticle patterns and double-staining patterns obtained during different stages of development.

Following heat shock, approximately 85% of class 1 stage embryos and 87% of class 2 stage embryos, hand selected for cuticle preparations (see Materials and Methods), yielded phenotypes similar to those shown. For En/*wg* staining, where embryos were not hand selected, the corresponding numbers were 68% and 72%. Other patterns observed were not stage specific.

Class 1 and class 2 phenotypes are distinguished by differential effects on *ftz* and *odd*

The initiation of *en* and *wg* stripes is under the control of pair-rule genes. Thus, we wished to determine how two different En/*wg* patterns could evolve from the Run-induced effects on pair-rule genes described earlier. The appearance of novel *en* stripes in class 1 but not class 2 embryos can be explained, in part, by differences in the resolution of *ftz* and *odd-skipped* (*odd*) stripes. Previous experiments have suggested that *ftz* encodes an activator and that *odd* encodes a repressor of the even-numbered *en* stripes (DiNardo and O'Farrell, 1987; Manoukian and Krause, 1992). In wild-type embryos, Ftz stripes narrow at their posterior edges during gastrulation and germ band extension (Fig. 6A-D). At the same time, *odd* stripes (blue), which initially overlap completely with Ftz (brown), resolve at both edges, leaving single-cell-wide *odd* stripes that only overlap with the posterior edges of the Ftz stripes (Fig. 6B,D). At this time, *en* expression (blue) initiates at the anterior edge of each Ftz stripe, where *odd* is absent (Fig. 6A,C). This transition progresses in an anterior-to-posterior fashion allowing the anteriormost *en* stripes to initiate prior to the posteriormost *en* stripes.

In class 1 embryos, Ftz initiates in a solid block that fills the entire trunk of the embryo (shown in Fig. 2). By gastrulation, this block of expression somehow resolved into seven wide stripes (Fig. 6E,F). At this time, *odd* expression was limited to very weak and narrow stripes positioned slightly posterior to the center of each Ftz stripe (Fig. 6F). Subsequently, *en* was expressed at both the anterior and posterior margins of each Ftz stripe, where *odd* appeared to be absent (Fig. 6E). In HSR embryos carrying a homozygous *ftz* mutation (*ftz*[w20]), all En stripes were absent (data not shown). Hence, all En stripes in class 1 embryos are Ftz-dependent.

In class 2 embryos, Run induction coincided with the time that Ftz stripes had already resolved or were in the process of narrowing. Further resolution was halted, again resulting in abnormally wide stripes (Fig. 6G, H). In this case, *odd* stripes overlapped with the posterior margins of each Ftz stripe (Fig. 6H). As expected, *en* was activated where *odd* was absent, at the anterior margin of each Ftz stripe (Fig. 6G). Hence the presence or absence of novel *en* stripes in class 1 and class 2 embryos appeared to be dependent upon the width of Ftz stripes and the positioning of *odd* within these stripes.

The expression of *wg* in class 1 and 2 embryos is consistent with these proposed relationships and helps to explain the strikingly different phenotypes of class 1 and 2 embryos. In class 1 embryos, the anterior edges of the widened *wg* stripes contacted the novel *en* stripes that were initiated at the posterior edges of Ftz stripes (see Fig. 4). In class 2 embryos, expression of *odd* at the posterior edges of the Ftz stripes prevented similar contacts between *en*- and *wg*-expressing cells (Figs 4, 6). Maintenance of *en* and *wg* expression has been shown to be dependent upon expression of these two genes in adjacent cells (DiNardo et al., 1988; Martinez-Arias et al., 1988; Heemskerk et al., 1991). Therefore, we suggest that the juxtaposition of *en*- and *wg*-expressing cells in class 1 embryos, although in an inverted relationship, permitted their continued expression and resulted in the mirror-image pattern shown in Fig. 5B.

