

Autoregulation of a segmentation gene in *Drosophila*: combinatorial interaction of the *even-skipped* homeo box protein with a distal enhancer element

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Autoregulation has been implicated in the expression of many patterning genes in *Drosophila*, but the molecular details of this process are largely unknown. In the case of the segmentation gene *even-skipped* (*eve*), autoregulation is important for the specification of sharp stripes of gene expression at the onset of gastrulation. Here, we use a combination of DNA binding and P-transformation assays to characterize the *cis*- and *trans*-acting factors responsible for autoregulation. We show that *eve* autoregulation is mediated, at least in part, by a 100-bp minimal autoregulatory sequence (MAS) located ~5 kb upstream from the *eve* transcription start site. Multimerization of a 200-bp DNA fragment that encompasses the MAS drives optimal autoregulatory activity, comparable to that obtained with the native distal enhancer element located between -5.9 and -5.2 kb. The MAS contains two *eve* protein-binding sites, as well as binding sites for two nuclear factors present in early embryos. Directed mutagenesis of these binding sites suggests that both the *eve* protein and nuclear factors are essential for autoregulation. These results provide evidence that the *eve* protein acts combinatorially with other transcription factors to enhance its own expression.

[Key Words: Autoregulation; *Drosophila*; *even-skipped*; homeo box protein; segmentation]

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Drosophila segmentation is governed by a hierarchy of interactions among a set of ~25 regulatory genes that are active in the early embryo (Nüsslein-Volhard and Wieschaus 1980; for review, see Akam 1987; Ingham 1988). One of these is the pair-rule gene *even-skipped* (*eve*), which encodes a homeo box protein that is critical for initiating the metamerized organization of the embryo (Harding et al. 1986; Macdonald et al. 1986; Frasch et al. 1987). *eve* is unique among the pair-rule genes in that weak (or hypomorphic) alleles delete alternating segments, while strong (or null) alleles result in the complete loss of segment boundaries within middle body regions of mutant embryos (Nüsslein-Volhard et al. 1985). Genetic studies have identified a total of four target genes that are either directly or indirectly regulated by *eve*, including the segment polarity genes *engrailed* (*en*) and *wingless* (*wg*), as well as the pair-rule genes *fushi tarazu* (*ftz*) and *eve* itself (Harding et al. 1986; Macdonald et al. 1986; Frasch et al. 1988; Ingham et al. 1988). Previous studies have established that *eve* encodes a sequence-specific transcription factor (Hoey and Levine 1988; Biggin and Tjian 1989; Han et al. 1989), which is likely to interact directly with one or more of the genet-

ically defined target genes of *eve* (Harding et al. 1986; Macdonald et al. 1986; Frasch et al. 1988).

To understand how the *eve* protein functions as a transcription factor *in vivo*, we have focused on the process of *eve* autoregulation. *eve* is initially expressed in a series of seven broad, symmetric stripes along the length of precellular embryos. During a period of only 30 min, these stripes are refined so that each includes two to three cells and show clear anterior-posterior polarity by the onset of gastrulation. A sharp on/off border forms at the anterior edge of each stripe, with peak levels of *eve* protein in the anterior-most cell. The formation of these asymmetric *eve* stripes is the earliest manifestation of segment polarity, with the anterior border of each stripe defining parasegment boundaries by activating *en* (Lawrence et al. 1987). The refinement of the *eve* stripes depends on *eve*⁺ gene activity. In *eve*⁻ embryos, the stripes fail to sharpen and there is a premature loss of *eve* expression (Frasch et al. 1988; Lawrence and Johnston 1989). Genetic studies suggest that *eve* autoregulation is complex and depends on the activities of at least two other pair-rule genes, *hairy* (*h*) and *runt* (*run*) (Frasch and Levine 1987; Frasch et al. 1988; Goto et al. 1989). *eve* and *h* are positive regulators, while *run* exerts a strong negative effect on *eve* expression.

Promoter fusion studies have shown that the initiation of individual *eve* stripes depends on separate *cis*-

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regulatory elements, which are controlled (directly or indirectly) by the products of the gap genes (Goto et al. 1989; Harding et al. 1989; Stanojevic et al. 1989). In contrast, the refinement of all seven stripes depends on a common *cis*-regulatory element that is located in a far distal region of the *eve* promoter, which maps between -5.9 and -5.2 kb upstream from the transcription start site. This distal element can drive seven stripes of expression from a heterologous promoter in P-transformed embryos (Goto et al. 1989; Harding et al. 1989). Such expression was observed only in *eve*⁺ embryos and disappeared in *eve*⁻ mutants. These observations prompted the proposal that the distal sequences correspond to an autoregulatory element, which mediates the refinement of the *eve* stripes by the direct interaction of the *eve* protein. Further dissection of the distal autoregulatory element led to the identification of a 200-bp sequence that is sufficient to drive seven stripes of expression in response to *eve*⁺ activity (Harding et al. 1989). However, this expression was found to be reduced substantially as compared with that obtained from the full-length autoregulatory element.

Here we show that a normal level of late *eve* expression can be achieved by multimerizing this 200-bp sequence, suggesting that autoregulation involves some type of cooperative mechanism. We have further narrowed the limits of the minimal autoregulatory sequence (MAS) to a 100-bp region of the *eve* promoter, located between -5.35 and -5.25 kb. The 100-bp MAS is sufficient to produce stripes of expression in P-transformed embryos when multimerized and attached to an *hsp70-lacZ* fusion gene. In vitro-binding assays have revealed two clusters of *eve* protein-binding sites within the MAS. Base substitutions that disrupt these binding sites cause a corresponding reduction in autoregulatory activity *in vivo*. These results suggest that *eve* autoregulation involves the direct interaction of the *eve* protein with its own promoter, via the binding sites identified within the MAS. In addition, we have characterized two additional nuclear factor-binding sites within the MAS. Point mutations that abolish the in vitro-binding activity of either site also cause severe reductions in autoregulatory activity. We propose that the *eve* protein acts combinatorially with these two nuclear factors to activate its own expression. One of the factors may correspond to *tramtrack* (*ttk*), a zinc finger protein that is expressed in early embryos (Harrison and Travers 1990; D. Reed and J.L. Manley, pers. comm.).

Results

The *cis*-regulatory elements identified within the *eve* promoter are summarized in Figure 1, on the basis of studies done by Harding et al. (1989) and Goto et al. (1989). Individual *eve* stripes are initiated by separate *cis*-regulatory elements, while the maintenance and refinement of the stripes in older embryos depends on an autoregulatory element located between -5.9 and -5.2 kb. A 1.6-kb or 900-bp DNA fragment spanning this element was shown to induce striped expression of a het-

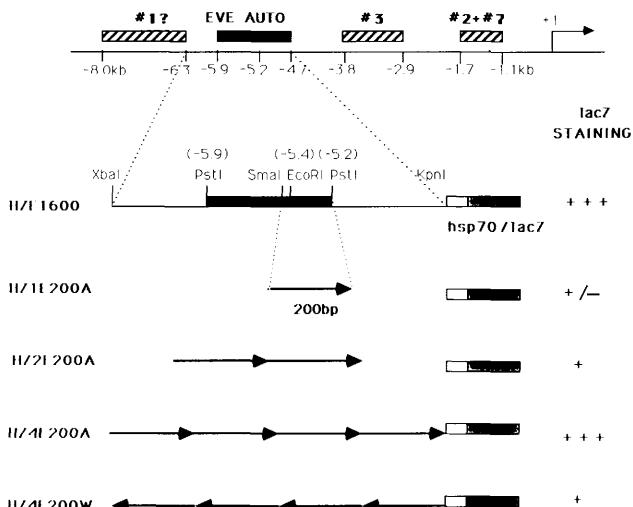


Figure 1. Summary of *eve* promoter elements and multimerization of autoregulatory sequences. (Top line) A map of the *eve* promoter and the limits of four different *cis*-regulatory elements identified in previous studies (Goto et al. 1989; Harding et al. 1989). The hatched boxes indicate stripe initiation elements, with the numbers indicating *eve* expression stripes. Autoregulatory sequences were mapped to a distal region, between -5.9 and -5.2 kb upstream from the transcription start site (+1). A 1.6-kb DNA fragment that spans this region (from -6.3 to -4.7 kb) was shown to induce strong expression (+++) of the heterologous *hsp70* minimal promoter, when attached to the *lacZ* reporter gene and introduced into embryos via P-element transformation. A 200-bp *Sma*I-*Pst*I fragment (from about -5.4 to -5.2 kb) within the autoregulatory element was shown to induce only very weak (+/-) expression from the *hsp70* promoter in P-element transformants. Multiple copies of this fragment were placed upstream of the *hsp70-lacZ* gene fusion, and their activities were examined in P-transformed embryos by monitoring the expression of the *lacZ* reporter using an anti- β -galactosidase antibody. The horizontal arrows correspond to the 200-bp *Sma*I-*Pst*I fragment and their orientation within the P-expression vector relative to the *hsp70* minimal promoter. The rectangles correspond to the *hsp70-lacZ* gene fusion, with the stippled region indicating the limits of the bacterial *lacZ* gene and the open region representing the *hsp70* promoter. The activities obtained with a two-copy promoter fusion (H2/2E200A) were stronger than that obtained with just one copy. A four-copy promoter (HZ4E200A) containing the fragments in the correct orientation gave very intense expression (+++), whereas much weaker expression (+) was observed when they were placed in opposite orientation (HZ4E200W).

erologous *hsp70* promoter when expressed in embryos via P-element transformation (Goto et al. 1989; Harding et al. 1989). Strong expression of the *lacZ* reporter gene was observed in wild-type embryos but disappears in *eve*⁻ embryos. Smaller fragments from the autoregulatory element were used in the P-transformation assays in an effort to identify the minimal sequences that can mediate autoregulation. The smallest piece of DNA that was found to work is 200 bp in length and maps between -5.4 and -5.2 kb (Harding et al. 1989). However, expression with this fragment is significantly weaker than that obtained with the full-length autoregulatory ele-

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ment. Other DNA fragments in the distal region completely failed to mediate autoregulation, including a 370-bp fragment located between -5.9 and -5.5 kb.

