Concentration-dependent activities of the *even-skipped* protein in *Drosophila* embryos

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The *Drosophila* pair-rule gene *even-skipped* (*eve*) encodes a homeo-domain-containing protein (Eve) that is required for the development of both odd- and even-numbered parasegments. We have used a heat shock-inducible *eve* transgene to study the regulatory functions of Eve in vivo. Transcripts encoded by eight other segmentation genes were monitored for changes in distribution and abundance following short pulses of ectopic Eve expression. Two tiers of response times appeared to distinguish between genes that were direct [*fushi tarazu* (*ftz*), *odd-skipped* (*odd*), *runt* (*run*), *paired*, and *wingless*] and indirect [*eve*, *hairy*, and *engrailed* (*en*)] targets of Eve. Genes that appeared to be directly regulated by Eve were differentially repressed in a concentration-dependent fashion. Interestingly, the *run* and *ftz* genes could also be activated by Eve during a brief 20- to 30-min stage in development. The delayed actions upon the *eve* and *en* genes appeared to be mediated by *run* and *odd*. As in *eve*⁻ embryos, these effects on segmentation gene expression patterns caused defects in both odd- and even-numbered parasegments. Four sequential phenotypes could be induced, each of which was attributable to the altered expression of a unique subset of target genes.

[Key Words: *even-skipped*; segmentation; pair-rule genes; segment polarity genes]

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Segmentation in *Drosophila* is embryonically controlled by a hierarchy of interactions among several classes of genes (Nüsslein-Volhard and Wieschaus 1980; Nüsslein-Volhard et al. 1985). Information is relayed in a temporal progression from the coordinate genes to the gap genes, then from the gap genes to the pair-rule genes and finally, from the pair-rule genes to the segment polarity genes (for review, see Ingham 1988). The *even-skipped* (*eve*) gene is considered to be a member of the pair-rule genes (Nüsslein-Volhard and Wieschaus 1980). Weak *eve* mutations (hypomorphs) fit the pair-rule gene criteria, causing deletions of alternate segment-wide regions (Nüsslein-Volhard and Wieschaus 1980). Unlike the other pair-rule genes, however, *eve* null alleles completely abolish segmentation within the trunk of the embryo (Nüsslein-Volhard et al. 1985). Thus, *eve* appears to be a particularly important member of the pair-rule class of genes.

In correspondence to the severity of the *eve* null phenotype, *eve* is expressed in a dynamic fashion throughout the trunk of the embryo (Frasch et al. 1987). Low levels of uniformly distributed *eve* protein (Eve) resolve into an anterior-to-posterior gradient which, in turn, resolves into a 7-stripe pattern of expression, followed by a 14-stripe pattern of expression. Eve performs both early and late functions during this 2- to 3-hr period (Goto et al. 1989). During the time that Eve is expressed in seven

stripes, it functions as a primary pair-rule gene, interpreting spatial cues provided by the gap genes and relaying this information to the other pair-rule genes (for review, see Pankratz and Jäckle 1990). Eve expression at this time defines the odd-numbered parasegmental primordia (Lawrence et al. 1987). As the Eve stripes begin to resolve, Eve is required for proper initiation of the segment polarity genes *engrailed* (*en*) (DiNardo and O'Farrell 1987; Lawrence et al. 1987) and *wingless* (*wg*) (Ingham et al. 1988) in odd-numbered parasegments. These two genes define anterior and posterior parasegmental identities, respectively.

An important issue that remains to be resolved is whether gene interactions such as these are direct or indirect. For example, is Eve a direct activator of the *en* gene, or does it regulate an intermediary gene whose product regulates *en*? Eve contains a DNA-binding homeo domain (MacDonald et al. 1986; Hoey and Levine 1988) and functions as a sequence-specific transcriptional repressor in transfected tissue-culture cells (Han et al. 1989) and in transcriptionally competent extracts (Biggin and Tjian 1989). Although Eve appears to function exclusively as a repressor in vitro, expression patterns of several genes in wild-type and mutant embryos (see below) suggest that Eve may function as both a repressor and an activator in vivo.

We wished to distinguish between direct and indirect

targets of Eve, and to determine whether Eve acts as a transcriptional repressor, an activator, or both. We have addressed these questions by providing short pulses of ectopic Eve expression at different stages of embryogenesis and monitoring potential Eve target genes for changes in their patterns of expression. Among the genes that we tested, those that might be direct targets of Eve repression include the pair-rule genes *fushi-tarazu* (*ftz*) (Carroll and Scott 1986; Hiromi and Gehring 1987), *odd-skipped* (*odd*) (DiNardo and O'Farrell 1987; Coulter and Wieschaus 1988), *runt* (*run*) (Ingham and Gergen 1988), and *paired* (*prd*) (Baumgartner and Noll 1991), and the segment polarity gene *wg* (Ingham et al. 1988). Genes postulated to be directly activated by Eve include the *eve* gene itself (Frasch et al. 1988), the pair-rule gene *hairy* (*h*) (Ingham and Gergen 1988), and the segment polarity gene *en* (DiNardo and O'Farrell 1987).

The results of this study indicate that only a subset of these genes are direct targets of Eve regulatory activities. In general, our data suggest that Eve acts as a transcriptional repressor, except during its earliest stages of expression. By regulating different target genes at different developmental stages, ectopic Eve could induce four different mutant phenotypes. On the basis of these results, we suggest that Eve functions as a concentration-dependent morphogen, with sequential regulatory roles in both the odd- and even-numbered parasegments.