As noted earlier, expression of *wg* in these domains was possible due to the lack of the *wg* repressors Ftz and Eve. The lack of Eve expression at this time also indicates that the resolution of *ftz* expression in class 1 embryos, from a solid block of expression into seven wide stripes, cannot be due to the actions of Eve. Similarly, it is unlikely that this resolution process is due to the actions of *h*, another repressor of *ftz*, since *h* expression was also absent during the time that *ftz* stripes were being resolved (data not shown). Hence, the repression of *ftz* in these regions is most likely regulated by a gene that was not followed in this study.

DISCUSSION

Using a heat-shock-inducible *run* transgene, we have attempted to dissect the regulatory roles of *run* during the process of segmentation. Short pulses of ectopic Run caused rapid changes in the expression patterns of both pair-rule and segment polarity genes when induced during a 50-60 minute period of embryogenesis. By monitoring the expression of these and other segmentation genes, shortly after Run induction and during later stages of embryogenesis, we were able to determine the basis of the two cuticular phenotypes that ensued. The mechanisms underlying these phenotypes suggest how Run functions in wild-type embryos and how the phenotype of *run⁻* embryos occurs. We propose that Run is required to establish polarity in both odd- and even-numbered parasegments.

Regulatory roles of Run in odd-numbered parasegments

Fig. 7 illustrates the spatial relationships between several of the pair-rule gene expression domains that are relevant to this study. The initial 7 stripe pattern of *eve* establishes the domains of the odd-numbered parasegments (Lawrence et al., 1987). At the blastoderm stage of development, stripes of Run overlap with the posterior half of each *eve* stripe (Kania et al., 1991). During gastrulation, *eve* expression is lost within these regions of overlap (Frasch et al., 1987; Warrior and Levine, 1990). In the presence of ectopic Run, the anterior regions of *eve* stripes were repressed as well, suggesting that Run is a negative regulator of *eve*. This negative relationship is consistent with the expansion of *eve* stripes previously observed in *run⁻* embryos (Frasch and Levine, 1987; Ingham and Gergen, 1988).

We have established temporal criteria that appear to distinguish between direct and indirect gene interactions following ectopic gene expression (Manoukian and Krause, 1992). These criteria suggest that the rapid repression of *eve* mediated by ectopic Run (within 15-30 min) is direct. Based upon these results, we suggest that Run plays a direct role in *eve* stripe narrowing and in establishing the anterior-to-posterior gradient within each Eve stripe.

In *run⁻* embryos, most *en* stripes appear to initiate, but they are of unequal spacing and width (DiNardo and O'Farrell, 1987; Martinez-Arias and White, 1988). Since the *en* regulators Ftz and Eve are misexpressed in *run⁻* embryos (Ingham and Gergen, 1988), the role of Run in *en* regulation has been difficult to determine. For this reason, and due to the subclassification of *run* as a primary pair-rule gene, a direct role in *en* regulation has generally been discounted. Here we made the surprising discovery that ectopic Run was capable of repressing odd-numbered *en* stripes within 15-30 minutes of induction, suggesting a direct interaction between the two genes. Since Run is normally restricted to the posterior half of the odd-numbered parasegments, we suggest that Run acts in wild-type embryos to restrict *en* from these regions. Thus, Run appears to establish polarity within the odd-numbered parasegments by at least two different mechanisms, first by repression of *eve* and second, by repression of *en*, both in the posterior regions of the odd-numbered parasegments.

Regulatory roles of Run in the even-numbered parasegments

In the even-numbered parasegments, *run* stripes overlap with the anterior half of each *ftz* stripe (Kania et al., 1990). Loss of *ftz* expression in *run⁻* embryos has suggested that *run* is a positive regulator of *ftz*. However, the reappearance of *ftz* stripes in *run⁻/h⁻* double mutant embryos indicated that *run* is not the only positive regulator of *ftz*, and that its positive effects may be mediated indirectly by repressing *h* (Carroll and Vavra, 1989), which is a negative regulator of *ftz* (Carroll and Scott, 1986; Hiromi and Gehring, 1987; Ish-Horowicz and Pinchin, 1987).

