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Autoregulatory and gap gene response elements of the *even-skipped* promoter of *Drosophila*

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The pair-rule gene *even-skipped* (*eve*) plays a key role in the regulatory hierarchy governing segmentation in *Drosophila*. Here we describe the use of P-transformation and *eve* promoter fusions to identify *cis* elements that regulate the periodic seven-stripe *eve* pattern. A distal region of the *eve* promoter, located between −5.9 and −5.2 kb, controls autoregulation. Sequences from this region will induce striped expression of a heterologous *hsp70* basal promoter in the presence, but not absence, of endogenous eve^+ products. Autoregulatory activity was localized to a 200-bp region of the distal *eve* promoter. We also provide evidence that individual *eve* expression stripes are regulated by separate *cis* sequences. *eve* promoter sequences located between −4.7 and −3 kb upstream of the transcription start site are important for the initiation of stripe 3, whereas sequences between −1.7 and −0.4 kb are needed for stripes 2 and 7. It is possible that these latter regions are directly regulated by the products of gap genes.

Key words: even-skipped/Drosophila/gap genes/autoregulation/stripes/homeobox

Introduction

The *Drosophila* segmentation process is governed by a hierarchy of interactions among >20 different regulatory genes that are expressed in the early embryo (Nusslein-Volhard and Wieschaus, 1980; see Akam, 1987; Ingham, 1988 for reviews). These genes fall into three classes: the gap genes, pair-rule genes and segment polarity genes (Nusslein-Volhard and Wieschaus, 1980). The gap genes subdivide the embryo into broad regions that each include several adjacent segment primordia. The gap genes control the segmentation pattern indirectly, by regulating the expression of the pair-rule genes (Carroll and Scott, 1986; Ingham *et al.*, 1986; Frasch and Levine, 1987). The pair-rule genes subdivide each gap domain into individual segments by controlling the expression of the segment polarity genes (Harding *et al.*, 1986; Howard and Ingham, 1986; Macdonald *et al.*, 1986; DiNardo and O'Farrell, 1987; Ingham *et al.*, 1988; Frasch *et al.*, 1988). It is likely that a number of these regulatory interactions occur at the level of transcription, since proteins encoded by at least seven of the genes contain either the homeobox or zinc finger DNA binding motif (i.e. McGinnis *et al.*, 1984; Scott and Weiner, 1984; Rosenberg *et al.*, 1986).

The pair-rule gene *even-skipped* (*eve*) plays a key role in the segmentation hierarchy since eve^- embryos lack all middle body segments (Nusslein-Volhard *et al.*, 1985). This severe mutant phenotype results primarily from a failure to initiate the segment polarity gene *engrailed* (*en*) (Harding *et al.*, 1986; Macdonald *et al.*, 1986). *en* is initially expressed in a series of 14 stripes in the middle body region of wild-type, gastrulating embryos (DiNardo *et al.*, 1985; Fjose *et al.*, 1985; Kornberg *et al.*, 1985). These sites of *en* expression define the posterior compartment of each segment, and are required throughout embryonic and larval development for the maintenance of segment borders (Lawrence and Morata, 1976; Kornberg, 1981). eve^- embryos do not express any of these *en* stripes, and therefore lack all segment borders. Previous genetic circuitry studies suggest that *eve* regulates *en* expression in concert with a second pair-rule gene, *fushi tarazu* (*ftz*) (Howard and Ingham, 1986). *eve* also influences the *ftz* pattern, and the loss of all 14 middle body stripes in eve^- embryos results from its dual role in regulating both *ftz* and *en* (Frasch *et al.*, 1988).

Since *eve* exerts such a strong influence on the segmentation process it is of interest to determine the basis for its regulated pattern of expression during early development. The *eve* protein first appears at ~2 h after fertilization, and at this time expression is detected in all of the embryonic nuclei. In a period of ~30 min this general staining pattern evolves into the seven-stripe pattern that defines the odd-numbered parasegments (Frasch *et al.*, 1987; Lawrence *et al.*, 1987). Upon their initial appearance, each expression stripe is broad and encompasses at least five cells. Adjacent stripes are separated by about three cells that show lower levels of expression. The early *eve* pattern is actually composed of a series of continuous bell-shaped peaks and troughs of expression along the anterior−posterior axis (Frasch *et al.*, 1987). During gastrulation, there is a refinement of the pattern such that each *eve* stripe narrows and includes only two or three of the cells within the original stripe. At this time, each embryonic cell shows a clearer on/off state of *eve* expression. Expression is transient, and each of these stripes disappears within the next hour of development.

Quite a lot is known about the *trans*-regulation of the wild-type *eve* pattern during development. *eve* antibody and RNA probes have been used to analyze *eve* expression in each of the known segmentation mutants in *Drosophila* (Harding *et al.*, 1986; Frasch and Levine, 1987). These studies suggest that separate classes of segmentation genes are responsible for the initiation and refinement of the *eve* pattern. Initiation depends on region-specific factors that autonomously regulate individual *eve* stripes. The gap genes are likely to encode such factors since mutations in each of

the five gap genes disrupt a specific subset of the seven *eve* stripes (Frasch and Levine, 1987). For example, stripes 4, 5 and 6 are absent in the gap mutant *knirps*, but stripes 1, 2, 3 and 7 appear normal.

The gap genes are essential for the initiation of *eve* expression. In contrast, mutations in each of the eight pair-rule genes and nine segment polarity genes do not significantly disrupt the early *eve* pattern (Frasch and Levine, 1987). However, three of the pair-rule genes are needed later in development, and participate in the refinement of the *eve* pattern during gastrulation. Mutations in two of these genes, *eve* and *hairy*, cause a premature loss of *eve* expression, while mutations in the third, *runt*, cause *eve* to be over-expressed.

One of the central issues of the segmentation process is how gap genes establish periodic patterns of pair-rule gene expression. The five gap genes act on broad, overlapping regions along the length of the early embryo, and show relatively simple patterns of expression (e.g. Jackle *et al.*, 1986). It has been proposed that borders of overlap between adjacent gap domains play an important role in the initiation of pair-rule stripes (Meinhardt, 1986). Pair-rule promoters appear to make sophisticated 'on/off' choices in response to different combinations of gap gene products. As a first step towards solving this complex problem it is important to identify a pair-rule promoter that is directly regulated by gap gene products. There are a total of eight pair-rule genes, and each of the five that have been examined shows a periodic pattern of expression (Hafen *et al.*, 1985; Ingham *et al.*, 1985; Harding *et al.*, 1986; Kilchherr *et al.*, 1986; Macdonald *et al.*, 1986; Gergen and Butler, 1988; D. Coulter and E.Wieschaus, personal communication). It is conceivable, but unlikely, that all eight pair-rule genes are directly regulated by gap gene products. Genetic epistasis studies suggest that only a few 'early acting' pair-rule genes directly respond to the gap genes, and that the periodic patterns of most pair-rule genes (i.e. 'late acting') are established in response to the early genes (Carroll and Scott, 1986; Howard and Ingham, 1986; Frasch and Levine, 1987; reviewed in Ingham, 1988). Previous genetic studies suggest that *eve* is an early acting gene, and consequently an analysis of its promoter should provide information concerning the mechanisms of gap gene function.