Results

Expression of *eve* in HSEVE embryos

Fly lines containing P-element-mediated insertions of a heat shock promoter (*hsp70*)-controlled *eve* gene (PHSEVE) were obtained from Gary Struhl (Columbia University; see Materials and methods). Two PHSEVE-transformed lines were used: one with the PHSEVE construct inserted into the second chromosome (HSEVE³²), and the other with an insert in the third chromosome (HSEVE^{19B}). Both inserts gave equivalent results in a va-

riety of different genetic backgrounds. Our first goal was to test these lines for heat shock-inducible Eve expression. Figure 1B shows that 30 min after a short 4-min heat shock, Eve was immunologically detected in all nuclei of HSEVE embryos. Note that the underlying pattern of seven stripes was still visible, indicating that the levels of ectopic Eve were probably similar to the levels of endogenous Eve.

It has been suggested that Eve plays a direct role in the activation of its own promoter (Jiang et al. 1991). To test whether ectopic Eve could activate the endogenous *eve* gene, we looked at *eve* mRNA expression after administering 3- to 4-min heat shock pulses to embryos aged between 2 and 3 hr after egg laying (AEL). Figure 1D shows an embryo fixed 15 min after the initiation of heat shock. At this time, the ectopically induced *eve* transcripts that had been distributed evenly over most of the embryo surface were already on the decline. In embryos fixed 30 min after heat shock, the heat shock-induced *eve* transcripts were no longer detectable (Fig. 1E). Transcripts of the endogenous *eve* gene, however, continued to be expressed in a relatively normal seven-stripe pattern of expression. To our surprise, in similarly staged embryos (2.5–3 hr AEL) that had been heat-shocked 45 min before fixation, *eve* transcripts were very weak or undetectable (Fig. 1F). This autorepression was not observed if embryos were older than 2.5 hr AEL at the time of heat shock. The 45-min delay before the loss of endogenous *eve* expression suggests that this repression was indirect (see below for further discussion). Thus, not only was ectopic Eve incapable of activating the endogenous *eve* gene outside of its normal domains of expression, but it caused a premature loss of expression within the domains in which it is normally expressed.

In this experiment and the experiments that follow, the effects described were Eve specific because extended heat shocks (up to 10 min) had no effects on wild-type embryos (data not shown), and the effects of other heat shock-inducible pair-rule genes varied, depending on the

Figure 1. *eve* expression in HSEVE embryos. Embryos aged 2–3 hr AEL were heat shocked for 4 min and stained for *eve* protein (A,B) or *eve* transcripts (C–F). (A,C) Wild-type patterns of protein and mRNA, respectively. Twenty minutes after the initiation of heat shock, Eve was detected in all nuclei, with the underlying pattern of seven stripes still visible (B). (D–F) *eve* transcripts at 15 (D), 30 (E), and 45 (F) min after the initiation of heat shock. The embryos shown were all fixed at a similar developmental stage (~2.5–3 hr AEL).

particular pair-rule gene that was induced (A.S. Manoukian and H.M. Krause, unpubl.).

Repression of ftz by ectopic Eve

By use of heat shock-inducible transgenes, it is possible to vary the levels of ectopic gene expression by altering the parameters of heat shock. This permits an assessment of expression levels that are functionally relevant. One of the suspected targets of Eve is the *ftz* gene. Figure 2A shows the correlation between the levels of ectopic Eve induced by different durations of heat shock and the subsequent effects on *ftz* gene expression. Embryos aged 2.5 to 3 hr AEL at the time of heat shock were fixed 30 min later and double-stained for Eve protein (brown) and *ftz* transcripts (blue). Consistent with the hypothesis that Eve is a repressor of the *ftz* gene (Carroll and Scott 1986; Frasch and Levine 1987; Hiromi and Gehring 1987; Lawrence and Johnston 1989), stripes of *ftz* expression diminished in intensity and width as the abundance of ectopic Eve increased. Total repression of *ftz* transcription occurred when heat shocks were ~4 min or longer.

To address the question of whether Eve is a direct or indirect regulator of *ftz* gene expression, we monitored the levels of Eve and *ftz* transcripts over the course of an hour following a 4-min heat shock. A very short temporal delay between the rise in levels of Eve and the subsequent loss of *ftz* transcripts would favor a direct interaction between the two genes. Embryos aliquoted from a common pool of heat-shocked embryos were fixed at 5- to 10-min intervals following heat shock. The levels of Eve and *ftz* transcripts in equivalent numbers of embryos were quantitated with the aid of secondary antibodies coupled to alkaline phosphatase (AP) and by monitoring AP activity colorimetrically.

Figure 2B shows that ectopic Eve expression could first be detected at 5- to 10-min and peaked at ~25 min, after the initiation of heat shock. At their peak, the levels of ectopic Eve were approximately three to four times the levels of Eve detected in non-heat-shocked embryos (or in heat-shocked wild-type embryos; not shown). If *eve* stripes normally occupy 20–30% of the surface of a blastoderm embryo (where the majority of nuclei are localized), this overall increase in Eve abundance by three- to fourfold should bring the interstripe levels of protein close to the levels that are expressed in the endogenous stripes. This is consistent with the levels of Eve staining in the HSEVE and wild-type embryos shown in Figure 1 (cf. A and B).