At first consideration, our results with HSR embryos appear to be inconsistent with this interpretation. Expansion of *ftz* gene expression was observed as expected. However, this expansion occurred prior to the loss of *h* gene products. We tested this further by co-expressing *run* and *h* in the same embryos, both under the control of heat shock promoters. Once again, expression of *ftz* expanded to fill the entire trunk of the embryo, despite the ectopic expression of
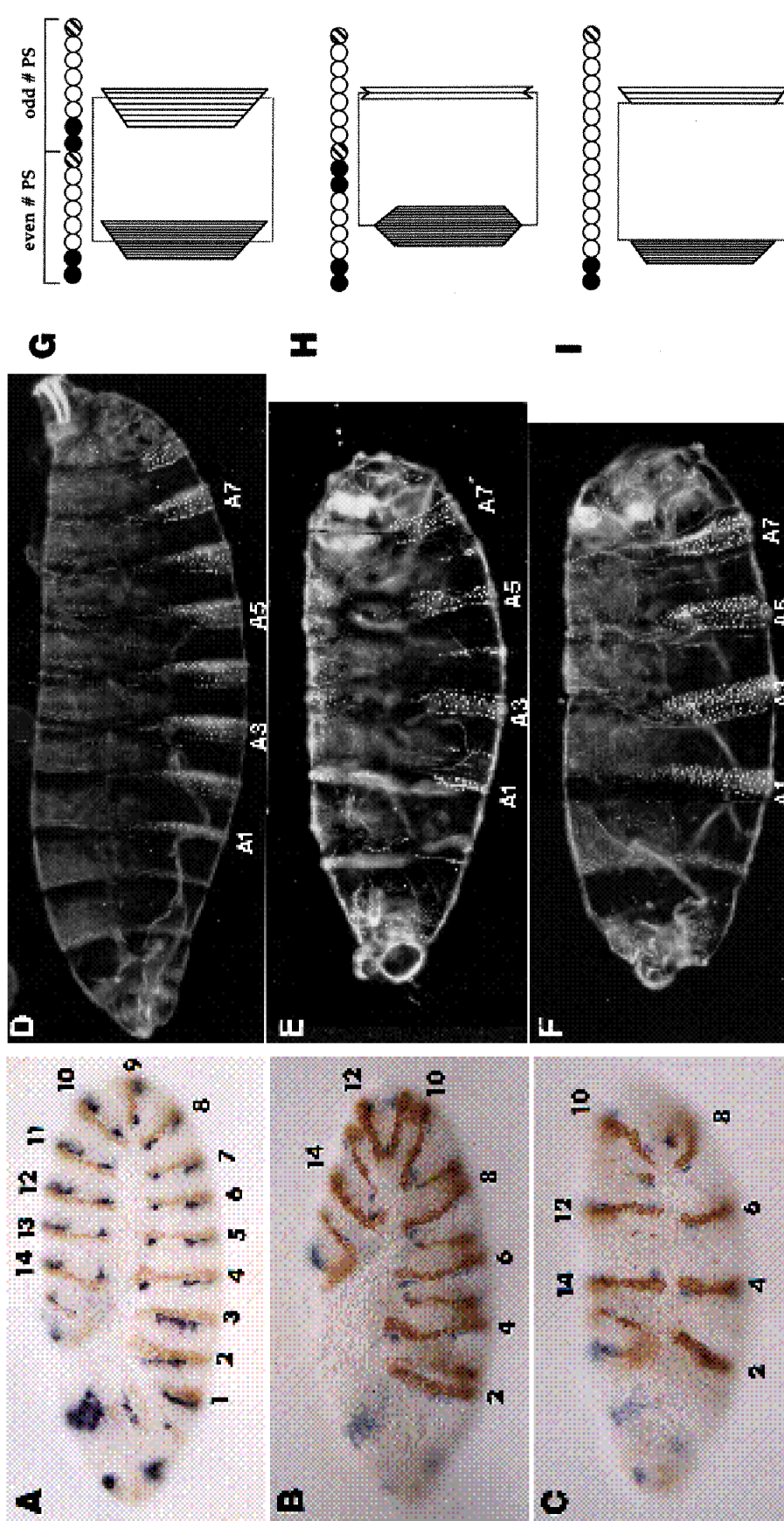
Fig. 5. Ectopic Run induces two different mutant phenotypes. Two mutant patterns were observed in HSR organisms, both in terms of *en* and *wg* expression (A–C) and in the cuticles of pharate larvae (D–F). These patterns are summarized in the diagrams to the right (G–I). The top panels (A, D, G) are non-heat-shocked controls. The middle panels (B, E, H) result from 6 minute heat shocks at 2:20-2:50 AEL (class 1). The lower panels (C, F, I) result from heat shocks administered