Here we identify *cis* control elements within the *eve* promoter that might mediate interactions with *trans*-acting factors encoded by gap and pair-rule genes. Different DNA fragments from the 5′ end of the *eve* transcription unit were attached to the reporter gene *lacZ*, and integrated into the *Drosophila* genome by P-element-mediated germ line transfer (Rubin and Spradling, 1982). The activities of the different promoter fragments were analyzed in transformed embryos using anti-β-galactosidase antibodies and a histochemical staining method. We show that a distal region of the *eve* promoter, located between −5.9 and −5.2 kb upstream from the transcription start site, mediates autoregulation by *eve* products. A 200-bp DNA fragment from this region of the *eve* promoter is sufficient to direct autoregulation when attached to the basal promoter of the *hsp70* gene. We also identify possible gap gene response elements within the *eve* promoter. One is located between −4.7 and −3 kb and is required for the initiation of stripe 3. The initiation of stripes 2 and 7 depends on sequences located between −1.7 and −0.4 kb.

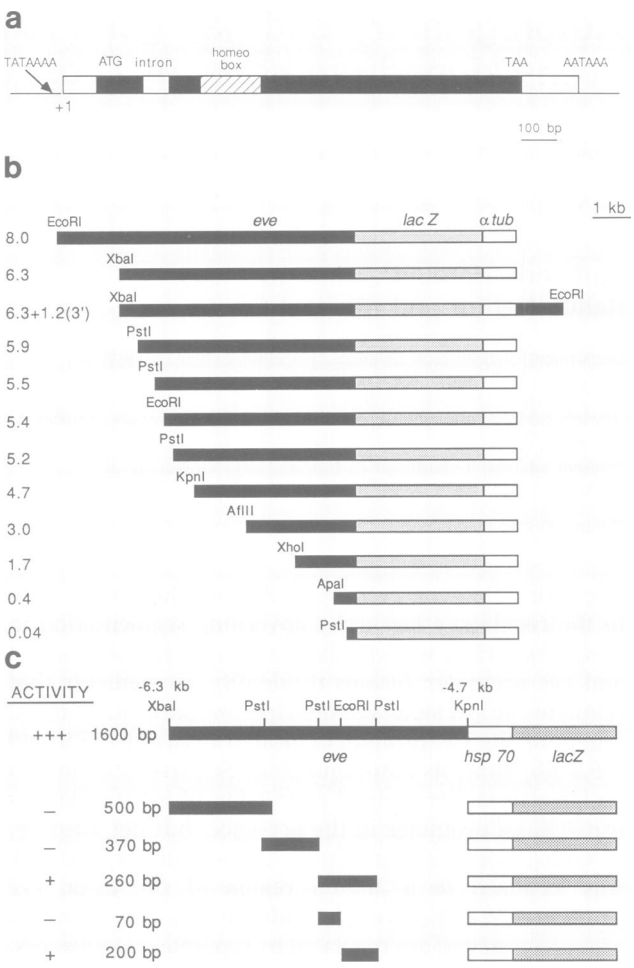

Fig. 1. Summary of *eve*−*lacZ* fusion promoters. (**a**) The *eve* transcription unit. The gene is ~1.4 kb in length, and is interrupted by a single intron of only 71 bp. The mature mRNA includes a 99-bp untranslated leader sequence and an ~160-bp trailer sequence. The 180-bp homeobox sequence is located within the 5′ half of the gene. 'ATG' refers to the initiating methionine, '+1' corresponds to the transcription start site, and 'TAA' is the terminating codon. There is a well-conserved 'TATA' box located 28 bp upstream from the transcription start site (details in Frasch *et al.*, 1987). (**b**) *eve*−*lacZ* fusions. *eve* promoter sequences are shown in black, *lacZ* coding sequences in grey, and 3′ α-tubulin sequences in white. The numbers on the left correspond to the amount of *eve* 5′ flank (in kb) used for each of the *lacZ* fusions; the restriction sites that were used to prepare each construct are also indicated. The α-tubulin sequence contains a strong polyadenylation site (Lawrence *et al.*, 1987). The *eve* coding sequence was fused in-frame with *lacZ* at codon 22 (see Materials and methods). (**c**) Heterologous *eve*−*hsp70* promoters. Different fragments from the distal *eve* promoter were attached to the *hsp70* basal promoter. *eve* promoter sequences are indicated in black, the basal *hsp70* promoter is in white, and the *lacZ* coding sequence is grey. The largest heterologous promoter includes a 1.6-kb *eve* promoter fragment that contains sequences from −6.3 to −4.7-kb upstream of the *eve* transcription start site. Subclones from this region were also inserted in the *hsp70*−*lacZ* vector (see Materials and methods). The left-most column indicates whether the heterologous promoter was active in mediating autoregulation (see Figure 5). The smallest active fragment is 200 bp in length. The 5′ site of this fragment corresponds to a *Sma*I site that maps ~10 bp upstream from the *Eco*RI site indicated in the map. The 3′ end corresponds to the 3′-most *Pst*I site in the map.