A decrease in the abundance of *ftz* transcripts in heat-shocked embryos was first detected ~15 min after the beginning of heat shock and was essentially complete within 30 min of the initiation of heat shock (Fig. 2C). The estimated half-life of *ftz* transcripts (6 min; Edgar et al. 1986) is consistent with the rapid degradation profile of *ftz* transcripts in HSEVE embryos depicted in Figure 2C. If Eve is a direct repressor of *ftz* transcription, then *ftz* transcripts should be reduced in abundance by 50%, ~6 min after Eve reaches sufficient levels to repress the

ftz gene completely. In Figure 2C, it can be seen that *ftz* expression was reduced by 50% ~23 min after the initiation of heat shock and that 6 min earlier (17 min after the initiation of heat shock), Eve was approximately two to three times the normal levels of endogenous Eve protein. As discussed above, these levels of ectopic Eve are probably similar to or less than the levels of Eve that are normally confined to the seven stripes. Hence, these results favor the hypothesis that Eve is a direct regulator of the *ftz* gene. Because the expression patterns of *ftz* promoter-*lacZ* fusion genes were also repressed within a similar time frame (data not shown), this negative regulation by Eve is likely to be mediated by regulatory elements located upstream of the *ftz*-coding region.

Ectopic Eve acts in a concentration-dependent fashion

To determine whether other pair-rule genes would also respond to ectopic Eve within the same time frame as *ftz*, and whether Eve would also act as a repressor of these genes, we performed whole-mount in situ hybridizations using sequences from the genes *h*, *run*, *prd*, *ftz*, and *odd* as probes (Fig. 3). The embryos shown were heat-shocked in a single batch for 3 min and fixed 30 min later. The only genes that were obviously affected within the 30-min recovery period, and with this duration of heat shock, were the genes *ftz*, *run*, and *odd*. All three genes were repressed but with different levels of efficiency. This dosage-dependent variation in sensitivity was also apparent when heat shocks were varied in duration (not shown). For example, heat shock durations of only 2 min did not affect *ftz* and *run* expression but were still sufficient to repress *odd*. When the duration of heat shocks was increased to 4 min or longer, all three genes were completely repressed. Four-minute inductions also repressed the *prd* gene within the same 15- to 30-min recovery period (determined by visual examination only). In contrast, the *h* and endogenous *eve* genes were not affected within this 30-min period, even when heat shock pulses were as long as 10 min. These results suggest that Eve is a direct regulator of *ftz*, *run*, *odd*, and *prd* and that each of these genes is differentially repressed by different levels of EVE. The 30- to 45-min delay in the response time of the *h* and endogenous *eve* genes suggests that they are not direct targets of Eve.

Differential repression of ftz and odd alters en expression

We wished to determine the consequences of the ability of Eve to repress *odd* at levels that did not repress *ftz*. This was tested by double-labeling for Ftz (brown) and either *odd* or *en* transcripts (blue). Before gastrulation, we found that Ftz and *odd* stripes were completely overlapping (not shown). At gastrulation, the Ftz stripes were one to two cells wider than the *odd* stripes (Fig. 4A). Cells that expressed Ftz and not *odd* were located where the *ftz*-dependent *en* stripes initiated (Fig. 4B). The cells that express *ftz* and *en* also express low levels of Eve (Frasch et al. 1987). Taken together, these observations

Figure 2. Effects of increasing Eve expression on *ftz*. (A) Embryos shown from *top* to *bottom* were heat shocked at 2.5–3 hr AEL for 0, 2, 3.5, and 5 min, respectively, and fixed 30 min later. In the non-heat-shocked embryo (*top*), the domains of Eve (brown) and *ftz* mRNA (blue) were mutually exclusive. In the heat-shocked embryos, *ftz* stripes narrowed and disappeared as the levels of Eve expression increased. (B,C) Embryos were aliquoted following a 4-min heat shock at 36°C and fixed at 5-min intervals. In half of the embryos (B) Eve was detected immunologically; in the other half (C), *ftz* transcripts were detected by whole-mount in situ hybridization. Signals were quantitated as described. Levels of AP activity are indicated (y-axes) for embryos fixed at the times shown (x-axes). (□) Values obtained with non-heat-shocked embryos; (♦) values obtained with heat-shocked embryos. These relative values were determined by dividing all A_{405} values by the average of A_{405} levels detected in non-heat-shocked embryos. The time after heat shock when *ftz* transcripts were diminished by 50% is indicated by a dotted line in B, and the abundance of Eve 6 min earlier is indicated similarly in A. Error bars indicate the variability in values obtained in two separate experiments.

Figure 3. Dosage-dependent repression of pair-rule genes in HSEVE embryos. Whole-mount in situ hybridizations were carried out with digoxigenin-labeled probes to detect wild-type (A,C,E,G,I) and HSEVE (B,D,F,H,J) pair-rule gene expression patterns: *h* (A,B); *prd* (C,D); *ftz* (E,F); *run* (G,H); and *odd* (I,J). The HSEVE embryos (right) were heat-shocked together for 3 min at 36°C between 2.5 and 3 hr AEL, before dividing them up for hybridization with the different probes. Note the difference in sensitivities relative to the wild-type expression patterns shown at left.

suggest the possibility that *ftz* encodes an activator of *en*, that *odd* encodes a negative regulator of *en*, and that low levels of Eve might act to repress *odd* so that *en* can be activated by Ftz.