Multimers of the 200-bp fragment restore full autoregulatory activity

It is possible that the *eve* autoregulatory element contains multiple copies of a functionally equivalent sequence. To test this possibility we inserted tandem copies of the 200-bp fragment into the HZ50 expression vector (see Fig. 1). HZ50 is a P-transformation vector that contains the minimal *hsp70* promoter attached to the *lacZ*-coding sequence (Hiromi and Gehring 1987). Initially, we compared the activities of promoters containing $1\times$, $2\times$, and $4\times$ copies of the 200-bp autoregulatory fragment. These fusion genes were introduced into the germ line via P transformation, and their relative activities were determined by analyzing transformed embryos with anti- β -galactosidase antibodies. The results of these experiments are shown in Figure 2.

Fusion genes containing two tandem copies of the 200-bp fragment (Fig. 2B) consistently showed much more intense reporter gene expression than that obtained with just one copy of the fragment (Fig. 2, cf. A with B). The one-copy promoter (HZ1E200A) drives weak and even sporadic expression of the *lacZ* reporter. Expression is primarily restricted to dorsal-lateral epidermis, and over half of the transformed lines show no expression at all. In contrast, two copies of the fragment (HZ2E200A) resulted in consistently strong expression in all five transformed lines that were tested. Moreover, expression was found to include both the mesoderm and ectoderm. The heterologous promoter containing four tandem copies in the normal orientation (HZ4E200A) showed intense *lacZ* expression (Fig. 2C), even stronger than the staining obtained with the full-length autoregulatory element (Fig. 2D).

The demonstration that multimers of the 200-bp fragment cause a dramatic increase in expression suggests that the authentic autoregulatory element is composed of multiple copies of a functionally similar sequence (see Discussion). Surprisingly, four copies of the 200-bp fragment in opposite orientation (HZ4E200W) give much weaker expression (Fig. 2E) than the same promoter containing the fragments in the correct orientation (Fig. 2D). This orientation dependence might indicate the occurrence of complicated interactions between enhancer-binding proteins and the basal transcription machinery.

Factor binding sites in the eve autoregulatory element

To determine whether the *eve* protein might act directly on the autoregulatory element we performed footprint assays using a full-length *eve* protein made in bacteria (Hoey and Levine 1988). The 200-bp autoregulatory DNA fragment was end-labeled with ^{32}P , incubated with increasing amounts of *eve* protein, and digested with DNase I (Fig. 3A). Two regions of protection were identified, called EVE-P (proximal) and EVE-D (distal). The *eve* protein appears to bind with somewhat higher affin-

ity to EVE-P than EVE-D (see Fig. 3A, lane 4). Each region contains both high- and low-affinity binding sites. Comparative footprint assays indicate that these sites have lower affinities for the *eve* protein than the A-T-rich binding sites identified in previous studies (data not shown). Inspection of the EVE-P and EVE-D sequences does not reveal extensive homology with previously identified *eve*-binding sites (Hoey and Levine 1988; Hoey et al. 1988), except that they both possess an AT-rich core (Fig. 4A). Nonetheless, we believe that the EVE-D and EVE-P regions are significant since they contain at least four tightly clustered *eve*-binding sites within a span of only 80 bp. Using the binding conditions described in Materials and methods we estimate an average of no more than one *eve* site in a random 500-bp DNA fragment.

Genetic studies suggest that *eve* activates its own expression, yet both cotransfection assays and in vitro transcription experiments have revealed only a repressor activity (Biggin and Tjian 1989; Han et al. 1989). Perhaps *eve* must interact with "cofactors" to function as an activator. To determine whether the 200-bp autoregulatory fragment might contain binding sites for additional transcription factors we have performed DNA-binding assays using whole nuclear extracts prepared from early *Drosophila* embryos (Fig. 3B). DNase I footprint assays identified three regions that are protected by increasing amounts of nuclear extract, called DENF1, DENF2, and DENF3 (summarized in Fig. 4A). The DENF1 and DENF3 sites share some similarities with the GAGA factor-binding sites (Biggin and Tjian 1988; Soeller et al. 1988), whereas DENF2 contains a TCCT core sequence that is related to factor-binding sites identified in proximal regions of the *eve* promoter (Read et al. 1990). The zinc finger protein *ttk* has been shown to bind these proximal sequences (D. Read and J.L. Manley, pers. comm.), and a *ttk* fusion protein gives a pattern of protection for the DENF2 site that is identical to that obtained with crude nuclear extracts (J. Jiang and D. Read, unpubl.). Thus, it is likely that DENF2 corresponds to *ttk*.

In vivo relevance of factor-binding sites—deletions

As a first step toward determining the role of factor-binding sites in autoregulation, we analyzed the effects of deletions on the expression of promoter fusions in P-transformed embryos (summarized in Fig. 4). We have shown that two copies of the 200-bp autoregulatory fragment drive strong and consistent expression of *hsp70-lacZ* gene fusions (Fig. 2B), indicating that this heterologous promoter could be used as a starting point for analyzing the effects of various mutations on autoregulation. For most of the experiments described below, deletions were made within the 200-bp fragment, and two tandem copies of the mutagenized fragment were placed in the HZ50 vector (see Fig. 4B). To generate appropriate deletions, in vitro mutagenesis was done to create four *BclI* restriction sites along the length of the 200-bp fragment (arrows in Fig. 4; for details, see Materials and