Results

eve promoter fusions

The *eve* transcription unit is 1.4 kb in length, and is interrupted by a single 71-bp intron (Macdonald *et al.*, 1986;

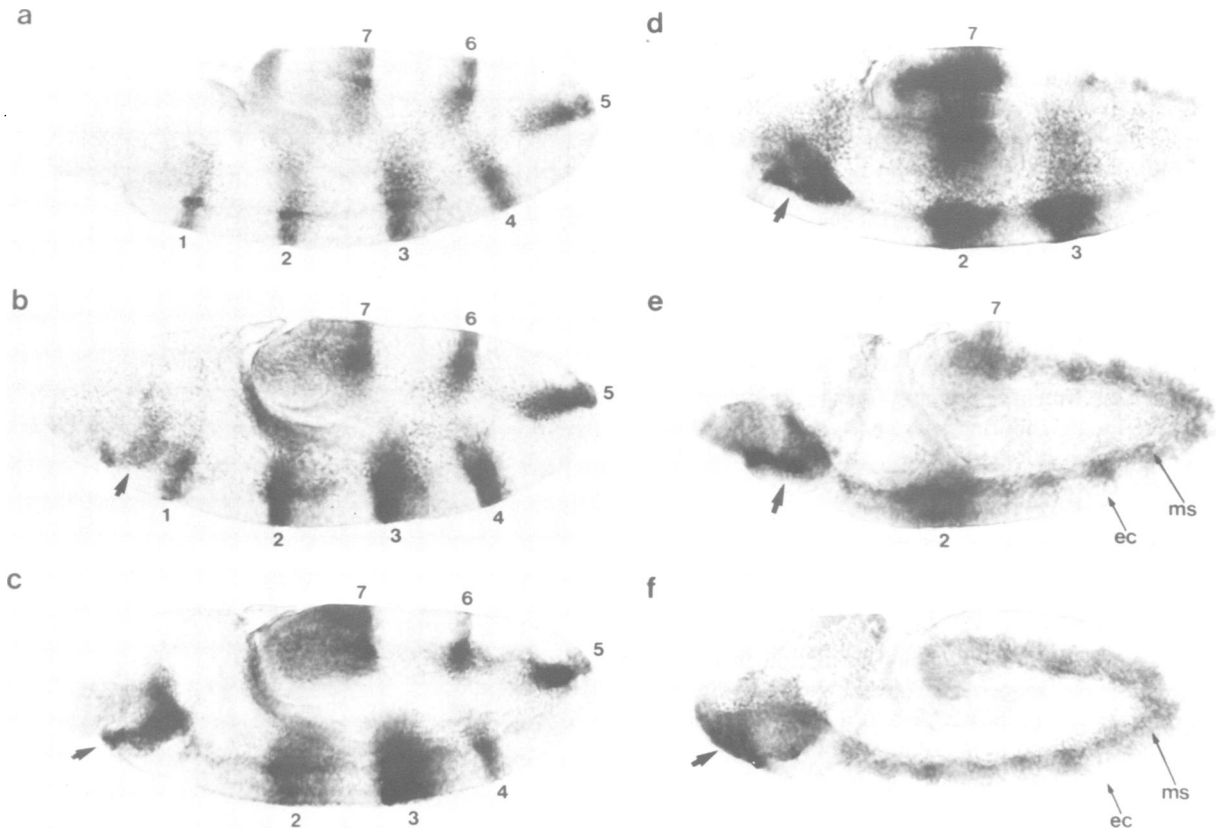

Fig. 2. Expression of *eve* promoters during germ band elongation. Whole mount preparations of embryos collected from ry^+ strains carrying different *eve*−*lacZ* promoter fusions. Embryos were stained with anti-β-galactosidase, and are oriented so that anterior is to the left and dorsal is up. (**a**) The 8-kb *eve* promoter. A normal seven-stripe pattern is observed. Note that an eighth site of staining is observed just posterior to stripe 7. This corresponds to the presumptive anal plate, which is a normal site of *eve* expression in older embryos. Staining of the anal plate is obscured in embryos carrying smaller *eve* promoter fusions, due to a posterior broadening of stripe 7. (**b**) The 5.9-kb *eve* promoter. A nearly normal staining pattern is observed. Stripes 2, 3 and 7 appear somewhat broader than normal [compare with (a)], and an ectopic site of staining is observed at the anterior midgut invagination (arrow). (**c**) The 5.5-kb *eve* promoter. Stripe 1 is absent, and there is a reduction in the levels of stripes 4, 5 and 6. The broadening of stripes 2, 3 and 7 is somewhat more marked than that observed for the 5.9-kb promoter [compare with (b)]. (**d**) The 5.2-kb *eve* promoter. Stripes 4, 5 and 6 are lost. Weak staining can be seen in the mesodermal regions where these stripes normally appear. (**e**) The 3-kb *eve* promoter. Only stripes 2 and 7 are detected. There is a general staining of the mesoderm, which is probably due to enhancer sequences present within the *rosy* P-transformation vector. (**f**) The 400-bp *eve* promoter. None of the expression stripes is observed. There is a general staining of the mesoderm and the anterior midgut invagination.

Frasch *et al.*, 1987) (summarized in Figure 1a). *eve* encodes a 1.3-kb mRNA, which specifies a 42-kd protein composed of 376 amino acid residues. An *eve* promoter−*lacZ* fusion that includes 6.3 kb of *eve* 5′ flank was shown to be sufficient to direct a nearly normal seven-stripe pattern of expression in advanced-stage embryos (Lawrence *et al.*, 1987). The fusion includes the *eve* transcription start site and 99-bp untranslated leader sequence, and ends at codon 22 of the protein coding sequence. We have used a similar promoter fusion, containing 5.2 kb of 5′ flank as a starting point for analyzing the *in vivo* expression of different *eve* 5′ fragments (Figure 1b). Sequential deletions of the 5.2 kb promoter were prepared, as well as several larger promoters which include additional 5′ sequences. The largest promoter that was assayed contains 8 kb of 5′ flank, and the smallest includes only the first 42 bp of the 5′ end. Each of the *eve* promoter−*lacZ* fusions was inserted downstream of the 3′ end of the *rosy* gene in derivatives of the Carnegie 20 P-element transformation vector (Rubin and Spradling, 1982; see Materials and methods). The *rosy* gene and *eve*−*lacZ* fusions were inserted in the same orientation of transcription within the P-vector. Several independent transformed lines were analyzed for each of the fusions shown in Figure 1. Embryos were collected from these lines and whole mount preparations were stained with an anti-β-galactosidase antibody.

Expression patterns in advanced-stage embryos

The 8-kb *eve*−*lacZ* promoter directs an essentially normal seven-stripe pattern of expression in embryos that have completed germ band elongation (Figure 2a). Double staining with a mixture of anti-*eve* and anti-β-gal antibodies show that these stripes coincide with the wild-type *eve* pattern (data not shown). The 6.3-kb and 5.9-kb promoters give slightly abnormal patterns of expression; the 5.9-kb pattern is shown as an example (Figure 2b). Stripes 1, 4, 5 and 6 appear normal, but stripes 2, 3 and 7 are somewhat broader than the others. All of the promoters that were assayed direct an ectopic site of β-gal expression in the anterior midgut invagination (AMG; arrow), which is probably an artifact of the transformation vector (see below).