To test this hypothesis, we attempted to induce levels of ectopic Eve that could repress *odd* without repressing Ftz, with the expectation that *en* might be activated in all Ftz-expressing cells. When HSEVE embryos were heat-shocked at ~2.5 hr AEL for 2–3 min and fixed 30 min later, the *odd* stripes that overlap with Ftz were repressed (Fig. 4C). Although some Ftz stripes changed in width, their levels of expression were close to normal. Consequently, embryos fixed 15 min later expressed *en* in all of the *ftz*-expressing cells rather than at the anterior edges only (Fig. 4D). Hence, these data are consistent with *ftz* encoding an activator of *en*, *odd* encoding a negative regulator of *en*, and low levels of Eve contributing to Ftz-dependent *en* activation through repression of *odd*. These results illustrate the importance of Eve concentration levels in wild-type embryos. Furthermore, they suggest that Eve may not only play an important role in the odd-numbered parasegments but in the even-numbered parasegments as well.

Eve activity varies during different stages of embryogenesis

The results presented above were obtained with embryos that were heat-shocked between 2.5 and 3 hr AEL (at cellular blastoderm). Because *eve* expression begins well before this and continues for 1–2 hr afterward, we wished to determine whether the regulatory properties of ectopic Eve would vary if induced earlier or later. Figure 5 shows the effects of ectopic Eve on the pair-rule genes *h*, *odd*, and *run* when 4-min heat shocks were administered either between 2 and 2.5 hr AEL (beginning of cellularization) or between 2.75 and 3.25 hr AEL (beginning of gastrulation). In each case, embryos were fixed 30 min after the initiation of heat shock to select for primary effects.

Not surprisingly, there was no effect on *h* expression at either time, and expression of the *odd* gene was repressed with equal efficiency during both the early and late time intervals. In contrast, whereas the *run* gene was repressed with late heat shocks, it was ectopically activated with early heat shocks. This ectopic activation during early embryogenesis was also observed with *ftz*, although it was not as extensive and was restricted to a shorter time interval (data not shown). Because this early activation was complete within the same time interval that *ftz*, *run*, *odd*, and *prd* had been repressed by later heat shocks, we also classified it as a primary response. This suggests that Eve may be capable of acting as both a repressor and an activator, depending on the stage of embryogenesis and the gene being regulated.

Effects of ectopic Eve on *en* and *wg* expression

Initiation of expression of the segment polarity genes *en* and *wg* begins at the time that *eve* changes from a 7- to a 14-stripe pattern of expression (~3–3.5 hr AEL). The 14 *en* stripes overlap with the 14 *eve* stripes, and *wg* stripes appear as the seven wide *eve* stripes begin to narrow. The nature of these overlaps, taken together with the loss of all *en* stripes and the widening of *wg* stripes in *eve* amorphs, suggests that *eve* may be a positive regulator of *en* and a negative regulator of *wg*. We induced ectopic Eve expression at the time that *en* and *wg* expression are normally initiated to address the question of whether these interactions are direct or indirect. Figure 6 shows embryos stained for either *en* or *wg* transcripts 30 min after the initiation of a 4-min heat shock. No effect was observed on the initiating pattern of *en* expression in the presence of ectopic Eve. In contrast, repression of *wg* transcripts was observed as early as 15 min and was complete within 30 min of heat shock, even with heat shock pulses as short as 2 min. These criteria suggest that Eve is a direct and potent repressor of *wg* expression and that it is not a direct regulator of *en*.

Ectopic Eve generates four mutant phenotypes

Changes in the patterns of *en* expression were only observed with recovery times in excess of 30 min. These

Figure 4. Differential repression of *ftz* and *odd* is important for *en* expression. Embryos were double-stained for *ftz* protein (Ftz) (brown) and either *odd* (A,C) or *en* (B,D) transcripts (blue). (A,B) Wild-type embryos fixed at 3.25 and 3.5 hr AEL; (C,D) similarly staged HSEVE embryos fixed 45 and 60 min after heat shock. Note that the *odd* transcripts (blue) that overlap with Ftz in A, are absent in C. Consequently, *en* transcripts that partially overlap with Ftz stripes in B are completely overlapping with Ftz in D.

delayed changes in *en* expression patterns varied, depending on the timing of ectopic Eve induction. Four different patterns were observed when heat shocks were administered between 2 and 3.5 hr AEL. Heat shocks within this 1.5-hr interval also generated four different segmental phenotypes that were evident in preparations of mature embryo cuticles. Figure 7 shows each of the four *en* expression patterns and the corresponding cuticular phenotypes.

The embryos shown in the left-hand panels of Figure 7 (A–E) were double stained for *en* protein (En) and *wg* transcripts at 5.5–6 hr AEL. At this stage, the patterns of En (brown) and *wg* (blue) are indicative of the cuticular phenotypes that will ensue. The patterns shown are also indicative of the early patterns of En and *wg* that we observed at 3.5 hr AEL (data not shown). In wild-type embryos, 14 evenly spaced stripes of En can be seen, each with a stripe of *wg* immediately in front (Fig. 7A). Each En/*wg* interface represents a parasegmental boundary. The odd-numbered En stripes mark the anterior boundaries of the “*eve*-dependent” (odd-numbered) parasegments, and the even-numbered En stripes mark the anterior boundaries of the “*ftz*-dependent” (even-numbered) parasegments.