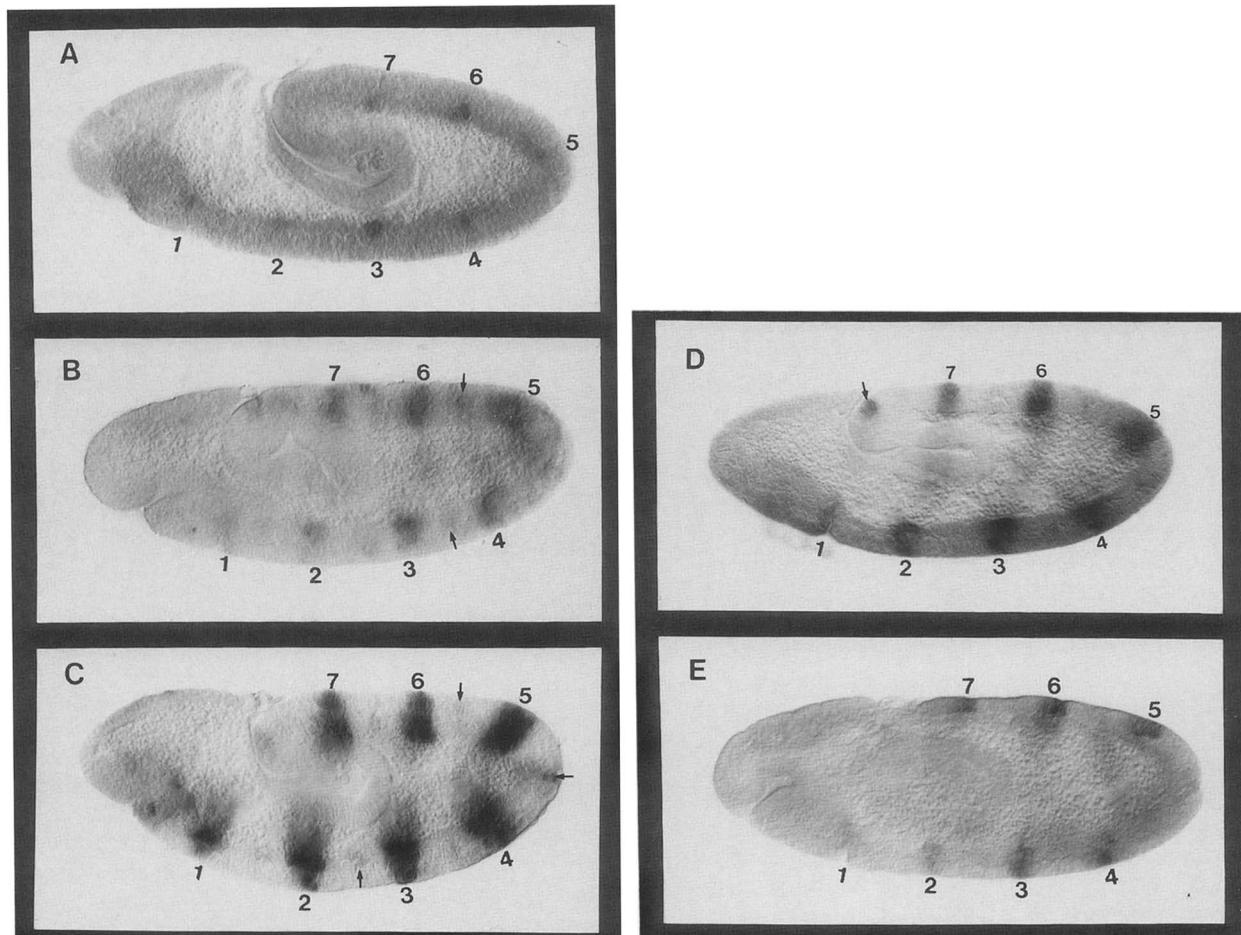


Figure 2. Expression patterns of heterologous promoters in P-transformed embryos. All embryos are oriented so that anterior is to the left and dorsal is up. Embryos were collected from P-transformed lines and stained with anti- β -galactosidase antibodies. The embryos shown here are ~4–6 hr old and have undergone germ-band elongation. Note the increase in reporter gene expression with promoters containing one, two, and four copies (A, B, and C, respectively). (A) One copy of the 200-bp fragment (HZ1E200A); (B) two copies of the fragment (HZ2E200A); (C) four copies of the fragment (HZ4E200A); (D) the 1.6-kb fragment that contains the entire autoregulatory region (from -6.3 to -4.7 kb). Note that expression is not quite as strong as that observed for four copies of the 200-bp fragment (cf. with C). (E) Four copies of the 200-bp fragment in opposite orientation. Note that expression is no stronger than that seen with two copies of the fragment in the correct orientation. The numbers refer to the 7-stripe *eve* pattern. Arrows indicate weak expression in interstripes. The arrow in D shows expression in the proctodeum, which does not correspond to one of the original *eve* stripes but is a normal site of *eve* expression in older embryos (Frasch et al. 1987).

methods). A 106-bp DNA fragment that includes the region between *BcII* sites 1 and 4 can mediate autoregulation when six tandem copies are placed in the HZ50 expression vector. The construction of this promoter (HZ6E100) is summarized in Figure 4B, and the staining pattern obtained in P-transformed embryos is shown in Figure 5A. The 106-bp fragment is the minimal sequence found to be active in autoregulation assays, and is referred to as the MAS. Note that the six-copy MAS promoter drives seven weak “spots” of expression (arrows in Fig. 5A), in addition to the seven strong stripes. This pattern is also obtained with the two- and four-copy 200-bp promoters (see Fig. 2) and is similar to the endogenous *eve* pattern that is observed in wild-type embryos at this stage (Frasch et al. 1987).

Sequences located 5' of the MAS appear to be important for optimal autoregulatory activity. Six copies of the

MAS drive a level of expression comparable to just two copies of the 200-bp autoregulatory fragment (cf. Fig. 2B with Fig. 5A). One interpretation of this finding is that DENF3 is required for optimal expression since the MAS lacks this binding site (see Fig. 4B). However, removal of the DENF3 region from the 200-bp autoregulatory element (2EΔ3'; Fig. 4B), has no effect on the levels of expression (Fig. 5C). In contrast, deletion of sequences 5' to *BcII* site 1 (the 2EΔ5' promoter) causes a substantial reduction in expression (Fig. 5B). It is possible that this reduction is due to the loss of factor-binding sites that map in the 5' region, which were missed in our in vitro-binding assays. A lack of sensitivity of this assay is suggested by our failure to identify the EVE-D and EVE-P regions using whole nuclear extracts.

Internal deletions within the limits of the MAS suggest that some or all of the factor-binding sites are im-

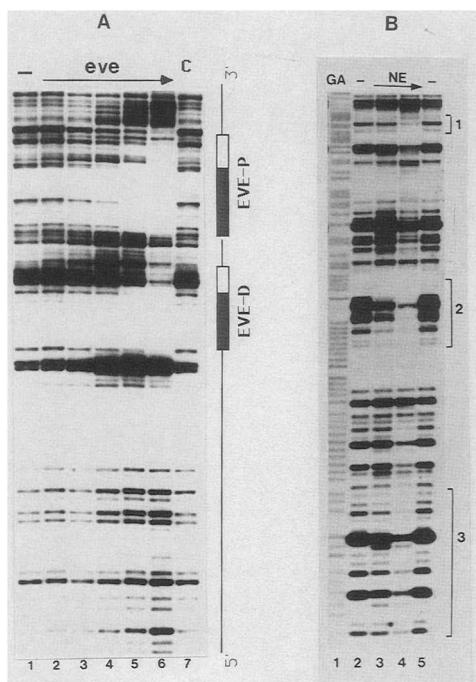


Figure 3. DNA-binding assays with the 200-bp autoregulatory fragment. (A) DNase I footprint assay with *eve* protein extract from bacteria. The DNA fragment was ^{32}P -labeled on the 5' end and incubated with various bacterial extracts prior to digestion with DNase I. (Lane 1) no added protein; (lanes 2–6) successive twofold increases in the amount of *eve* protein extract; (lane 7) total protein extract from bacteria expressing the T7 vector without *eve*-coding sequences. The boxes at right of the autoradiogram indicate the regions of protection with increasing amounts of the *eve* protein, called EVE-D (distal region) and EVE-P (proximal region). The solid regions of the boxes indicate sites of relatively high affinity, whereas the open regions indicate sites with lower affinity. Note that only the high-affinity sites within EVE-D and EVE-P are protected in lane 5, but a twofold increase in the amount of *eve* protein expands the region of protection into lower affinity sites (lane 6). (B) DNase I footprint assay with a nuclear extract obtained from 4- to 10-hr embryos. The 200-bp autoregulatory fragment was ^{32}P -labeled on the noncoding strand, incubated with increasing amounts of nuclear extract (NE). (Lane 1) The G + A sequence of the DNA fragment using the Maxam-Gilbert sequencing method; (lanes 2 and 5) no added protein; (lane 3) addition of 5 μg of nuclear extract; (lane 4) addition of 25 μg of nuclear extract.

portant for autoregulation. The MAS includes two *eve*-binding sites, as well as DENF1 and DENF2 (see Fig. 4B). Deletion of the sequences located between *BcII* sites 1 and 2 (2E Δ 12) completely abolished the autoregulatory activity of the 200-bp fragment (data not shown). This 44-bp deletion removed the EVE-D- and DENF1-binding sites, suggesting that one or both are essential. The DENF2 site was removed by deleting sequences located between *BcII* sites 3 and 4 (2E Δ 34). The 2E Δ 34 promoter gave barely detectable levels of reporter gene expression, indicating that DENF2 is probably important for autoregulation (data not shown). An unexpected result was obtained when the EVE-P site was removed by deleting sequences between *BcII* sites 2 and 3 (2E Δ 23). A heterol-

ogous promoter bearing two copies of this deleted fragment appeared to direct a very weak 14-stripe expression pattern (data not shown). To better visualize these stripes, we placed four tandem copies of the fragment (4E Δ 23) in the HZ50 expression vector (Fig. 5D). Each of these stripes is weaker than any of the primary stripes observed with promoters containing four copies of the intact 200-bp autoregulatory fragment (i.e., Fig. 2B). The dramatic alteration in the expression pattern obtained with the 4E Δ 23 promoter suggests that the EVE-P region might be exerting both a positive and negative effect on the autoregulatory element. It appears to be required for optimal expression of the seven-stripe *eve* pattern, and it also seems to play a role in suppressing expression in interstripe regions. Alternatively, it is conceivable that the 14-stripe pattern is due to the altered spacing of the remaining factor-binding sites, rather than the loss of EVE-P per se.

In vivo relevance of factor-binding sites—point mutations

To determine whether the results of the deletion analysis are due to the loss of individual factor-binding sites or altered spacing among the remaining sites, we generated point mutations within EVE-D, EVE-P, DENF1, and DENF2 (see Fig. 6). Base substitutions were made in the context of the 200-bp autoregulatory element. Two copies of the mutagenized fragment were placed in the HZ50 expression vector and used for P-transformation assays. The large sizes of EVE-D and EVE-P suggest that they contain multiple *eve* protein-binding sites (see summary, Fig. 6). The *eve* protein recognizes a 10-bp core consensus sequence, while EVE-D and EVE-P are >20 and 30 bp in length, respectively. Clustered base substitutions were made in what appear to be the core-binding sites. For EVE-D, we targeted the ATTA sequence since this is a motif found in the vast majority of homeo box recognition sites that have been identified (for review, see Levine and Hoey 1988). A 6-nucleotide substitution was made that converts the sequence ATTATG to GGATCC, thereby completely changing the core ATTA motif (Fig. 6). DNase I footprint assays indicate that these substitutions reduce binding of the *eve* protein by a factor of at least two- to fourfold and also change the overall pattern of binding (Fig. 7, cf. B with A). Despite this reduced binding, the mutation has no apparent effect on in vivo activity. Reporter gene expression driven by the mutagenized promoter is about as intense as that observed for the wild-type fragment (Fig. 8, cf. E with A). This result suggests that the EVE-D site is not essential for autoregulation, although it appears to function redundantly with the EVE-P region (see below).