at 2:50-3:10 AEL (class 2). Embryos in A-C were double labeled for *en* protein (brown) and *wg* transcripts (blue) at 5.5-6 hours AEL. The diagrams in G-I represent an interval of two parasegments. In these diagrams, *en*-expressing cells are denoted by solid black circles, while *wg*-expressing cells are denoted by hatched circles.
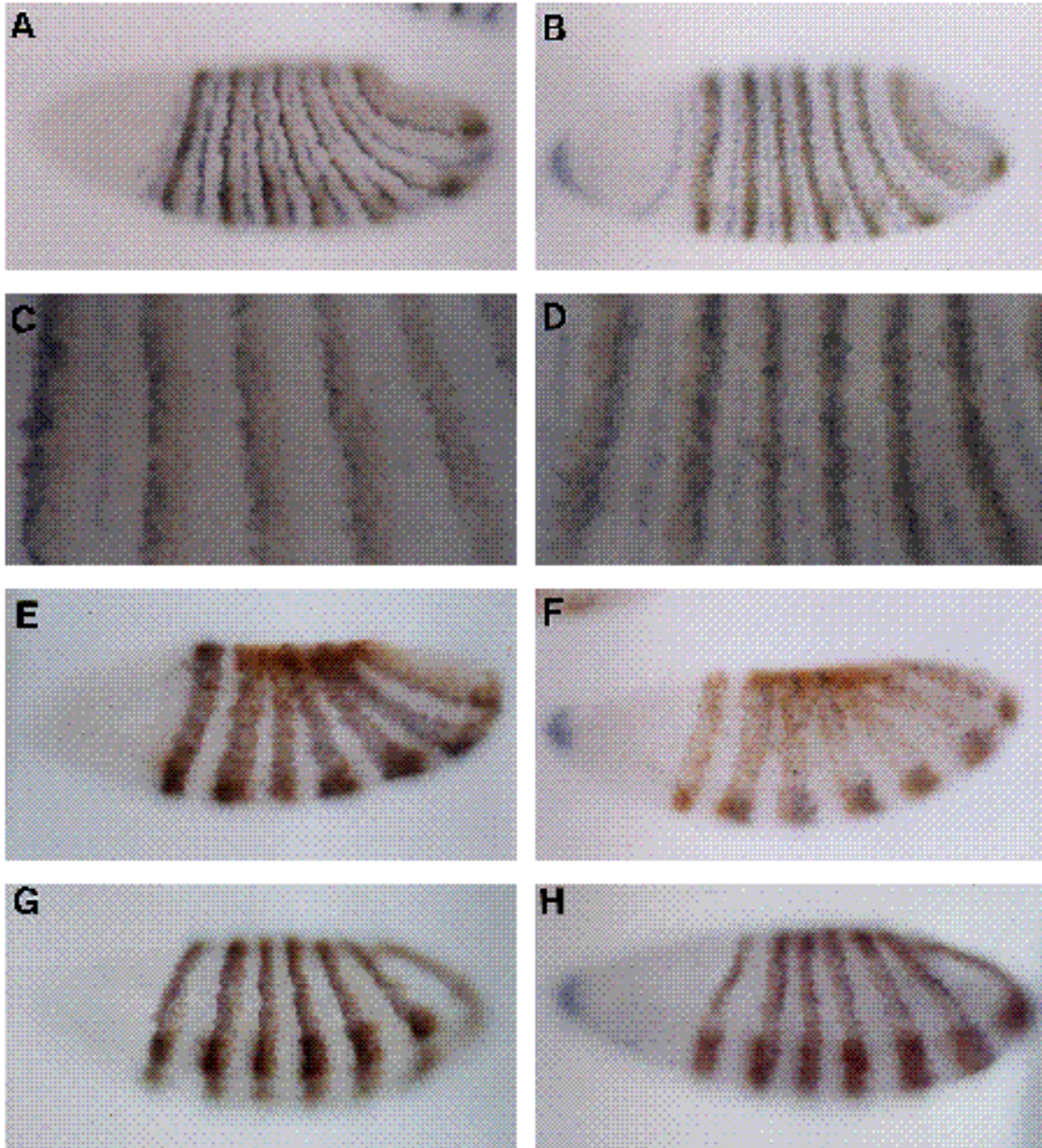
Fig. 6. Differential regulation of *ftz* and *odd* in class 1 and 2 embryos and the generation of class 1 and 2 *en* expression patterns. Wild-type (A, B, C, D) and HSR (E, F, G, H) embryos were double labeled either for *ftz* protein (brown) and *en* transcripts (blue), shown in the left-hand panels (A, C, E, G) or *ftz* protein (brown) and *odd* transcripts (blue), shown in the right-hand panels (B, D, F, H). All embryos shown are approximately 3.5 hours AEL in age. C and D are at higher magnification. Note that *en* expression consistently appears where *ftz* protein is present and *odd* transcripts are absent.