When embryos were heat-shocked between 2 hr and 2 hr and 20 min AEL, the odd-numbered En stripes shifted posteriorly (Fig. 7B). Closely juxtaposed En stripes

tended to fuse together, forming a single stripe of En with a single stripe of *wg* in front (e.g., stripe 3/4 in Fig. 7B). This fusion of En stripes was observed more frequently with stronger heat shocks (>3 min) and in embryos fixed at later stages of development. The regions that were deleted by these fusions corresponded to the odd-numbered (*eve*-dependent) parasegments. This is in agreement with the pair-rule cuticular phenotype shown in Figure 7G. The regions still remaining in this cuticle correspond to the even-numbered (*ftz*-dependent) parasegments.

Heat shock pulses of 2–3 min administered at 2 hr and 20 to 2 hr and 40 min AEL generated the novel pattern of En and *wg* stripes shown in Figure 7C. Seven wide En stripes were flanked on both sides by stripes of *wg* expression. This pattern resulted from widening of the even-numbered (*ftz*-dependent) En stripes, as shown previously in Figure 4, and loss of the odd-numbered En stripes. Seven wide stripes of *wg* appeared between the widened stripes of En ~1 hr after heat shock (not shown) and then split, leaving two narrow stripes adjacent to the En stripes on either side (Fig. 7C). This generated a pattern of mirror-image symmetries in which lines of symmetry passed, either down the center of each En stripe or midway between each En stripe. With stronger heat shocks, all En stripes narrowed and then disappeared, with the narrowest stripes disappearing first.

Figure 5. Eve regulatory activities vary at different stages of embryogenesis. Embryos aged at either 2.5 (A–F) or 2.75–3.25 (G–L) hr AEL were heat shocked for 3 min and fixed 30 min after the initiation of heat shock. Embryos in the *top* row show in situ detection of *h* transcripts (A,B,G,H); embryos in the *middle* row were probed for *odd* transcripts (C,D,I,J); and embryos in the *bottom* row were probed for *run* transcripts (E,F,K,L). Wild-type expression patterns (A,C,E,G,I,K) are shown to the *left* of each heat-shocked embryo (B,D,F,H,J,L).

The corresponding cuticular phenotype (Fig. 7H) also exhibited patterns of mirror-image symmetry. With weak heat shocks, the odd-numbered abdominal denticle belts were replaced by mirror-image duplications of the first one or two rows of denticle belt bristles, and the even-numbered denticle belts were replaced by duplications of one or two rows of posterior denticle belt bristles. Stronger heat shocks caused further deletions of first the anterior and then the posterior denticle belt duplications, leaving only naked cuticle (not shown).

The third pattern of *En* and *wg* (Fig. 7D) was much like the 2-hr to 2-hr and 20-min AEL pattern. Heat shocks administered between 2 hr and 40 min and 2 hr and 50 min AEL also caused pairing and fusion of *En* stripes. However, in this case, fusions were between *En* stripes 2 and 3, 4 and 5, etc. Whereas the odd-numbered *En* stripes appeared to shift posteriorly in the early pattern, they

appeared to shift anteriorly in this pattern. As might be expected, a pair-rule cuticular phenotype, which was the opposite of the 2-hr to 2-hr and 20-min phenotype, resulted (Fig. 7I). In this case, the even-numbered (*ftz*-dependent) parasegments were deleted.

The fourth mutant phenotype occurred when heat shocks were administered at 2 hr and 50 min to 3 hr and 20 min AEL. In contrast to the earlier heat shocks, *wg* expression generally failed to initiate and did not reappear at later stages of embryogenesis (Fig. 7E). This repression of *wg* was probably not a factor with earlier heat shocks owing to the removal of ectopic *Eve* before the time that *wg* expression was normally initiated. Although *En* stripes initiated normally with heat shock pulses at this time, they began to disappear shortly afterward. This gave rise to a cuticular phenotype (Fig. 7J), which appeared to be the opposite of the 2-hr and 20-min

Figure 6. Transcription of the segment polarity genes *en* and *wg* in wild-type and HSEVE embryos. The expression patterns of *en* (A) and *wg* (C) in wild-type embryos at the onset of expression are highlighted by 14-stripe transcription patterns. The *en*-expressing embryos shown are slightly older than the *wg*-expressing embryos because *en* expression is initiated shortly after *wg*. In HSEVE embryos, which were fixed 30 min after the initiation of a 4-min heat shock, *en* expression was unaffected (B) while *wg* expression was repressed (D).

Figure 7. Effects of ectopic Eve on segmental patterning. Embryos were double-stained for *en* protein (En), and *wg* transcripts in HSEVE embryos were fixed at 5.5–6 hr AEL (A–E). The different En/*wg* patterns are as follows: (A) Wild-type En/*wg* expression; (B) embryo heat-shocked for 4 min at 2 hr to 2 hr and 20 min AEL; (C) embryo heat-shocked for 3 min at 2 hr and 20 min to 2 hr and 40 min AEL; (D) embryo heat-shocked for 3 min at 2 hr and 40 min to 2 hr and 50 min AEL; (E) embryo heat-shocked for 3 min at 2 hr and 50 min to 3 hr and 10 min AEL. Corresponding cuticle patterns (F–J) are shown for larvae that received heat shocks at the same stages as the embryos shown at *left*: No heat shock (F); heat shock between 2 hr and 2 hr and 20 min AEL (G); 2 hr and 20 min and 2 hr and 40 min (H); 2 hr and 40 min and 2 hr and 50 min (I); and 2 hr and 50 min and 3 hr and 20 min (J). The abdominal denticle belts are numbered. Note that germ band extension was retarded by Eve induction at 2 hr and 20 min to 2 hr and 40 min AEL (C).

to 2-hr and 40-min AEL phenotype; instead of naked cuticle, these embryos yielded lawns of denticles.