Truncated *eve* promoters that delete sequences located between −5.9 and −4.7 kb disrupt the expression of stripes 1, 4, 5 and 6, but do not substantially alter the expression of stripes 2, 3 and 7. *eve* expression of stripe 1 is not detected in transformants carrying the 5.5- or 5.4-kb promoter, and

stripes 4, 5 and 6 are strongly reduced in intensity (Figure 2c). The deletion of an additional 300 bp of 5′ flank (the 5.2-kb promoter) eliminates expression of stripes 4, 5 and 6 (Figure 2d). Both the 5.5- and 5.2-kb promoters give essentially normal expression of stripes 2, 3 and 7, as does the 4.7-kb promoter (data not shown).

The proximal region of the *eve* promoter, including the first 4.7 kb of 5′ flank, is important for the expression of stripes 2, 3 and 7. Only stripes 2 and 7 are expressed by the 3- and 1.7-kb promoters, while stripe 3 is lost (Figure 2e). The 400- and 42-bp promoters do not drive expression of any of the stripes (Figure 2f). Note that the shorter *eve* promoters show a general staining of the mesoderm (i.e. Figure 2e and f), which is probably due to an enhancer element within the neighboring *rosy* gene contained within the transformation vector (H.J.Doyle, C.Rushlow and M. Levine, submitted).

Expression patterns in early embryos

The activities of the 8-, 6.3- and 5.9-kb promoters in advanced-stage embryos suggest that they contain all of the *cis* regulatory elements needed for normal *eve* expression. However, their activities in early embryos indicate that they lack essential promoter sequences. The 8-kb promoter directs normal expression of stripes 2, 3 and 7 in cellularizing embryos, whereas stripe 1 is reduced in expression and stripes 4, 5 and 6 are undetectable (Figure 3a). Only stripes 2, 3 and 7 are observed for the 6.3- and 5.9-kb promoters in early embryos (Figure 3b). There is a delay in the appearance of the other stripes, which appear during gastrulation. The 5.2- and 4.7-kb promoters give the same early staining pattern as that observed for the larger promoters (Figure 3c). However, the 5.2- and 4.7-kb promoters fail to express stripes 1, 4, 5 and 6 even during advanced stages of development (see Figure 2c).

The 3- and 1.7-kb promoters give the same staining pattern in both early and advanced-stage embryos. Only stripes 2 and 7 are observed, and there is a general staining of the ventral surface (Figure 3d). The ventral staining corresponds to the mesodermal expression seen in older embryos (i.e. Figure 2d). This staining of the presumptive mesoderm is particularly intense for the 400- and 42-bp *eve* promoters (Figure 3e). There is evidence that the ventral staining results from regulatory sequences within the neighboring *rosy* gene that can act over a considerable distance to influence the activity of basal *eve* promoter fragments (H.J.Doyle, C. Rushlow and M.Levine, submitted). Neither the 400-bp nor the 42-bp *eve* promoter gives any of the expression stripes either early or late in development (Figure 3d).

eve autoregulation

Previous studies have shown that eve^+ gene activity is required for the maintenance and refinement of the *eve* pattern during gastrulation and germ band elongation (Frasch *et al.*, 1988). *eve* exerts a positive effect on its own expression in that eve^- embryos show a premature loss of *eve* products. In order to determine what influence eve^+ products might exert on the activities of the *eve* promoter fusions, we have analyzed the expression of several of these fusions in eve^- embryos.

The 6.3-kb promoter gives an essentially normal seven-stripe pattern in advanced-stage, wild-type embryos (see Figure 2a). However, only stripes 2, 3 and 7 are detected when this same promoter is crossed into an eve^- back-