h (A. S. M. and H. M. K., data not shown; C. Tsai and J. P. Gergen, pers. comm.). One possible explanation for this apparent discrepency is that Run may act, not only to repress *h* expression, but also to interfere at some level with *h* protein function such that it becomes incapable of repressing the *ftz* gene. Alternatively, the relative levels or activities of Run in these embryos may simply be higher than those of Hairy.

The activation of *ftz* that we observed in HSR embryos (30-45 minutes AHS) correlated closely with the prior loss of *eve* expression (15-30 minutes AHS). We have previously shown that Eve appears to be a direct repressor of *ftz* (Manoukian and Krause, 1992). Indeed, double labeling of Eve and *ftz* in HSR embryos showed that *ftz* transcripts expanded as Eve disappeared (data not shown). Thus, for the expansion of *ftz* in HSR embryos, repression of *eve* appeared to be more important than repression of *h*. In *run⁻* embryos, the expansion of *h* expression (Ingham and Gergen, 1988)
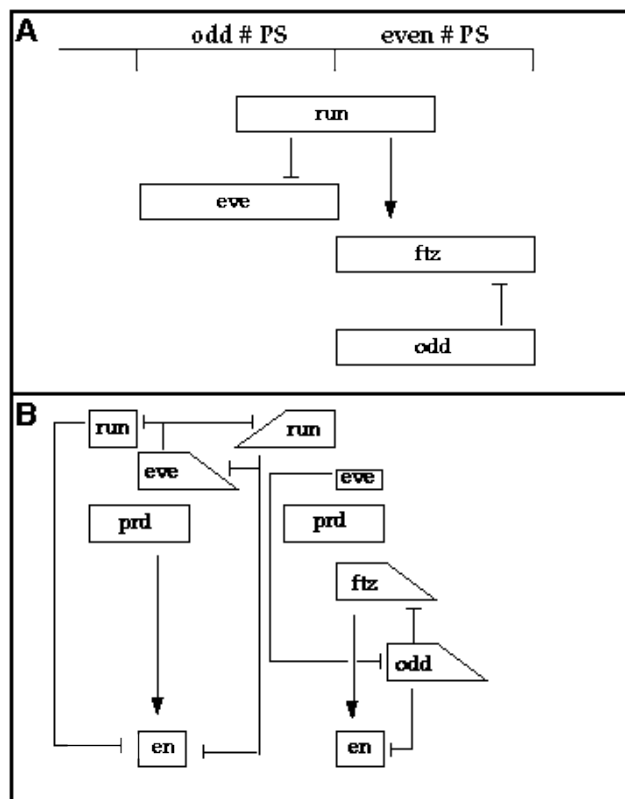
Fig. 7. Summary of expression patterns and regulatory relationships of segmentation genes during wild-type *Drosophila* development. (A) Expression patterns and regulatory interactions that occur at the onset of cellular blastoderm (2:30-2:50 AEL); (B) relative expression patterns and regulatory relationships during early gastrulation (3:10-3:30 AEL). The interval shown in each panel corresponds to two parasegments. Arrows indicate positive regulatory interactions whereas T-shaped arrows indicate negative regulatory interactions. Note the number of interactions that are negative. In B, 14 stripe patterns of *run* and *odd* are depicted. This pattern of *odd* expression can be seen in Fig. 6, and the pattern shown for *run* stems from our unpublished data.