Discussion

Null mutations of *eve* disrupt both odd- and even-numbered parasegments. Our results with a heat shock-inducible *eve* transgene indicate that Eve provides multiple regulatory cues in both sets of parasegments and that these functions are sequential. The window of time during which ectopic Eve generated segmental phenotypes—1.5 hr—is considerably longer than the 15- to 30-min window of sensitivity obtained with the pair-rule gene *ftz* (Struhl 1985), and can be subdivided into four phases, each characterized by a specific phenotype. The segmental phenotypes caused by earlier Eve inductions were apparent reciprocals of those generated by later inductions. These phenotypes and their apparent reciprocity can be rationalized in terms of the observed effects on the expression of downstream target genes.

Direct vs. indirect targets of Eve regulatory activities

By providing short pulses of Eve expression and following the levels of potential target gene products, we distinguished two different response times. Changes in the expression patterns of five of the eight genes tested (*ftz*, *run*, *odd*, *prd*, and *wg*) could be detected within 15 min of a short 2- to 4-min heat shock and were complete within 30 min of heat shock. The remaining three genes (*eve*, *h*, and *en*) showed a delayed response between 30 and 45 min of ectopic Eve induction. A quantitative analysis of Eve and *ftz* expression levels following heat shock indicated that the rapid 15- to 30-min response time was consistent with a direct regulatory interaction between the two genes. A similar response time observed with *ftz* promoter-*lacZ* fusion genes indicates that this negative regulation was mediated via sequences located upstream of the *ftz*-coding region. The other four genes that responded within the same 15- to 30-min interval may also be direct targets of Eve, whereas the three genes that responded later are probably not.

These response times may be of general use in distinguishing between other direct and indirect gene interactions, although several caveats must be considered. For example, heat shocks are believed to alter rates of transcription and translation. Interestingly, we did not observe any obvious effects on the expression of genes monitored in this study (even with 15- to 20-min heat shocks). Translation of ectopic *eve* transcripts also appeared to be unaffected by heat shock, as ectopic Eve accumulated at a constant rate, even during heat shocks as long as 1–2 hr (data not shown). We did, however, note short delays in development that were proportional to the duration of heat shock. These were minimal with the 2- to 4-min heat shocks used in this study. Another consideration is the size and half-life of transcripts being monitored. In this study all of the transcripts were similarly sized, and those that have been tested have similar half-lives (Edgar et al. 1989). Another concern is that

some proteins may be active at very low levels of expression. This would shorten the time that is necessary between successive gene interactions.

Regulation of the en and eve genes

The loss of *en* and *eve* transcripts in *eve* mutant embryos had suggested previously that Eve could be a direct activator of these two genes (DiNardo and O'Farrell 1987; Frasch et al. 1988). However, their delayed response to ectopic Eve argues against this possibility. Our results with *en* indicate that Eve may activate it indirectly by repressing other repressors. One candidate is the product of the *odd* gene, because Eve rapidly repressed *odd*, and this repression was followed by activation of *en* in cells that had expressed *odd* previously. This indirect circuitry is also consistent with the observation that half of the 14 *en* stripes that disappear in *eve*[−] embryos reappear in *eve*[−]/*odd*[−] double mutant embryos (DiNardo and O'Farrell 1987). The reappearance of only the even-numbered *en* stripes in *eve*[−]/*odd*[−] embryos suggests that Eve represses other negative regulators of *en* in the odd-numbered parasegments. One of these may be the product of the *run* gene, because ectopic *run* rapidly represses the odd-numbered *en* stripes (first and second phenotypes; A.S. Manoukian and H.M. Krause, in prep.) and Eve was a potent repressor of *run* (after 2.5 hr AEL).

The lack of a direct response to ectopic Eve by the endogenous *eve* gene was more of a surprise. In *eve*[−] embryos, stripes of expression driven by the *eve* promoter fail to resolve normally and are then lost prematurely (Frasch et al. 1988). Jiang et al. (1991) proposed that this regulation was direct because they identified a regulatory element upstream of the *eve* transcription start site that contained Eve-binding sites and required *eve* gene activity. To abolish this *eve*-dependent activity, all Eve-binding sites within a truncated form of the element had to be destroyed. Jiang et al. (1991) favored redundancy of these sites to explain the need to remove them completely. Our data suggest that Eve acts indirectly by regulating the expression of an intermediary gene. The product of this gene may no longer be capable of regulating the extensively mutated element constructed by Jiang and co-workers. As with *en*, *run* is a candidate for such a gene, because *run* appears to be a negative regulator of *eve* (Ingham and Gergen 1988; A.S. Manoukian and H.M. Krause, in prep.) and ectopic Eve was an effective repressor of *run*. Alternatively, the failure of ectopic Eve to activate the endogenous *eve* gene in the interstripe regions might be explained by the absence of a necessary cofactor or by the presence of an overriding repressor.