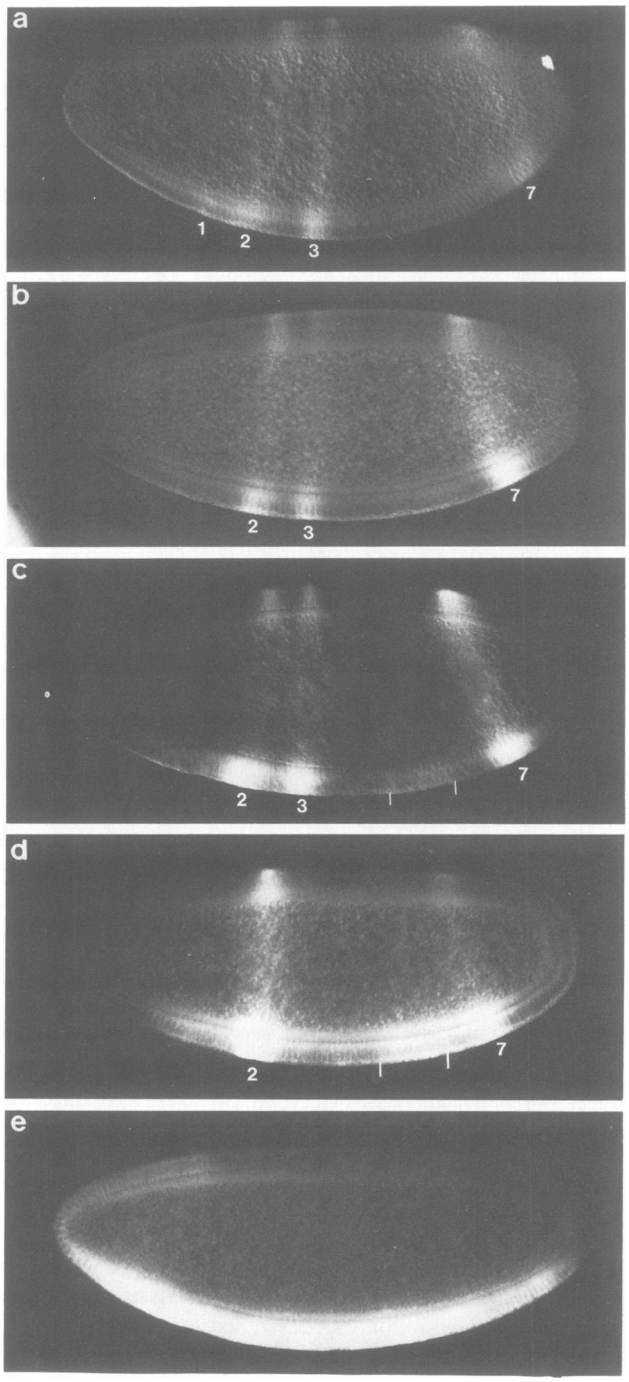

Fig. 3. Expression of *eve* promoters in cellular blastoderm embryos. Embryos are oriented so that anterior is to the left and dorsal is up. (**a**) The 8-kb *eve* promoter. Stripes 2, 3 and 7 are normal in appearance. Stripe 1 is reduced in expression as compared with wild-type *eve* products, and stripes 4, 5 and 6 are not observed. (**b**) The 5.9-kb *eve* promoter. Stripes 2, 3 and 7 are strongly stained but the other stripes are not detected. (**c**) The 5.2-kb *eve* promoter. The pattern is virtually identical to that observed for the 5.9-kb promoter [compare with (b) above]. The 6.3- and 5.5-kb *eve* promoters also display this pattern of expression (data not shown). (**d**) The 3-kb *eve* promoter. Only stripes 2 and 7 are detected. These are superimposed on generally strong staining of the ventral surface (presumptive mesoderm). The ventral staining is not quite uniform, and is somewhat stronger in the region normally occupied by stripes 5 and 6 (brackets). (**e**) The 400-bp *eve* promoter. None of the expression stripes is observed. Instead, there is strong staining of the ventral surface. Photomicrographs were prepared by printing brightfield color transparencies, thereby giving a darkfield image. The same printing method was also used for Figures 5 and 6.

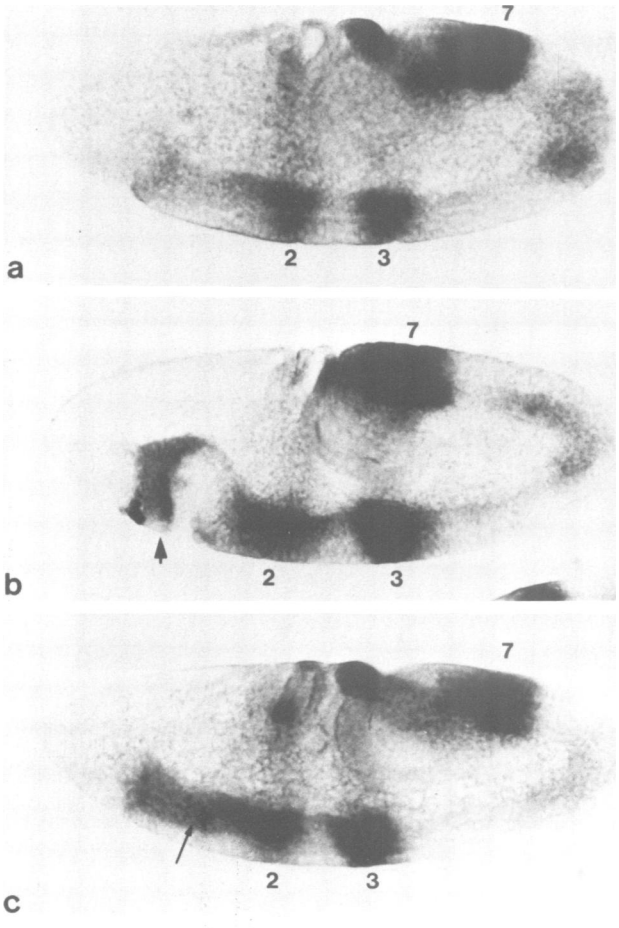

Fig. 4. Expression of *eve* promoters in eve^- embryos. The age and orientation of the embryos are the same as in Figure 2. (**a**) The 6.3-kb *eve* promoter in an eve^- embryo. Stripes 2, 3 and 7 are strongly stained. Stripe 1 is absent, and stripes 4, 5 and 6 are barely detectable. Weak, variable staining is seen in the mesoderm, which corresponds to the normal locations of stripes 5 and 6. Very weak staining of stripe 4 is occasionally seen. (**b**) The 5.2-kb *eve* promoter in a wild-type embryo. The pattern is very similar to that seen in (a), except that the staining of the anterior midgut invagination (arrow) is more intense. (**c**) The 5.2-kb *eve* promoter in an eve^- embryo. The pattern is similar, but not identical to that observed in wild-type embryos [compare with (b) above]. The most obvious difference is that there is a continuous band of staining extending from the anterior midgut to stripe 2 in the mesoderm of eve^- embryos.

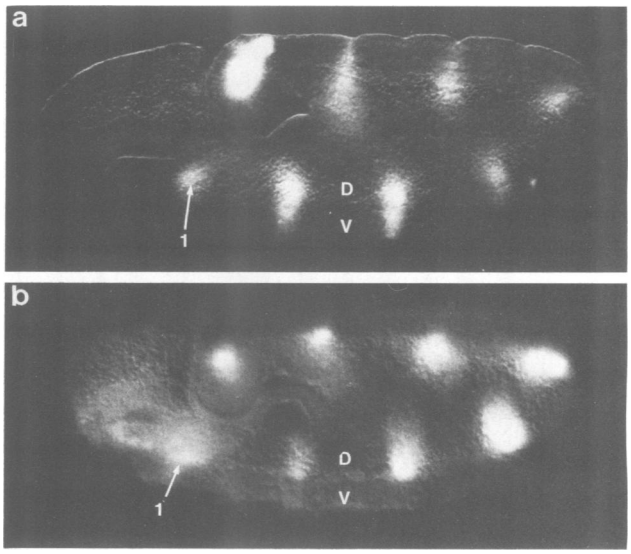

Fig. 5. Activities of heterologous promoters containing *eve* autoregulatory sequences. Age and orientation of the embryos are as in Figure 2. (**a**) The 1.6-kb *eve−hsp70* heterologous promoter. Seven stripes of staining can be seen. Each stripe includes both dorsal (D) and ventral (V) tissues of the germ band. The strongest staining corresponds to the presumptive anal plate, which is posterior to stripe 7. (**b**) The 260-bp *eve−hsp70* heterologous promoter. Seven stripes of expression are detected, but in contrast to the 1.6-kb heterologous promoter (above), staining is restricted to dorsal, not ventral, tissues. Staining of the presumptive anal plate is also restricted to fewer cells as compared with the staining seen for the 1.6-kb promoter.

ground (Figure 4a). This pattern of expression is similar to that obtained with the 5.2-kb promoter in either wild-type embryos (Figure 4b) or eve^- embryos (Figure 4c). This observation suggests that the 6.3-kb promoter, but not the 5.2-kb promoter, contain sequences regulated by eve^+ products to give stripes 1, 4, 5 and 6. Both promoters contain *cis* regulatory elements responsible for the initiation of stripes 2, 3 and 7, which appear even in the absence of eve^+ products.