Two different clusters of base substitutions were made within the EVE-P region. The first that we tested converts the sequence ATTT to GCTC (Fig. 6), which is nestled in a region of EVE-P that is similar to the mammalian octamer motif (ATTTGCAT; for review, see Levine and Hoey 1988). Footprint assays indicate that these base changes abolish binding of the *eve* protein to

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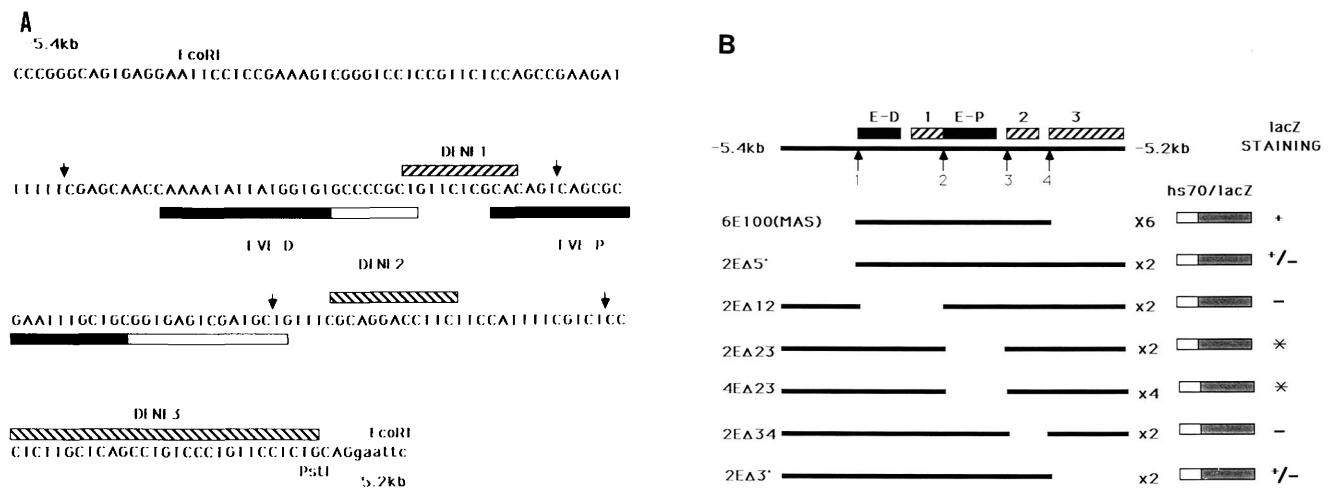


Figure 4. Summary of deletions in the 200-bp autoregulatory fragment. (A) Nucleotide sequence of the 200-bp autoregulatory element. The bars show the limits of factor-binding sites identified within this sequence. The three nuclear factor-binding sites are indicated by hatched bars above the sequence and are called DENF (*Drosophila* embryo nuclear factor) 1–3. The two *eve*-binding sites are indicated by the bars below the sequence. The solid regions show high-affinity sites, while the open regions indicate low-affinity sites. Vertical arrows show the sites where *BclI* sites were created by in vitro mutagenesis. (B) The top line corresponds to the autoregulatory element, and the bars represent the two *eve*-binding sites and three nuclear factor-binding sites. (E-D) EVE-D; (E-P) EVE-P. Numbers correspond to DENF1, etc. A total of four *BclI* restriction sites were created in the DNA fragment by in vitro mutagenesis, and their locations are indicated by vertical arrows, 1–4, arrayed from distal to proximal. Note that the *BclI* sites occur between factor-binding sites. Internal deletions were created within the autoregulatory fragment by digesting templates containing just one or two of the *BclI* sites and religating. Each mutagenized template was multimerized and inserted into the HZ50 expression vector. The series of horizontal lines shows the mutagenized fragments, lacking the indicated region of the 200-bp fragment. The numbers to the right indicate the numbers of copies inserted, in tandem, into the HZ50 expression vector. + and – (right) indicate relative activities of the heterologous promoters in P transformants. +/- corresponds to extremely weak staining, which is restricted to dorsal epidermis. The asterisks (*) indicate novel expression patterns of 14 stripes, rather than the normal 7-stripe pattern. The smallest DNA fragment that is able to direct autoregulatory activity extends from *BclI* sites 1 to 4 and corresponds to the MAS. Six copies of this 100-bp fragment direct weak expression in P-transformed embryos.

the mutagenized site within EVE-P (Fig. 7C). However, the mutation acts only locally, and binding is not altered in other regions of EVE-P. This mutation has no effect on the seven-stripe *eve* pattern (Fig. 8C), although the interstripes are more intense than those seen with comparable wild-type promoters (i.e., Fig. 8A). This result is consistent with the deletion analysis, indicating that EVE-P is involved in suppressing interstripe expression. The second mutation made in EVE-P converts the sequence CAGCG to GGATC (Fig. 6; SEP1). This region was selected for mutagenesis since it shares some similarity with the so-called class II *eve* recognition sequence, which includes the consensus TCAGCACCG (Hoey et al. 1988). The mutation abolishes binding to this site (data not shown) but has no effect on in vivo activity (Fig. 8B).

The limited effects of the two EVE-P mutations led us to create the double mutant (SEP3; Fig. 6), which knocks out both the TCAGCACCG-like site and the octamer-like element. This mutagenized DNA fragment shows a severe reduction in the binding of *eve* protein to the EVE-P region (Fig. 7D). Nonetheless, it is quite active in vivo and drives a *lacZ* expression pattern similar to the SEP2 mutation (see Fig. 8C), whereby interstripe expression is somewhat more intense than normal. This derepression of the interstripes might be due to impaired binding of the homeo box protein *en*, since *en*⁻ embryos show a

14-stripe *eve* pattern that persists during advanced stages when *eve* is normally off (Harding et al. 1986).

To test the possibility that the EVE-D and EVE-P regions function redundantly, we analyzed the effects of the triple mutant (NE; Fig. 6) on autoregulation. This mutation contains disruptions in the core EVE-D region, as well as in both of the high-affinity EVE-P-binding sites. Promoters containing this mutagenized fragment show a substantial reduction in expression (Fig. 8F) as compared with either the wild-type promoter (Fig. 8A) or promoters containing disruptions in just single sites (Fig. 8B–D). The embryo shown in Figure 8F displays the strongest staining observed for any of the transformed lines, and it is possible that this residual expression is due to the activities of the remaining weak *eve*-binding sites within the EVE-D and EVE-P regions. The virtual loss of autoregulatory activity suggests that the two *eve*-binding regions identified here play an important role in *eve* expression and represent bona fide *eve* regulatory sites.

The effects of mutagenizing the DENF1 and DENF2 sites suggest that they are also essential for autoregulation. Clustered 6-nucleotide substitutions were made in each site. For DENF1, the sequence TGTTCCT was converted to GTCGAC (SNF1; Fig. 6), which results in reduced binding to nuclear extracts (see Fig. 7E). This disruption of the DENF1 site causes a severe reduction in