Eve as a concentration-dependent morphogen

In wild-type embryos, the levels of Eve vary dramatically. Initially, expression is very low and then resolves into an anterior-to-posterior gradient. Following their formation, the seven stripes polarize with the highest levels of expression at the anterior edges. Finally, at the

14-stripe phase, the stripes alternate between weak and strong. Our results indicate that these different levels determine the scope of target gene regulation. Each of the five genes that *Eve* repressed responded to a different level of the ectopic protein: *odd* and *wg* by very low levels of *Eve*, *ftz* and *run* by intermediate levels, and *prd* only by very high levels. The importance of this differential regulation was demonstrated effectively by the relative balance between the levels of *Eve*, *ftz*, and *odd* and the subsequent effects on *en* expression. With low levels of *Eve*, the loss of *odd* allowed expansion of the even-numbered *en* stripes into all *ftz*-expressing cells. At slightly higher levels of *Eve*, *ftz* was also repressed and *en* expression was lost altogether.

Possible roles for *Eve* as a transcriptional activator

In addition to acting as a dosage-dependent repressor, *Eve* had the apparent ability to act as a temporally restricted activator. When *Eve* was induced before cellularization (2 hr to 2 hr and 20 min AEL), *ftz* and *run* were activated ectopically rather than being repressed. The rapid response of these two genes suggests that this activation was direct. Examples of transcription factors that can act as both activators and repressors include the proteins PRTF (pheromone/receptor transcription factor), which regulates yeast haploid cell mating type (Tan and Richmond 1990), and the glucocorticoid receptor (Diamond et al. 1990). In both cases, the basic activity of these proteins could be altered by specific interactions with other proteins. In a similar fashion, *Eve* may sometimes act as a gene-specific transcriptional activator as well as a repressor, perhaps through specific interactions with different stage- and promoter-specific factors.

Changing regulatory roles of the *eve* gene

Each of the four phenotypes generated by ectopic *Eve* is summarized in Figure 8. The similarities of these phenotypes to known pair-rule and segment polarity mutant phenotypes, taken together with changes in the expression patterns of these genes upon *Eve* induction, suggest possible mechanisms for each phenotype. We attribute the reciprocity of the first and third phenotypes to the respective loss of either the *eve*- or *ftz*-dependent parasegments. Loss of the *eve*-dependent parasegments appeared to be caused by indirect repression of the endogenous *eve* gene and the odd-numbered stripes of *en* owing to the ectopic activation of *run*. Loss of the *ftz*-dependent parasegments in the third phenotype is probably the result of the direct repression of *ftz*. The second and fourth phenotypes also retained either the even- or odd-numbered parasegments, respectively. However, unlike the first and third phenotypes, they were subject to mirror-image duplications owing to the widening or loss of *en* and *wg* stripes. In the 2-hr and 20-min to 2-hr and 40-min phenotype, *en* stripes expanded owing to the repression of *odd*. Expansion of *wg* was probably the result of the expansion of *run* and the subsequent repression of *eve*. In contrast, the fourth phenotype appeared to be

Figure 8. Summary of *En/wg* expression patterns and associated cuticle patterns in HSEVE embryos. The top panel portrays the approximate expression patterns of the segmentation genes addressed in this study between parasegments (PS) 5 and 8. The borders of each parasegment are defined by the juxtaposition of *en* and *wg* stripes (denoted by dotted lines). Segmental borders and ventral denticle belt features are shown at the bottom of the panel. The wedge-shaped stripes of *eve*, *ftz*, and *odd* denote the narrowing of these stripes that occurs during gastrulation. The narrow stripes of *eve* and *odd* that appear at gastrulation are shown as unlabeled boxes. (Panels 1–4) The four mutant *En/wg* and cuticle patterns that evolved in HSEVE embryos and the times at which they occurred.

caused by the direct repression of *wg* and the subsequent loss of *en*, which has been shown to be *wg* dependent during early stages of expression (Martinez-Arias et al. 1988; Heemskerk et al. 1991).

These experiments suggest that the endogenous *eve* gene is required to perform multiple regulatory roles in both the odd- and even-numbered parasegments. The earliest role of *Eve* may be to assist in the activation of *run* and *ftz*, as evidenced by its ability to activate these genes ectopically. Shortly thereafter, *Eve* appears to act as a repressor of these two genes as well as *odd*, thereby establishing the odd-numbered parasegments. The narrowing of the seven *Eve* stripes then permits *wg* expression at the posterior edges of the odd-numbered parasegments. Initiation of *en* stripes follows at the 14-stripe phase of *Eve* expression, with the 7 weaker stripes of *Eve* required to repress *odd* in the even-numbered parasegments and higher levels to repress both *odd* and *run* in the odd-numbered parasegments.

Opposing roles of Eve and Ftz

Although Eve and Ftz define reciprocal sets of parasegments, Eve appears to function primarily as a transcriptional repressor, whereas in vitro and tissue culture studies suggest that Ftz acts as a transcriptional activator (Jaynes and O'Farrell 1988; Han et al. 1989; Winslow et al. 1989; Okhuma et al. 1990). In the embryo, both genes are positive regulators of *en* and negative regulators of *wg*. Perhaps Ftz performs these functions in a manner that is complementary to that of Eve, acting as a direct activator of *en* and an indirect repressor of *wg*.