Direct evidence for an autoregulatory element was obtained by analyzing the activities of different distal *eve* promoter fragments attached to the basal promoter of the *hsp70* gene (see Figure 1c). This basal promoter includes the *hsp70* TATA box and untranslated leader sequence fused to the *lacZ* gene. A heterologous promoter that includes a 1.6-kb fragment from the −6.3-kb to −4.7-kb region of the *eve* promoter gives a seven-stripe pattern of expression in wild-type embryos (Figure 5a). These sites of expression coincide with the endogenous *eve* stripes. Thus, eve^+ products either directly or indirectly interact with this distal *eve* fragment to enhance expression of the *hsp70* basal promoter. An additional, eighth site of staining is observed within the presumptive anal plate (arrow, Figure 5a). Endogenous eve^+ products first appear in this region after the completion of germ band elongation.

Shorter fragments from the −6.3- to −5.2-kb region of the *eve* promoter were examined for autoregulatory activity (summarized in Figure 1c). A fragment from −6.2 to −5.9 kb does not give detectable staining, suggesting that it lacks autoregulatory sequences. Similarly, a 370-bp fragment from −5.9 to −5.5 kb also fails to direct autoregulation (data not shown). However, a 260-bp fragment from −5.5 to −5.2 kb, and a 200-bp fragment from −5.4 to −5.2 kb, give seven weak stripes of staining in wild-type embryos (Figure 5b). The autoregulatory activity of the 260-bp fragment is not as intense as that observed for the 1.6-kb fragment containing the −6.3- to −4.7-kb region of the *eve* promoter (compare Figure 5a and b); the 200-bp fragment possesses even weaker autoregulatory activity (data not shown). The reduced expression of the 260- and 200-bp heterologous promoters is primarily due to their inability to mediate autoregulation in ventral tissues; the 1.6-kb autoregulatory fragment directs expression in both dorsal and ventral regions of each stripe.

The 1.6-kb *eve−hsp70* heterologous promoter was crossed into several *eve* mutants (Figure 6). Expression from this promoter is virtually abolished when crossed into an eve^- background (Figure 6c), which suggests that the striped pattern shown in Figures 5a and 6a is a relatively direct effect of endogenous eve^+ products. None of the seven stripes is observed, although staining is detected in the presumptive anal plate (Figure 6d). Thus, it would appear that the *eve*

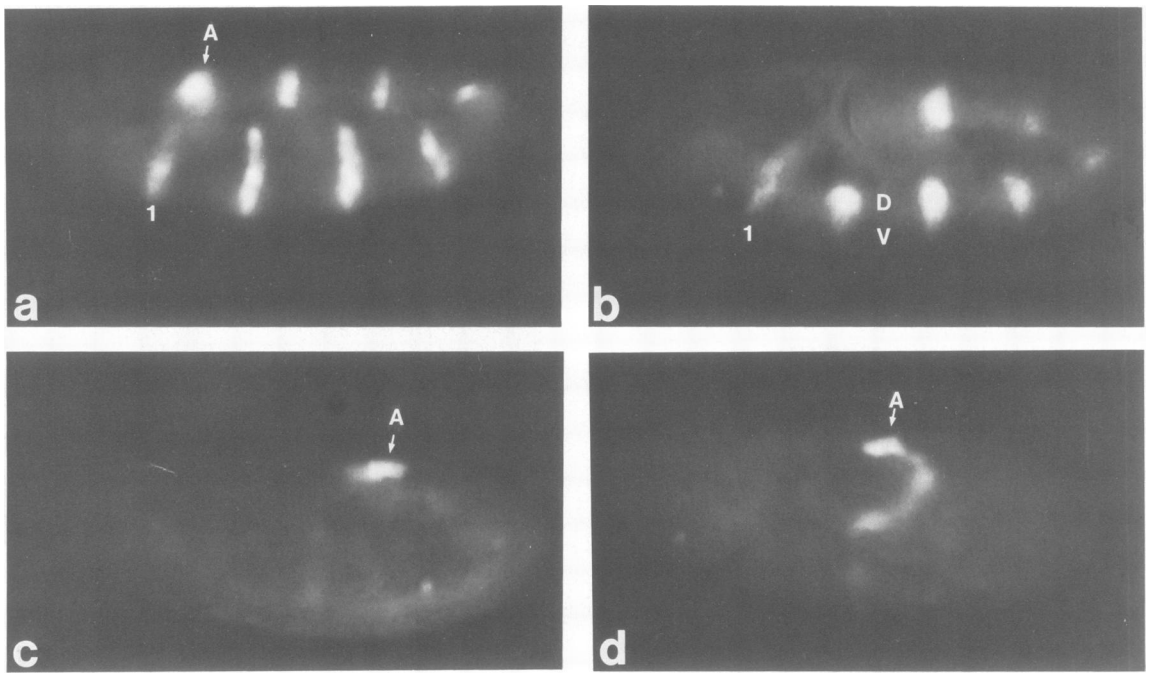

Fig. 6. Expression of a heterologous *eve−hsp70* promoter in *eve* mutants. The 1.6-kb *eve−hsp70* promoter was crossed into various *eve* mutants, and stained with anti-β-galactosidase antibody. (**a**) Expression in a wild-type embryo. All seven stripes of expression are observed; staining is also observed in the presumptive anal plate (A). (**b**) Expression in an embryo homozygous for the 3.77.17 allele. This mutation reduces, but does not abolish, eve^+ activity. Staining is restricted to dorsal tissues, and the stripes are not quite as sharp as those seen in wild-type embryos. (**c**) and (**d**) Expression in embryos homozygous for the null mutation, R13. None of the expression stripes is observed. However, staining persists in the presumptive anal plate.

autoregulatory region also contains a closely linked promoter element which directs expression in the anal plate. The 1.6-kb *eve−hsp70* heterologous promoter was also expressed in $eve^{3.77.17}$ mutants, which have been previously shown to disrupt the *eve* pattern (Frasch *et al.*, 1988). The $eve^{3.77.17}$ mutation reduces, but does not abolish, eve^+ activity. The expression stripes observed in 3.77.17 mutants do not encompass both ventral and dorsal tissues (Figure 6b), as they do in wild-type embryos (Figures 5a and 6a). Instead, expression is restricted to dorsal regions, similar to that observed for the 260- and 200-bp heterologous promoters in a wild-type background (Figure 5b).