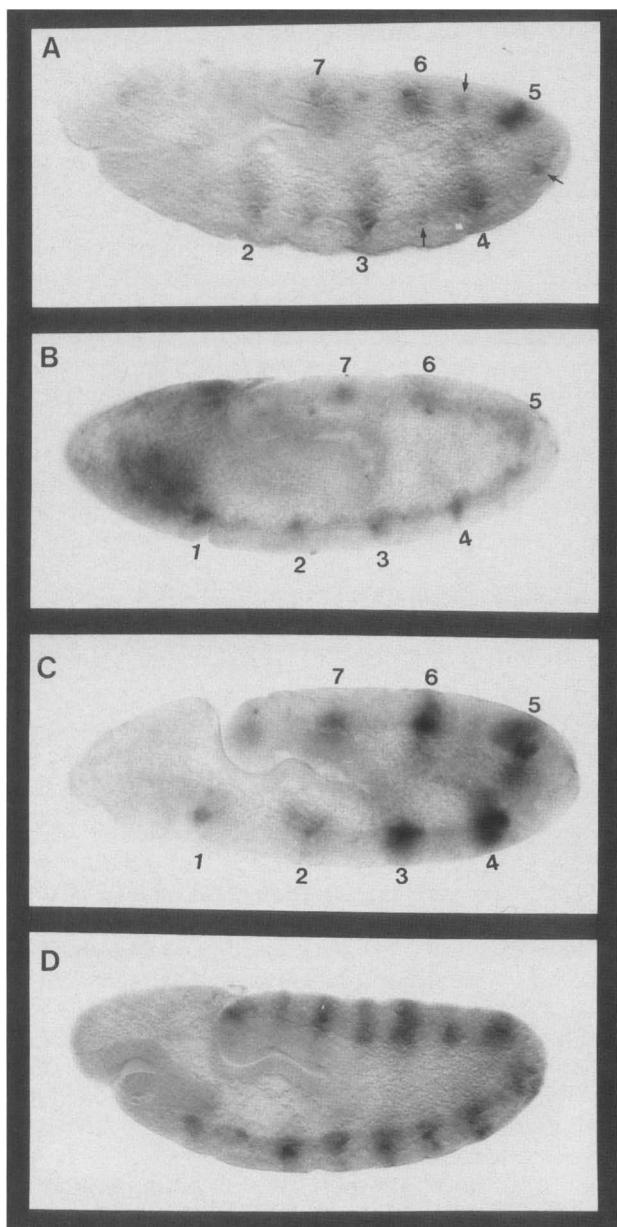


Figure 5. In vivo activities of deleted promoters. Embryos were collected from P transformants containing the expression vectors shown in Fig. 4B. Embryos were stained with anti- β -galactosidase antibodies and are oriented so that anterior is to the left and dorsal is up. The numbers refer to *eve* stripes, and the arrows indicate weak expression in interstripe regions. (A) Embryo expressing the HZ6E100 promoter, which includes six tandem copies of the 106-bp MAS. The pattern and intensity of expression are similar to that observed with two copies of the intact 200-bp fragment (see Fig. 2B). (B) The HZ2E Δ 5' promoter. This promoter lacks 60 bp from the 5' region of the 200-bp fragment, upstream of the *Bcl* 1 site. There is a substantial decline in expression, similar to that observed with just one copy of the intact 200-bp fragment (see Fig. 2A). (C) The HZ2E Δ 3' promoter. This lacks 3' sequences downstream from *Bcl* site 4, and removes the DENF3-binding site. The staining pattern is unaffected and appears quite similar to that obtained with two intact copies of the 200-bp fragment (see Fig. 2B). (D) The 4E Δ 23 promoter. This contains four tandem copies of a deleted fragment lacking sequences between *Bcl* sites 2 and 3, which removes the EVE-P region. A 14-stripe pattern is observed instead of the normal 7-stripe pattern.

autoregulatory activity (Fig. 8G). Similarly, substitution of the core CAGGAC sequence within DENF2 (SNF2; Fig. 6) nearly abolishes in vitro-binding activity (Fig. 7F) and causes a substantial reduction in autoregulatory activity in vivo (Fig. 8H). The near loss of in vivo activity resulting from mutations in the DENF1 and DENF2 sites is not due to altered binding of the *eve* protein to the EVE-D and EVE-P sites (data not shown). Instead, it would appear that the DENF1 and DENF2 factors act combinatorially with the *eve* protein to activate transcription.

Discussion

eve autoregulation is mediated by a distal enhancer element located between -5.9 and -5.2 kb upstream from the *eve* transcription start site. We have shown that mul-

timers of a 200-bp DNA fragment from this region function as well as or better than the full-length 700-bp autoregulatory element to activate transcription from a heterologous *hsp70* promoter. A 100-bp MAS was identified that contains two *eve*-binding sites, as well as two binding sites for nuclear factors present in early embryos. Mutations that interfere with the binding of any one of these factors cause a catastrophic loss in expression, which suggests a combinatorial mechanism for *eve* autoregulation, whereby the *eve* protein interacts with neighboring nuclear factors to function as a transcriptional activator.

Cooperative interactions among *eve* autoregulatory elements

Autoregulation has long been thought to play an impor-

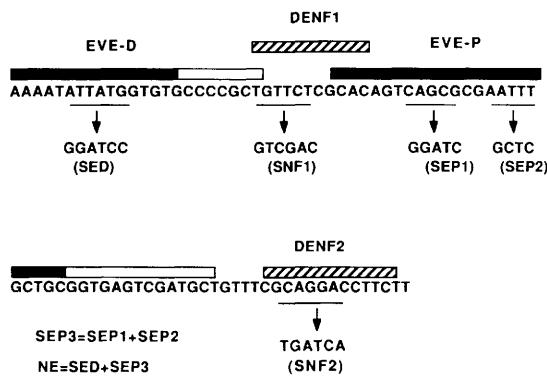


Figure 6. Summary of base substitutions in the autoregulatory element. The sequence shown here corresponds to an 85-bp region of the MAS, which contains the EVE-D-, EVE-P-, DENF1-, and DENF2-binding sites. The limits of these sites are indicated by the bars above the sequence. The filled regions of the EVE-D and EVE-P bars indicate high-affinity binding sites; the open regions denote low-affinity sites, which are protected only by high concentrations of the *eve* protein. Clusters of base substitutions were made using mutagenic oligonucleotides. The mutagenized sequence is indicated by the arrows. In some cases, double or even triple mutants were prepared. For example, the SEP3 mutant corresponds to the sum of the SEP1 and SEP2 mutations. NE is a triple mutant, which contains the SED mutation in EVE-D plus the SEP1 and SEP2 mutations in the EVE-P region. Two copies of the 200-bp fragment containing these various mutations were inserted in the HZ50 expression vector, and their *in vivo* activities were analyzed in P-transformed embryos.

tant role in transducing short-lived signals into stable patterns of gene expression during development (Meinhardt and Gierer 1980; Meinhardt 1986). Autoregulatory processes have been implicated in the expression of a number of patterning genes in *Drosophila*, including the gap gene *Krüppel* (*Kr*) (Warrior and Levine 1990), the pair-rule genes *eve* (Frasch et al. 1988; Goto et al. 1989; Harding et al. 1989) and *ftz* (Hiromi and Gehring 1987; Pick et al. 1990), and the homeotic selector genes *Deformed* (*Dfd*) (Kuziora and McGinnis 1988) and *Ultrabithorax* (*Ubx*) (Muller et al. 1989). The type of autoregulation exhibited by *Dfd* could be distinct from that observed for segmentation genes since it is stably expressed throughout embryogenesis and larval development (Chadwick and McGinnis 1987). In contrast, segmentation genes such as *Kr*, *ftz*, and *eve* are transiently expressed in the early embryo, and in these cases autoregulation is thought to be important for refining and maintaining expression during brief, critical periods of development. Despite the apparent differences between *Dfd* autoregulation and the type represented by *eve*, in both cases the situation is probably more complex than simple auto-feedback of the encoded protein with an autoregulatory element. There is evidence that both the *Dfd* and *eve* proteins act in concert with other spatially restricted factors to effect autoregulation (Frasch et al. 1988; Jack et al. 1988).

The refinement of the *eve* stripes might involve coop-

erative interactions among multiple, functionally equivalent sequences in the distal *eve* promoter. We have shown that multimers of short autoregulatory elements (200 or 100 bp) from the *eve* promoter are strongly activated by the *eve* protein. There is a dramatic increase in reporter gene expression as the 200-bp element is multimerized from one to two to four copies. Such cooperative interactions of the *eve* protein with distal promoter sequences could help generate a nonlinear response, causing the transition of a smooth gradient (early stripe) into a refined stripe with a sharp anterior border. This type of mechanism has been demonstrated for the activation of the gap gene *hunchback* (*hb*) by the *bicoid* (*bcd*) morphogen (Struhl et al. 1989; Driever et al. 1989). At least two types of cooperativity can be envisioned, including cooperative binding to DNA and/or cooperative interactions of the *eve* protein with general transcription factors active in proximal regions of the promoter (e.g., Lin et al. 1990). We favor the latter mechanism since there is no indication that the *eve* protein binds cooperatively to the EVE-D and EVE-P regions of the autoregulatory element. For example, mutations in EVE-D do not reduce binding to EVE-P, and vice versa (see Fig. 7B,D).

Identification of bona fide *eve* recognition sequences

Previous *in vitro* binding studies have led to the identification of numerous *eve* recognition sequences in several promoters, including *eve* (Hoey et al. 1988), *en* (Hoey and Levine 1988), *Ubx* (Biggin and Tjian 1989), and *Dfd* (M. Regulski and B. McGinnis, pers. comm.). Several of these sites were shown to mediate repression by *eve* in either transient cotransfection assays (Han et al. 1989) or *in vitro* transcription experiments (Biggin and Tjian 1989). However, in no case were any of the binding sites shown to mediate an authentic genetic interaction in the embryo. Here, we have identified *eve*-binding sites that are critical for function *in vivo*. These *eve* sites function redundantly since removal of the high-affinity sites in either EVE-D or EVE-P had little or no effect on expression. However, *in vivo* activity was severely reduced when both regions of binding were mutated. The simplest interpretation of these results is that the *eve* protein directly interacts with the EVE-D and/or EVE-P regions, although we cannot rule out the possibility that *eve* regulates the expression of an unknown intermediate regulatory factor which, in turn, binds to the *eve* autoregulatory element.