Clearly, transcriptional repressors can control patterning as effectively as transcriptional activators. Gene regulation by repressors may be particularly suited to genes that are activated by ubiquitous or broadly expressed activators. This would allow many different genes to be activated by a few widely distributed activators and resolved into complex patterns by specific repressors. This may be the case for most of the genes that are regulated by Eve.

Materials and methods*Construction and transformation of PHSEVE*

Construction of the plasmid PHSEVE and P-element transformation of flies were performed by Gary Struhl. Briefly, a *Hin*I restriction site located 21 bp upstream of the *eve* ATG and an *Ssp*I site located 50 bp downstream of the *eve* translation stop site were used to isolate the *eve*-coding region (for *eve* sequence, see MacDonald et al. 1986). After the addition of *Eco*RI linkers, the *eve* sequence was inserted into a vector that contains an *hsp70* promoter and *tubulin* gene 3'-untranslated sequences (Struhl 1989). The *hsp70-eve-tubulin* hybrid gene was inserted into Carnegie 20 and transformed as described previously (Struhl 1989).

mRNA and protein localization

In general, embryos were collected in cylinders over a 30-min interval and aged appropriately at 25°C before fixation. When correct staging of the embryos was critical, embryos were collected for 20 min and visually staged under halocarbon oil (Wieschaus and Nüsslein-Volhard 1986). The detection of transcripts in embryos was achieved by whole-mount in situ hybridization with the modifications described by Edgar and O'Farrell (1990). Antibody staining procedures were as described previously (Krause et al. 1988), except that PBTB (PBS buffer + 0.1% Tween 20 and 1% dry milk powder) was used for blocking and antibody incubations, and biotinylated secondary antibodies were detected with the Vectastain kit (Vector Laboratories). In general, double antibody/in situ stainings were performed as described, with in situ hybridizations done before the antibody stainings. Alternatively, antibody detection was performed first by using PBTH (filter-sterilized 1× PBS + 0.1% Tween 20, 100 µg/ml of heparin, 100 µg/ml of tRNA, 0.05 U/ml of RNasin) instead of PBTB, followed by in situ hybridization as described. All embryos were mounted in 80% glycerol, 20 mM Tris (pH 7.5). Embryos in which signals were detected by AP staining were subjected to dehydration in 90% and 100% ethanol, to remove background staining and to change the signal from purple to blue, and then rehydrated before mounting.

Protein and RNA measurements

Embryos for kinetic experiments were collected on apple juice plates that were placed in population cages for 30 min. The

embryos were then aged for 2.5 hr at 25°C, transferred to glass coverslips, covered with a thin layer of glycerol to distribute heat evenly, and heat-shocked by floating the coverslips on water for 4 min in a 36°C water bath. Embryos were then dechorionated and divided into equal portions. These were maintained at 25°C and fixed at appropriate intervals following heat shock. Protein or mRNA localization was carried out as described with secondary antibodies that were coupled to AP, and with the following modifications. Just before enzymatic detection, embryos were washed once in diethanolamine buffer [10 mM diethanolamine (pH 9.5), 0.5 mM MgCl₂]. The number of embryos per reaction was normalized by adding the equivalent of 50 µl of settled embryos to 0.5-ml microcentrifuge tubes. The reactions were developed by rocking for 1 hr at 25°C in 400 µl of AP reaction mix [a 5-mg tablet of *p*-nitrophenyl phosphate (PNP, Sigma) dissolved in 10 ml of diethanolamine buffer]. Reactions were stopped by adding an equal volume of 100 mM EDTA, and activity was determined by measuring the A₄₀₅ of each supernatant. We found that activities were linear with respect to time during the 1-hr incubation period and that anywhere from 10 to 100 µl of settled embryos gave proportional signals. At the end of the 1-hr period, embryos were recovered, washed in the usual AP staining buffer and then stained with the insoluble AP substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT). The AP reactions with PNP in diethanolamine had no detrimental effects on RNA or protein localization within embryos.

It was also possible to extract much of the insoluble purple AP substrate produced by the NBT/BCIP reaction from the embryos with 100% ethanol. This was performed by rinsing the embryos once in 100% ethanol and then extracting for 1 hr with occasional shaking at 65°C in 200 µl of ethanol. The ethanol was then diluted with 300 µl H₂O, and absorbance was measured at a wavelength of 550 nm. Quantitative results obtained by this method were similar to those obtained by using the soluble AP substrates. We found that for the soluble PNP reactions, antibodies that gave a weaker signal gave a more linear result. For Eve detection we used anti-Eve monoclonal antibodies obtained from N. Patel (Carnegie Institution of Washington). The ethanol extraction technique worked best with strong signals such as those generated with a polyclonal anti-Eve antibody obtained from M. Frasch. A higher percentage of the BCIP/NBT product could be ethanol extracted if the embryos were stained lightly rather than heavily.

Cuticle preparations

Embryos were collected for 20 min on apple juice plates and aged appropriately. Heat shocks were performed by transferring the embryos to glass coverslips, covering them with a thin layer of halocarbon oil, and floating the coverslips on water in a 36°C water bath. Following heat shock, embryos were examined under the microscope, and properly staged embryos were transferred to apple juice plates. After 24 hr at 25°C, unhatched embryos were dechorionated and then dissected from their vitelline membranes in Hoyer's medium (Wieschaus and Nüsslein-Volhard 1986). Clearing was carried out for 2–3 days at 65°C in Hoyer's medium.

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