may have a more significant role due to the total absence of Run. It should be noted that, although these and previous experiments indicate that Run activates *ftz* indirectly by repressing repressors, the possibility exists that Run may also act simultaneously as a direct activator of *ftz*.

The role that Run plays in the activation of *ftz* may also be viewed as one of establishing polarity. Normally, *ftz* stripes narrow at their posterior edges, beginning at gastrulation. In *run⁻* embryos, premature loss of *ftz* expression begins at about the same time, but is no longer limited to the posterior domains of expression (Ingham and Gergen, 1988). This loss of *ftz* expression at the time that *en* stripes normally initiate is a likely cause for the loss of polarity within even-numbered parasegments.

As shown in Fig. 7, an additional seven stripes of *run* expression, which have not been previously reported, were observed in the posterior regions of the even-numbered parasegments, beginning at gastrulation. The role of *run* in these expression domains has not been tested. However, the ability of ectopic Run to repress *eve* and odd-numbered *en*

stripes suggests these stripes of *run* may be required to prevent *eve* and *en* expression in these regions.

Level of action of the *run* gene product

The *run* gene encodes a nuclear protein which, unlike the other cloned pair-rule genes, does not appear to contain a recognizable DNA-binding domain (Kania et al., 1990). This raises obvious questions regarding the level of Run action. This question is all the more intriguing since *run* bears a high degree of homology to the human gene *AML1,* translocations of which are associated with a specific subtype of acute myeloid leukemia (Daga et al., 1992).

Studies with promoter-*lacZ* reporter genes indicate that most of the pair-rule and segment polarity gene expression patterns are regulated at the level of transcript initiation. This, and the fact that all of the other pair-rule genes appear to encode DNA-binding transcription factors, suggests that Run should act on its target genes at the level of transcript initiation. However, if Run cannot bind directly to DNA, a possibility suggested by the lack of a recognizable DNA-binding domain, then it would have to do so through actions upon, or interactions with, other DNA-binding proteins. Interestingly, Run contains a putative ATP-binding domain (Kania et al., 1990) which could be involved in modulating such interactions.

The ability of ectopic Run to repress expression of odd-numbered *en* stripes and not the even-numbered *en* stripes, is consistent with Run acting at the level of transcript initiation. A single form of *en* transcript has been identified, making it unlikely that Run could differentially alter the processing of this transcript in one set of parasegments and not another. Hence, we suggest that Run interacts with odd-numbered *en* stripe enhancer elements, either directly or through interactions with other DNA-binding transcription factors.

Similar interactions may be involved between Run and regulatory elements or regulators of the *eve* gene. As with *en*, Run did not repress all *eve* stripes equally, consistent with Run acting differentially upon different stripe-specific enhancers. The regulatory elements and factors that cue the expression of *h* stripe 1 may also interact directly with Run, since this stripe also responded rapidly and dramatically to ectopic Run.

Phenotypes generated by ectopic Run

The ability of ectopic Run to alter the expression patterns of both pair-rule and segment polarity target genes resulted in two different phenotypes (class 1 and class 2), each during a different 20-30 minute interval of development. Strong class 1 phenotypes appear to be reciprocals of the *run* amorphic phenotype (Gergen and Wieschaus, 1985). Both involve mirror-image duplications of non-deleted regions to replace regions that are deleted. In class 2 mutants, there were no duplications of the non-deleted regions.