Discussion

We have identified at least three distinct *cis* regulatory elements within the *eve* promoter, as summarized in Figure 7. The region between −5.9 and −5.2-kb is required for autoregulation by eve^+ products. The initiation of stripe 3 in early embryos depends on sequences located between −4.7 and −3 kb, while the −1.7 to −0.4-kb region is required for the initiation of stripes 2 and 7. The identification of separate *cis* sequences for individual stripes is consistent with previous genetic circuitry studies, which suggest that gap gene products are responsible for initiating the periodic pattern of *eve* expression (Frasch and Levine, 1987).

Autoregulatory element

The maintenance and refinement of the *eve* expression pattern during advanced stages of development depends on a direct or indirect autofeedback mechanism (Frasch *et al.*, 1988). This autoregulation is complex in that it is both tissue-specific, and spatially restricted. *eve* null mutants show a premature loss of expression in the ventral ectoderm, whereas the mesoderm and dorsal ectodermal tissues show essentially normal levels of expression. Progressively more posterior *eve* stripes are less disrupted by *eve* mutations. For example, *eve* expression stripe 1 is more sensitive to the loss

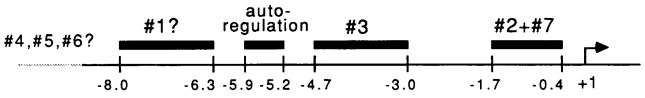

Fig. 7. Summary of *eve* promoter elements. The map shows the 5′ region of *eve*. Solid bars above the map indicate the locations of important regulatory sequences identified in this study. A total of three essential regions were identified. Sequences located between −5.9 and −5.2 kb are both necessary and sufficient for autoregulation. It is possible that redundant autoregulatory sequences reside in more proximal regions of the *eve* promoter, and act in concert with the distal sequences to provide optimal autoregulation. Sequences located between −4.7 and −3 kb are important for the initiation of stripe 3, whereas the region from −1.7 to −0.4 kb is necessary for the initiation of stripes 2 and 7. It has not been established whether the −4.7-kb/−3-kb or −1.7-kb/−0.4-kb regions are sufficient for initiation of stripe 3 or stripes 2 and 7. Sequences located between −8 and −6.3 kb increase the strength of stripe 1 in early embryos. It is possible that this region contains sequences important for the initiation of stripe 1. Alternatively, this region might include redundant autoregulatory elements that act in concert with the −5.9- to −5.2-kb interval to provide optimal autoregulation of stripe 1. Previous studies have shown that stripe 1 is the most sensitive to the loss of eve^+ products (Frasch *et al.*, 1988). Sequences needed for the initiation of stripes 4, 5 and 6 have not been identified. They do not appear to reside just downstream from the 3′ end of the *eve* coding sequence since a 6.3-kb *eve* fusion promoter that also contains 1.2 kb of the 3′ flanking region does not result in the early expression of stripes 4, 5 or 6. It is possible that the initiation of these stripes depends on sequences located upstream of −8 kb.

of eve^+ products as compared with stripes 2–7.

Sequences located between −5.9 and −5.2 kb mediate *eve* autofeedback. Deletions in this region can uncouple expression in ventral and dorsal tissues. The reduced expression of stripes 4, 5 and 6 seen with the 5.5-kb promoter is primarily due to the loss of staining in ventral, but not dorsal, tissues (see Figure 2b and c). This observation suggests that the region between −5.9 and −5.5 kb promotes *eve* autoregulation in ventral tissues, and dorsal expression depends on sequences located between −5.5 and −5.2 kb. Support for such tissue specificity stems from the staining patterns obtained with heterologous promoters. The heterologous *hsp70* promoter containing the entire *eve* autoregulatory region (from −6.3 to −4.7 kb) drives strong expression in both dorsal and ventral tissues in response to endogenous eve^+ products in wild-type embryos. However, a 260-bp (or 200-bp) distal *eve* DNA fragment (from −5.5 to −5.2 kb) directs expression only in dorsal, and not ventral, tissues. It is possible that expression in both tissues involves the co-operation of multiple elements within the autoregulatory region. The occurrence of multiple autoregulatory elements is also suggested by the staining pattern observed for the 1.6-kb heterologous promoter in weak *eve* mutants (i.e. Figure 6b). Reduction of eve^+ activity eliminates expression in ventral, but not dorsal, tissues.

The *eve* promoter might contain additional autoregulatory sequences that reside outside the region from −5.9 to −5.2 kb. The 1.6-kb heterologous promoter, which contains the entire distal autoregulatory region, gives weaker expression than the 8-, 6.3- and 5.9-kb *eve* fusion promoters. These latter promoters express stripes 1, 4, 5 and 6 by the onset of gastrulation, just after the appearance of high levels of endogenous eve^+ proteins. However, there is a significant delay in the expression of the heterologous promoter, which is not active until after the beginning of germ band elongation. Perhaps more proximal regions of the *eve* promoter contain autoregulatory elements that act in concert with the distal region to give optimal expression. Such proximal elements might be unable to mediate autoregulation without the distal sequences since truncated promoters smaller than 5.2 kb do not contain obvious autoregulatory activities.

The identification of an autoregulatory element within the distal *eve* promoter is similar to the situation previously reported for the *ftz* promoter (Hiromi *et al.*, 1985; Hiromi and Gehring, 1987). The region located between −6 and −4 kb upstream from the *ftz* transcription start site is required for maintaining optimal expression of *ftz*−*lacZ* promoter fusions. When this region is attached to the basal promoter of *hsp70*, it drives seven stripes of expression in response to endogenous ftz^+ products. It was proposed that the *ftz* protein might bind to one or more sites within the upstream element to promote positive autofeedback of expression (Hiromi and Gehring, 1987). A similar mechanism might pertain to *eve* autoregulation.

Gap gene response elements

We have shown that the initiation of different *eve* expression stripes can be uncoupled. *eve*−*lacZ* promoter fusions containing at least 4.7 kb of 5′ flank direct the correct initiation of stripes 2, 3 and 7 but not the other stripes. Even the largest promoter examined in this study (8 kb) fails to initiate stripes 4, 5 and 6, and directs only weak expression of stripe 1 in early embryos. This result suggests that the *cis* regulatory elements needed for stripes 4, 5 and 6 (and possibly 1) are located elsewhere, probably upstream of −8 kb.