It is not clear why certain *eve*-binding sites are regulated by the *eve* protein *in vivo* while others are not. For example, high-affinity *eve*-binding sites in the *en* promoter fail to direct reporter gene expression in response to the endogenous *eve* protein in P-transformed embryos (Vincent et al. 1990; R. Warrior and K. Han, pers. comm.). Comparative footprint assays indicate that the *eve* protein has two- to fourfold higher affinity for these sites than those identified in the *eve* autoregulatory element (J. Jiang and T. Hoey, unpubl.). There are several

Autoregulation of the homeo box gene eve

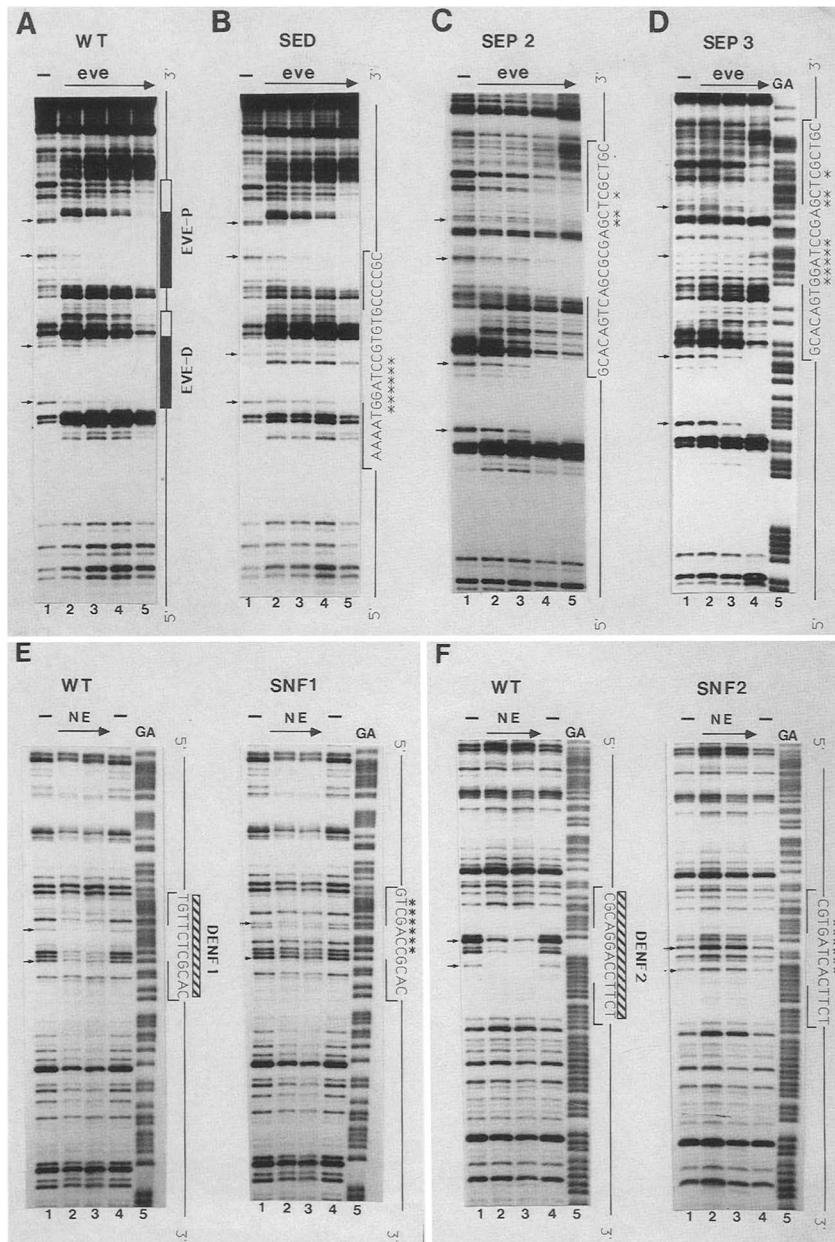


Figure 7. Base substitutions in the EVE-D, EVE-P, DENF1, and DENF2 sites disrupt DNA binding. DNase I footprint assays were done with the mutagenized DNA templates shown in Fig. 6. (A–D) Bacterial extracts containing a full-length eve protein were used in these assays, along with the appropriate 200-bp DNA fragment, 32 P-labeled on the 5' end. For A, B, and C, lanes 2–5 contain progressive twofold increases in the amount of eve protein. The gel shown in D contains fourfold increases in the amount of protein in lanes 2–4, and lane 5 shows the G + A sequence of the DNA fragment. In all cases, lane 1 is a negative control, lacking added eve protein. (A) The wild-type 200-bp fragment. The arrows at left indicate fragments that are protected by increasing amounts of the eve protein. The same bands are indicated by the arrows in the other panels, which show footprint assays with mutagenized templates. (B) The SED mutation, which changes the core ATTA sequence in the EVE-D region. At least two to four times more eve protein is needed to protect the bands indicated by the two arrows as compared with the wild-type template shown in A. The diagram at right shows the location of the mutagenized sequence (indicated by asterisks). Despite this reduced binding to EVE-D, there is no effect on the filling of the EVE-P region. (C) The SEP2 mutation, which changes sequences within the core "octamer-like" motif in a high-affinity region of EVE-P. This mutation causes a narrowing in the limits of protection obtained with eve protein. The top-most arrow indicates a doublet of bands adjacent to the mutagenized sequences, which were not protected even by the highest levels of eve protein that were used. In contrast, these bands are protected by even lower levels of eve protein in the wild-type and SED (EVE-D mutant) templates (see A and B). However, this mutation has little or no effect on binding to the region indicated by the second arrow from the top. (D) The SEP3 mutant, which contains substitutions in two different high-affinity regions of EVE-P. Even the highest concentrations of eve protein (lane 4) give little or no protection of the regions indicated by the top two arrows. However, binding to the low-affinity sequence just "above" the top-most arrow is still observed. Furthermore, despite the loss of binding to the high-affinity sites in EVE-P, binding of the eve protein to EVE-D appears normal. These results suggest that the eve protein does not bind to the autoregulatory element in a cooperative manner. (E) Wild-type (left) and mutagenized (right) DNA templates from the 200-bp autoregulatory region were used for DNase I protection assays. The DNA fragments were 32 P-labeled on the noncoding strand at the 3' end. Increasing amounts of nuclear extract from 0- to 4-hr embryos were used in lanes 2 and 3. The sequences to the right of the autoradiograms indicate the sites that were mutagenized in the DENF1 site (*). The small arrows (left) indicate bands within the DENF1 site. These bands are protected by at least a factor of 3 to 4 in the wild-type template but are barely changed with even high concentrations of nuclear extract in the mutagenized template. The – lanes show the DNase I pattern without added protein; GA lanes shows the G + A sequence of the fragment. (F) Wild-type (left) and mutagenized (right) templates that were incubated with increasing amounts of nuclear extract from 4- to 10-hr embryos. The fragments were 32 P-labeled on the noncoding strand at the 3' end. The sequences to the right of the autoradiograms indicate the base substitutions within the DENF2 site. The arrows identify bands that reside within the DENF2 site. The SNF2 mutation in the DENF2 site completely abolishes DNA binding.

arrow from the top. (D) The SEP3 mutant, which contains substitutions in two different high-affinity regions of EVE-P. Even the highest concentrations of eve protein (lane 4) give little or no protection of the regions indicated by the top two arrows. However, binding to the low-affinity sequence just "above" the top-most arrow is still observed. Furthermore, despite the loss of binding to the high-affinity sites in EVE-P, binding of the eve protein to EVE-D appears normal. These results suggest that the eve protein does not bind to the autoregulatory element in a cooperative manner. (E) Wild-type (left) and mutagenized (right) DNA templates from the 200-bp autoregulatory region were used for DNase I protection assays. The DNA fragments were 32 P-labeled on the noncoding strand at the 3' end. Increasing amounts of nuclear extract from 0- to 4-hr embryos were used in lanes 2 and 3. The sequences to the right of the autoradiograms indicate the sites that were mutagenized in the DENF1 site (*). The small arrows (left) indicate bands within the DENF1 site. These bands are protected by at least a factor of 3 to 4 in the wild-type template but are barely changed with even high concentrations of nuclear extract in the mutagenized template. The – lanes show the DNase I pattern without added protein; GA lanes shows the G + A sequence of the fragment. (F) Wild-type (left) and mutagenized (right) templates that were incubated with increasing amounts of nuclear extract from 4- to 10-hr embryos. The fragments were 32 P-labeled on the noncoding strand at the 3' end. The sequences to the right of the autoradiograms indicate the base substitutions within the DENF2 site. The arrows identify bands that reside within the DENF2 site. The SNF2 mutation in the DENF2 site completely abolishes DNA binding.

possible explanations to account for discrepancies between the efficiency of binding in vitro and activity in vivo. Perhaps *eve* is a very weak transcriptional activa-

tor, and only clusters of binding sites in exactly the correct orientation relative to the basal promoter work in vivo. Some support for this possibility comes from the