The phenotype of class 1 embryos is similar to those of *eve*/*odd* double mutants (Coulter and Wieschuas, 1988) and the class 2 pattern generated by HSEve embryos (Manoukian and Krause, 1992). All three phenotypes appear to arise similarly, due to loss of *eve* and *odd* expression coupled with a posterior expansion of *ftz* stripes. This leads to the formation of ectopic *en* stripes in the posterior regions

of even-numbered parasegments and loss of *en* stripes in the odd-numbered parasegments (refer to Fig. 5). In *run⁻* embryos, a phenotype that is opposite to this probably occurs due to the loss of *ftz* and *run* expression and a posterior expansion of *eve* stripes. A key aspect of both phenotypes is the expansion of alternate *wg* stripes. These expanded stripes must contact the ectopic *en* stripes in order for mirror-image duplications to occur.

In class 2 HSR embryos, the *eve*-dependent *en* stripes were also lost, but ectopic *en* stripes failed to initiate at the posterior edges of the *ftz* stripes. Hence, no duplications occurred and deletions encompassed the posterior regions of *ftz*-dependent parasegments and the anterior regions of *eve*-dependent parasegments. Phenotypes that are similar to the class 2 phenotype are generated by *eve* hypomorphs, HSF embryos (Struhl,1985; Ish-Horowicz et al., 1989), class 3 HSEve embryos (Manoukian and Krause, 1992) and with extra copies of the *run* gene (Gergen and Wieschaus, 1986). Each of these phenotypes is likely due to limited expansion of the *ftz* domains at the expense of the *eve* domains.

en regulation is largely controlled by repressors

Lawrence et al. (1987) previously noted that *en* initiation coincides with Ftz and Eve stripe narrowing. The lack of *en* expression in Eve and Ftz-expressing cells prior to this time, and the limitation of *en* stripes to the anteriormost row of cells in each Eve and Ftz stripe, suggested that *en* initiation may be dependent upon high levels of protein and sharp stripe boundaries. Alternatively, DiNardo and O'Farrell (1987) provided evidence to support the combinatorial model (Gergen and Wieschaus, 1985), suggesting that odd-numbered *en* stripes are activated by the combination of *eve* and *prd*, and that even-numbered *en* stripes are activated by the combination of *ftz* and the pair-rule gene *odd-paired*. However, neither model in its present state explains the timing and pattern of *en* stripe initiation.

Here we show that much of *en* stripe regulation can be explained by negative regulatory interactions (summarized in Fig. 7). We suggest that the *run*, *eve* and *odd* gene products all act as repressors. Of the genes pursued in this study, this leaves only *prd* and *ftz* as genes that may encode direct activators of *en*. Our data indicate that the activity of these activators is spatially restricted by the products of the *run* and *odd* genes. Wherever Run overlaps with Prd, or Odd with Ftz, *en* expression fails to occur. Thus, the actions of the two repressors, Run and Odd, are dominant over those of the activators Prd and Ftz.

The epistatic nature of these repressors requires a mechanism to restrict their expression from cells fated to express *en*. We have previously suggested that this is the function of Eve (Manoukian and Krause, 1992). At gastrulation, Eve exists in a pattern of 14 stripes. We suggest that the seven strong Eve stripes in the anterior regions of odd-numbered parasegments are required to repress Run, while the seven weak Eve stripes in the anterior regions of even-numbered parasegments are required to repress *odd* (Manoukian and Krause, 1992). Thus, Eve appears to have a net positive role in the expression of all 14 *en* stripes, despite acting as a repressor. This would explain the loss of all 14 *en* stripes that is observed in *eve* null mutant embryos and the reappearance of even-numbered *en* stripes in

eve/*odd* double mutant embryos (DiNardo and O'Farrell, 1987). Similarly, odd-numbered *en* stripes might reappear in *eve*/*run* double mutant embryos, unless the loss of *eve* leads to ectopic expression of *en* repressors such as *odd* or loss of *en* activators such as *prd*.

In summary, we find that much of *en* expression is controlled by negative and double negative gene interactions. Our results, however still do not fully explain the initiating pattern of *en* expression. The action of at least one more gene must be invoked to explain why odd-numbered *en* stripes appear long after Eve and Prd stripe borders are sharpened and favorable overlaps have formed. The factor encoded by this gene may either be a coactivator that appears later or yet another repressor that must be removed.

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