The expression of stripes 2, 3 and 7 can be uncoupled by deletions in the proximal promoter. The identification of a specific region within the *eve* promoter (−4.7 to −3 kb) that participates in the expression of only a single stripe (stripe 3) raises the possibility that there are different *cis* regulatory elements for each of the other stripes as well. Such an organization might simplify the task of understanding how gap genes specify pair-rule stripes. For example, the expression of *eve* stripe 3 depends on the gap genes *hunchback* (*hb*) and *Krupple* (*Kr*), but not the other three known gap genes (Frasch and Levine, 1987). Future studies will determine whether *hb* and/or *Kr* products directly interact with the −4.7- to −3-kb region of the *eve* promoter to establish the expression of this stripe. Furthermore, it will be necessary to determine whether either of the gap response elements (see Figure 7) can act autonomously when taken outside the context of an intact *eve* promoter. It is possible that the initiation of individual *eve* stripes depends on discrete, modular promoter elements. Alternatively, their expression might involve long-range co-operative interactions among multiple promoter elements. Perhaps the initiation of stripe 3 depends on interactions between distal (−4.7- to −3-kb) and proximal regions of the *eve* promoter.

The occurrence of separate *eve* promoter elements for the expression of specific stripes is similar to the situation reported for *hairy* (Howard *et al.*, 1988), but distinct from the organization of *ftz* (Hiromi *et al.*, 1985; Hiromi and Gehring, 1987). *eve* and *hairy* appear to correspond to early pair-rule genes since their initial patterns of expression are not disrupted by mutations in any of the known pair-rule and segment polarity genes (Frasch and Levine, 1987; Carroll *et al.*, 1988). Only gap mutants significantly alter their initial seven-stripe patterns of expression. In contrast, mutations in at least two pair-rule genes, *hairy* and *runt*, disrupt the establishment of the seven-stripe *ftz* pattern, suggesting that it is a late class pair-rule gene which is not directly regulated by gap gene products (Ingham, 1988). The initiation of the *ftz* pattern has been shown to depend on only a small region of the proximal promoter, including as little as ~400-bp of 5′ flank (Hiromi *et al.*, 1985; Y.Hiromi, personal communication). In no case has a truncated promoter been shown to uncouple the regulation of different *ftz* stripes. This apparently simple organization of the *ftz* promoter might reflect its relatively straightforward response to periodically distributed *hairy* and *runt* products. The more complicated organization of the *eve* and *hairy* promoters might indicate a greater sophistication in the mechanisms responsible for the initiation of pair-rule expression in response to crudely localized gap gene products.

Materials and methods

even promoter fusions

eve−*lacZ* promoter fusions were inserted into a derivative of the Carnegie 20 vector (Rubin and Spradling, 1983) that contains a unique *Not*I cloning site (called DM 30; Mismer and Rubin, 1987). Transformed lines were established by standard methods using the *rosy* gene as a selectable marker. DM 30 recombinants were coinjected with a helper P-element, pPi 25.7wc (Karess and Rubin, 1984), into cleavage stage embryos from the ry^{506} line (Lindsley and Grell, 1968). The 5.2-kb *eve*−*lacZ* fusion shown in Figure 1b was used as the starting point for preparing the other fusion promoters. The original 5.2-kb fusion was prepared by inserting a *Pst*I fragment from the

5′ end of *eve*, including sequences from −42 bp to −5.2 kb, into the pEL1 plasmid obtained from Dr P.Macdonald (see Lawrence *et al.*, 1987). pEL1 is identical to the 42-bp *eve−lacZ* fusion shown in Figure 1b, and contains the entire 99-bp untranslated leader sequence from the *eve* transcription unit as well as the first 22 codons of the coding sequence. The 8-, 5.9- and 5.5-kb fusions were prepared by inserting appropriate 5′ restriction fragments into the 5.2-kb *eve−lacZ* construct. The 6.3-kb *eve−lacZ* fusion shown in Figure 1b is the one described by Lawrence *et al.* (1987); the *Xba*I site derives from polylinker sequences and is not contained in the native *eve* promoter. A second 6.3-kb fusion promoter was prepared by adding appropriate 5′ sequences to our original 5.2-kb construct (see Figure 1b); this yields results identical to those obtained with the one prepared by Lawrence *et al.* (1987). The truncated series of promoters was prepared by deleting different 5′ fragments from the 5.2-kb construct. The 5′ restriction sites within the *eve* promoter that were used are indicated in Figure 1b. At least three different independent transformed lines were obtained for each *eve−lacZ* promoter fusion, and the staining patterns that are presented were observed in each of the different lines.

The eve^{-} strain used for the experiment shown in Figure 4 corresponds to the R13 allele (Nusslein-Volhard *et al.*, 1985). eve^{R13} homozygotes possess a null cuticular phenotype (the so-called 'lawn of denticle hairs') and do not express detectable levels of the *eve* protein (Frasch *et al.*, 1987).

The heterologous promoter fusions used for the experiments shown in Figure 5 were prepared with the *HZ50* gene (for details see Hiromi and Gehring, 1987). *HZ50* contains the basal promoter of the *hsp70* gene, including only −50-bp upstream from the cap site, fused in frame to the *lacZ* gene (at codon 7 of the *hsp70* coding sequence). The 3′ region of the fusion gene contains *hsp70* sequences, including the trailer sequence and polyadenylation site. The *hsp70−lacZ* fusion was inserted into the Carnegie 20 transformation vector, and has been shown to drive only weak, sporadic expression in embryos. Sequences from the distal, autoregulatory region of the *eve* promoter were inserted in the same transcription orientation as the *hsp70* promoter. All *eve−hsp70* heterologous promoters were inserted so that the *rosy* marker gene lies at the 5′ side.

Fixation and staining of embryos

Whole mount preparations of embryos were fixed and pretreated exactly as described by Frasch *et al.* (1987). A 1:700 dilution of a mouse anti-β-galactosidase serum was used as the primary antibody (purchased from Jackson Labs, Maine). A biotinylated horse anti-mouse IgG was used as a secondary antibody (diluted 1:750; purchased from Vector Labs, Burlingame, CA). Signal detection was done with an aggregate of streptavidin-biotinylated horseradish peroxidase, as described by the manufacturer (Vector Labs). Histochemical staining was done in a solution containing 50 mM citrate + ammonium acetate, pH 5.0, 0.015% H_2O_2, 0.5 mg/ml diaminobenzidine, 0.05% $NiSO_4$ for 3−30 min at room temperature. Stained embryos were dehydrated in 100% ethanol (2 × 5 min), cleared with Xylene (2 × 20 s), and mounted in Permount (purchased from Fisher Scientific). Photomicroscopy was done with Nomarski optics. The photomicrographs shown in Figures 2 and 5 were done by printing the negatives of the brightfield Nomarski images.

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Note added in proof

Similar results are reported by Goto *et al.* (1989) *Cell*, in press, although we disagree with their interpretation of the distal autoregulatory element.