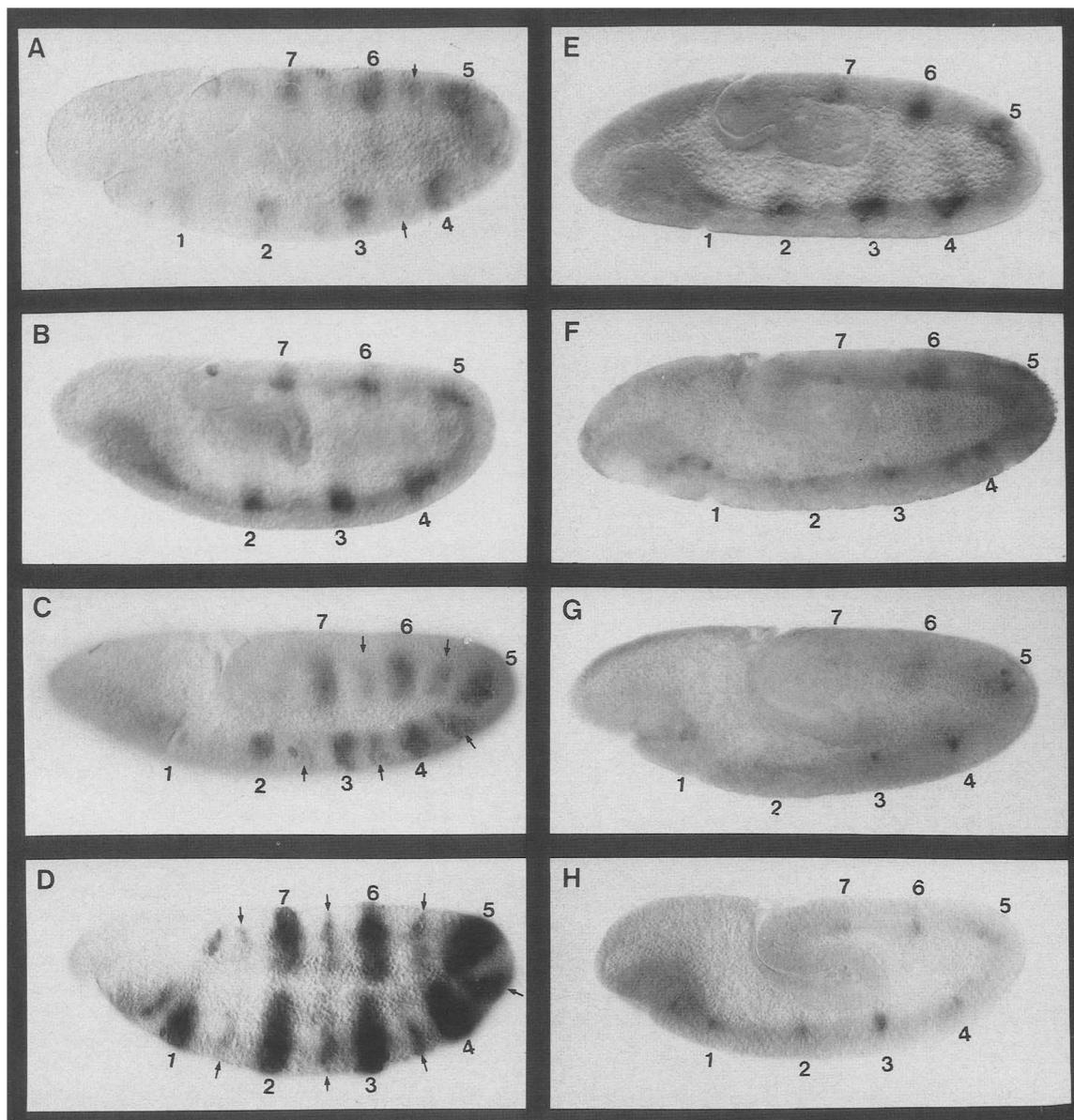


Figure 8. Effects of point mutations on in vivo expression. Embryos were collected from P transformants and stained with anti- β -galactosidase antibodies, and are oriented with anterior to the left and dorsal up. The numbers refer to *eve* expression stripes; the arrows indicate expression in interstripe regions. (A) Wild-type control, showing the staining pattern obtained with two copies of the normal 200-bp autoregulatory element. (B) The HZ2SEP1 promoter, which contains two copies of the SEP1 mutation in EVE-P. This mutation has little or no effect on expression. (C) The HZ2SEP2 promoter, which contains two copies of the SEP2 mutation (in the octamer-like element) in EVE-P. This mutation has no effect on the seven stripes of expression but causes an increase in interstripe expression. (D) The HZ4SEP2 promoter, which contains four copies of the SEP2 template. The seven stripes are strong and sharp, but there is a substantial elevation in the levels of interstripe expression. (E) The HZ2SED promoter, which contains two copies of the SED mutation in EVE-D. There is at most only a slight reduction in expression. (F) The HZ2NE promoter, which contains two copies of a triple mutant that impairs binding to EVE-D and the two high-affinity sites within EVE-P. There is a severe reduction in expression. (G) The HZ2SNF1 promoter, which contains two copies of the SNF1 mutation in the DENF1 site. There is a severe reduction in expression. (H) The HZ2SNF2 promoter, which contains two copies of the SNF2 mutation in the DENF2 site. There is a significant reduction in expression.

orientation dependence of *eve* autoregulatory sequences (see Figs. 1 and 2). Alternatively, it is conceivable that *eve* must act in concert with other factors bound to neighboring sites to function as a transcriptional activa-

tor. According to this “lock and key” model, a functional *eve* response element must contain a proper arrangement of binding sites both for the *eve* protein and neighboring cofactors (summarized in Fig. 9).

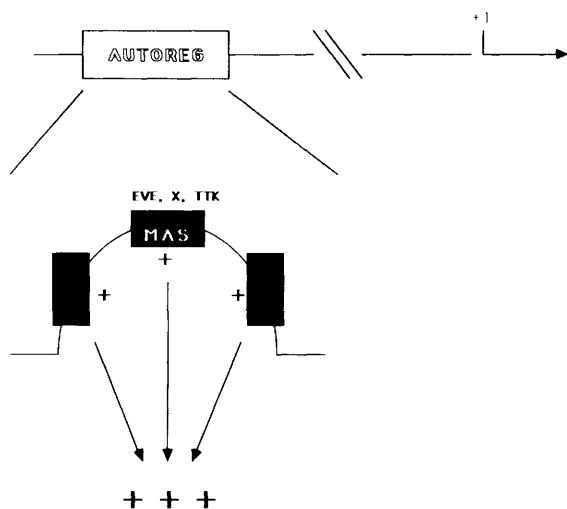


Figure 9. Summary of cooperative and combinatorial interactions underlying *eve* autoregulation. The top line represents the *eve* promoter; the autoregulatory element is located between -5.9 and -5.2 kb upstream from the transcription start site. We have presented evidence that this element might be composed of multiple copies of a 100-bp MAS, which is represented by the solid rectangle. Each copy of the MAS contains binding sites for *eve*, DENF1, and DENF2. It is possible that DENF2 corresponds to *ttk*. The third factor is denoted X and might correspond to the GAGA protein. Combinatorial interactions among these three proteins are responsible for each MAS mediating weak activation in response to the *eve* protein. Full activation depends on cooperative interactions among multiple MAS elements.

Combinatorial interactions between *eve* and nuclear factors

At least three proteins, *eve*, DENF1, and DENF2, are essential for *eve* autoregulation. DENF1 and DENF2 probably represent different proteins since their binding sites are totally distinct (see sequence, Fig. 4A). Mutations that reduce binding of any one of these factors cause a severe reduction or loss in *in vivo* activity. This catastrophic loss in expression suggests that *eve*, DENF1, and DENF2 act combinatorially to activate *eve* transcription. If the proteins were functioning in an additive manner, then the removal of any one binding site should cause a relatively mild reduction in expression. There are at least two types of models to account for combinatorial interactions among these proteins. First, interactions might permit cooperative binding of the *eve* protein to DNA. *eve* alone does not bind with very high affinity to the autoregulatory element, and perhaps it is targeted to these sites by appropriate protein-protein interactions with the neighboring DENF1 and DENF2 proteins. This mechanism has been demonstrated in the yeast mating-type system, where the binding of the homeo box protein α 2 to α -specific promoters depends on cooperative interactions with the general transcription factor MCM1 (Keleher et al. 1988). A nonexclusive alternative is that protein-protein interactions might cause conformational changes in the *eve*, DENF1, or DENF2

proteins, which unmasks their activation domains. Perhaps these proteins fail to function as effective transcriptional activators when in isolation.

To sort out the exact mechanism underlying combinatorial interactions among these proteins, it will be necessary to clone and characterize the DENF1 and DENF2 factors. It is possible that DENF2 corresponds to a zinc finger protein called *ttk*, which is expressed in early embryos (Harrison and Travers 1990). DENF1 contains some sequence similarities with the binding sites for a general factor called GAGA (Biggin and Tjian 1988; Soeller et al. 1988), which binds to a number of different promoters expressed in early embryos, including *Ubx* and *en*.

Materials and methods

Construction of *eve/hsp70* heterologous promoters

The starting plasmid (pBlu 1E) for most of our studies corresponds to a 200-bp *Sma*I-*Pst*I DNA fragment from a distal region of the *eve* promoter, between -5.4 and -5.2 kb upstream from the transcription start site, inserted into the pBluescript vector (Stratagene, La Jolla, CA). Previous studies have shown that this fragment is sufficient to drive weak autoregulatory activity (Harding et al. 1989). Multimerization, deletion, and base substitutions within this fragment were all done in the pBluescript vector, and the derivatives were then excised with *Xba*I-*Kpn*I and inserted into the HZ50 P-transformation vector (Hiromi and Gehring 1987). To construct a heterologous promoter containing two copies of the 200-bp fragment, we inserted a 200-bp *Eco*RI fragment into the pBlu 1E vector, which already contained one copy of the fragment. There is a native *Eco*RI site located 13 bp downstream from the *Sma*I site in the *eve* sequences, and the second *Eco*RI site is derived from the polylinker of the vector. The *Eco*RI fragment was blunted by filling in the ends with Klenow and cloned into the *Sma*I site of the pBlu 1E vector. Plasmids were selected that contained two tandem copies of fragment and were removed from the vector in a single fragment using *Xba*I-*Kpn*I. This 400-bp fragment was fused into the HZ50 vector to obtain the HZ2E00 construct (see Fig. 1). The four-copy promoters were prepared by ligating the 200-bp *Eco*RI into the unique *Eco*RI site of the pBluescript vector. A high ratio of insert to vector was used, and recombinant plasmids were screened for those that contain four tandem repeats. In general, we found that there was a selection against recombinants containing the fragment in "head-to-head" orientations. Tandem repeats in either orientation relative to the transcription start site were inserted separately into the HZ50 vector.

In vitro mutagenesis was done to create four separate *Bcl*I sites along the length of the 200-bp *Eco*RI fragment (see below). Templates containing individual and almost all pairwise combinations of these sites were used to create specific truncations and internal deletions within the 200-bp autoregulatory fragment. For example, to delete the EVE-P region, a 200-bp fragment containing only the *Bcl*I sites 2 and 3 was used (see map, Fig. 4). A pBluescript recombinant plasmid containing this template was digested with *Bcl*I (there are no such sites in the vector) and religated. The internally deleted DNA fragment was excised from the vector with *Sma*I-*Pst*I, filled in with T4 polymerase, and ligated into the unique *Sma*I site of the pBluescript recombinant containing one copy of the corresponding truncated fragment. To obtain four copies of the Δ 23 fragment, we removed the two-copy insert with *Xba*I-*Eco*RV and ligated it

into the *Xba*I–*Sma*I sites in a pBluescript plasmid already containing two copies of the Δ23 fragment.

To create terminal truncations within the 200-bp fragment, templates were used that contain either the *Bcl*I site 1 or 4. For deletion of the 5' region of the 200-bp fragment, a plasmid containing an insert with *Bcl*I 1 was digested with a *Bcl*I–*Bam*HI fragment [a *Bam*HI site is located 6 bp upstream from the *Sma*I site within the polylinker]. After digestion, the plasmid was religated, and the fragment was removed from the vector using *Xba*I and *Eco*RV. The *Xba*I site was filled in with Klenow, and the fragment was cloned into the unique *Eco*RV site of a pBluescript recombinant already containing the deleted 200-bp fragment lacking sequences between the *Bam*HI and *Bcl*I 1 sites. To remove 3' sequences from the 200-bp auto-regulatory fragment, a template was used containing *Bcl*I site 4. A recombinant plasmid containing this template was digested with *Xba*I–*Bcl*I, and this truncated fragment (lacking sequences 3' to the *Bcl*I site 4) was inserted into pBluescript cut with *Xba*I and *Bam*HI. The truncated fragment was removed from the recombinant plasmid by digesting with *Eco*RV and *Bam*HI. The *Bam*HI site was filled-in with Klenow and the fragment was inserted into the unique *Eco*RV site of a plasmid that already contained one copy of the truncated fragment.

Site-directed mutagenesis

Base substitutions in the binding sites were made with appropriate mutagenic oligonucleotides using the "muta-gene" kit purchased from Bio-Rad (Richmond, CA). Mutagenic oligonucleotides contained 9–10 bases of flanking sequence on either side of the mutagenized sequence. Mutations were made that created restriction sites to facilitate screening. For example, the mutagenic oligonucleotide GACTGTGCGGTCGACCCGG-GGCAC was used to disrupt the DENF1-binding site. The base substitutions are underlined and correspond to a *Sall* restriction site. Mutagenesis was done essentially as described in the instructions that came with the kit, except that single-stranded DNA templates were made directly from the pBluescript vector. After annealing the mutagenic oligonucleotide with the single-stranded template, polymerization was driven by T4 DNA polymerase and terminated by ligation with T4 ligase. The double-stranded templates were transformed into the DH5 strain of *E. coli*, and mutant templates were identified by screening mini-preparations of DNA for the restriction sites created by mutagenesis. In all cases, the identities of mutant templates were confirmed by DNA sequencing. For some of the templates, we created double mutants by using two different mutagenic oligonucleotides in the same polymerization reaction. For example, the triple mutant NE, which disrupts the EVE-D region and two binding sites within the EVE-P region, was made by annealing two different mutagenic oligonucleotides at the same time. One of the oligonucleotides generated the SED 1 substitutions in EVE-D, whereas the other created both the SEP1 and SEP2 substitutions in EVE-P (see Fig. 6).

P-element transformations and antibody staining

Heterologous fusion promoters were introduced into the *Drosophila* germ line via P-element-mediated transformation, as described by Rubin and Spradling (1982). The *rosy*⁵⁰⁶ line was used for all injections.

The expression patterns of the *lacZ* reporter gene were examined by staining whole-mount embryos with a 1 : 1000 dilution of a rabbit anti-β-galactosidase antibody (purchased from Organon Teknika, West Chester, PA). Histological staining was done with the Elite ABC kit from Vector Labs (Burlingame, CA)

using biotinylated anti-rabbit immunoglobulin antibodies and horseradish peroxidase conjugated to streptavidin.

Preparation of bacterial eve protein and embryo nuclear extracts

The *eve* protein was overexpressed in bacteria using the T7 promoter, exactly as described by Hoey et al. (1988). After induction, bacterial extracts were prepared in guanidine-HCl and renatured by dialysis as described by Hoey and Levine (1988).

Embryos were collected at 25°C from population cages of the Oregon-R strain and aged to give distributions of either 0–4 or 5–10 hr. To accumulate large amounts of material for extraction, staged embryos were kept at 4°C for as long as 3 days. Similarly aged embryos were pooled, dechorionated in sodium hypochlorite, and washed first in NaCl/Triton X-100 and finally in water. Nuclear extracts were prepared essentially as described previously (Biggin and Tjian 1988; Soeller et al. 1988). We found that nuclear extracts from 5- to 10-hr embryos contained higher DENF2-binding activity than extracts from 0- to 4-hr embryos. DENF1 activity is quite weak in 5- to 10-hr extracts and is somewhat higher in 0- to 4-hr extracts. Extracts prepared from 0- to 4-hr embryos were used directly for DNA binding, while 5- to 10-hr extracts were first fractionated in a heparin–agarose column, as described by Heberlein et al. (1985).

DNA-binding assays

DNase I footprint assays were done essentially as described previously (Heberlein et al. 1985; Hoey et al. 1988). Binding reactions were done with ~5 ng of ³²P-labeled DNA in 50 μl of a buffer containing 110 mM KCl, 47.5 mM HEPES (pH 7.9), 13.75 mM MgCl₂, 1 mM DTT, 17% glycerol, and 0.05% NP-40. After incubating for 60 min on ice, 50 μl of 10 mM MgCl₂ and 5 mM CaCl₂ was added to the reaction, followed by freshly diluted DNase I (purchased from Worthington). Binding reactions were done with the bacterial *eve* protein including 1 μg of poly[d(I-C)] as carrier, and the DNase I digestion was done for 2–3 min on ice using a final concentration of 0.4 μg/ml. Binding reactions with nuclear extracts contained 0.5 μg of poly[d(I-C)] and a total of ~50 μg of total protein extract. Because the crude extract contains a high level of exonuclease activity, MgCl₂ was omitted from the binding buffer. DNase I digestions were done with 10-fold more DNase I (than for the *eve* reactions), and digestions were done at room temperature for 1 min. DNase I reactions were stopped by adding 90 μl of 1% SDS, 20 mM EDTA, 200 mM KCl, 250 μg/ml of yeast tRNA. The samples were extracted twice with phenol–chloroform (1 : 1), ethanol-precipitated, and electrophoresed in 8% polyacrylamide–7.5 M urea gels. The DNA fragments used for footprinting were prepared by labeling with [γ -³²P]ATP and polynucleotide kinase, and were isolated from native polyacrylamide gels.

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