The EMBO Journal vol.11 no.11 pp.4047-4057, 1992

Regulation of *even-skipped* stripe 2 in the *Drosophila* embryo

Stephen Small, Adrienne Blair and Michael Levine

Biology Department, Center for Molecular Genetics, Bonner Hall, UCSD, La Jolla, CA 92093-0322, USA

Communicated by E.Hafen

In an effort to determine how crude gradients of transcriptional activators and repressors specify sharp stripes of gene expression in the early embryo, we have conducted a detailed study of *even-skipped* (*eve*) stripe 2. A combination of promoter fusions and P-transformation assays were used to show that a 480 bp region of the *eve* promoter is both necessary and sufficient to direct a stripe of *LacZ* expression within the limits of the endogenous *eve* stripe 2. The maternal morphogen *bicoid* (*bcd*) and the gap proteins *hunchback* (*hb*), *Kruppel* (*Kr*) and *giant* (*gt*) all bind with high affinity to closely linked sites within this small promoter element. Activation appears to depend on cooperative interactions among *bcd* and *hb* proteins, since disrupting single binding sites cause catastrophic reductions in expression. *gt* is directly involved in the formation of the anterior border, although additional repressors may participate in this process. Forming the posterior border of the stripe involves a delicate balance between limiting amounts of the *bcd* activator and the *Kr* repressor. We propose that the clustering of activator and repressor binding sites in the stripe 2 element is required to bring these weakly interacting regulatory factors into close apposition so that they can function both cooperatively and synergistically to control transcription.

Key words: *Drosophila*/*even-skipped* stripe 2/embryogenesis/transcription

Introduction

Interactions among the segmentation genes in *Drosophila* probably constitute the best characterized cascade of transcription factors known for any developmental process in a higher eukaryote. This cascade transduces broad gradients of maternal morphogens, such as *bicoid* (*bcd*), into highly refined patterns of gene expression that are crucial for the establishment of the segmented body plan. This process involves the progressive refinement in patterns of gene expression, whereby segmentation genes at each step in the hierarchy make relatively sharp on/off choices in response to more crudely distributed regulatory factors (reviewed by Ingham, 1988; Carroll, 1990; Pankratz and Jackle, 1990; Small and Levine, 1991).

The first evidence of a metameric body pattern is the expression of each of the primary pair-rule genes in a repeating series of seven transverse stripes in precellular embryos. We have examined the regulation of one of these primary pair-rule genes, *even-skipped* (*eve*) (Harding *et al.*, 1986; Macdonald *et al.*, 1986; Frasch *et al.*, 1987), to determine how stripes are formed. The *eve* protein is first detected during nuclear cleavage cycle 12, when it is uniformly distributed in all nuclei. By the onset of cycle 14 the protein is repressed at both poles and forms a sharp boundary in the region of the presumptive cephalic furrow. During a period of just 20–30 min this pattern gives way to a series of seven stripes, each spanning 5–6 nuclei (Frasch and Levine, 1987). There is considerable evidence from genetic studies that the generation of these stripes involves the gap class of segmentation genes (Frasch and Levine, 1987; Goto *et al.*, 1989; Stanojevic *et al.*, 1989, 1991; Small *et al.*, 1991). Each of the five best characterized gap genes is expressed in one or two broad domains that include several adjacent segment primordia (Gaul and Jackle, 1987; Tautz, 1988; Pignoni *et al.*, 1990). All five have been implicated in the establishment of the seven stripe pattern because mutations in any one of them disrupt the formation of a distinct subset of stripes. For example, in *Kruppel*− (*Kr*−) embryos *eve* stripes 2–6 are replaced by two broad bands, while in *giant*− (*gt*−) embryos stripes 1 and 2, and 5 and 6 are fused (Frasch and Levine, 1987).

Promoter fusion studies indicate that individual primary pair-rule stripes are regulated by separate *cis* elements. For *h*, *cis* elements have been identified for nearly all of the stripes (Howard *et al.*, 1988; Pankratz *et al.*, 1990; Howard and Struhl, 1990; Riddihough and Ish-Horowitz, 1991). The identification of stripe initiation elements is not as complete for *eve*, but discrete regions have been identified for stripes 2 and 3 (Goto *et al.*, 1989; Harding *et al.*, 1989). This organization of the *eve* and *h* promoters is quite distinct from the promoter of the secondary pair-rule gene, *ftz* (Hiromi *et al.*, 1985; Hiromi and Gehring, 1987; Dearolf *et al.*, 1989). For the most part, disruptions in the *cis* sequences responsible for the periodic *ftz* pattern do not uncouple individual stripes, but instead exert similar effects on all of the stripes (Dearolf *et al.*, 1989).

We have conducted a detailed study of *eve* stripe 2 since there is considerable information about both its *cis* and *trans* regulation. Promoter fusion studies have shown that a truncated *eve* promoter containing ~1.7 kb of 5′ flanking sequence is sufficient to drive the expression of a *LacZ* reporter gene within the limits of stripe 2 (Goto *et al.*, 1989; Harding *et al.*, 1989). There are anterior and posterior expansions of the stripe borders when this fusion gene is crossed into *gt*− and *Kr*− embryos, respectively (Small *et al.*, 1991). In *bcd*− and *hb*− embryos the stripe is abolished or reduced. These and other genetic studies (Frasch and Levine, 1987; Goto *et al.*, 1989) suggest the following model for stripe 2 regulation. The gap gene *hb* acts in concert with the maternal morphogen *bcd* to activate stripe 2 expression. The borders of the stripe are formed through selective repression by the gap gene *gt* in anterior regions and *Kr* in posterior regions.

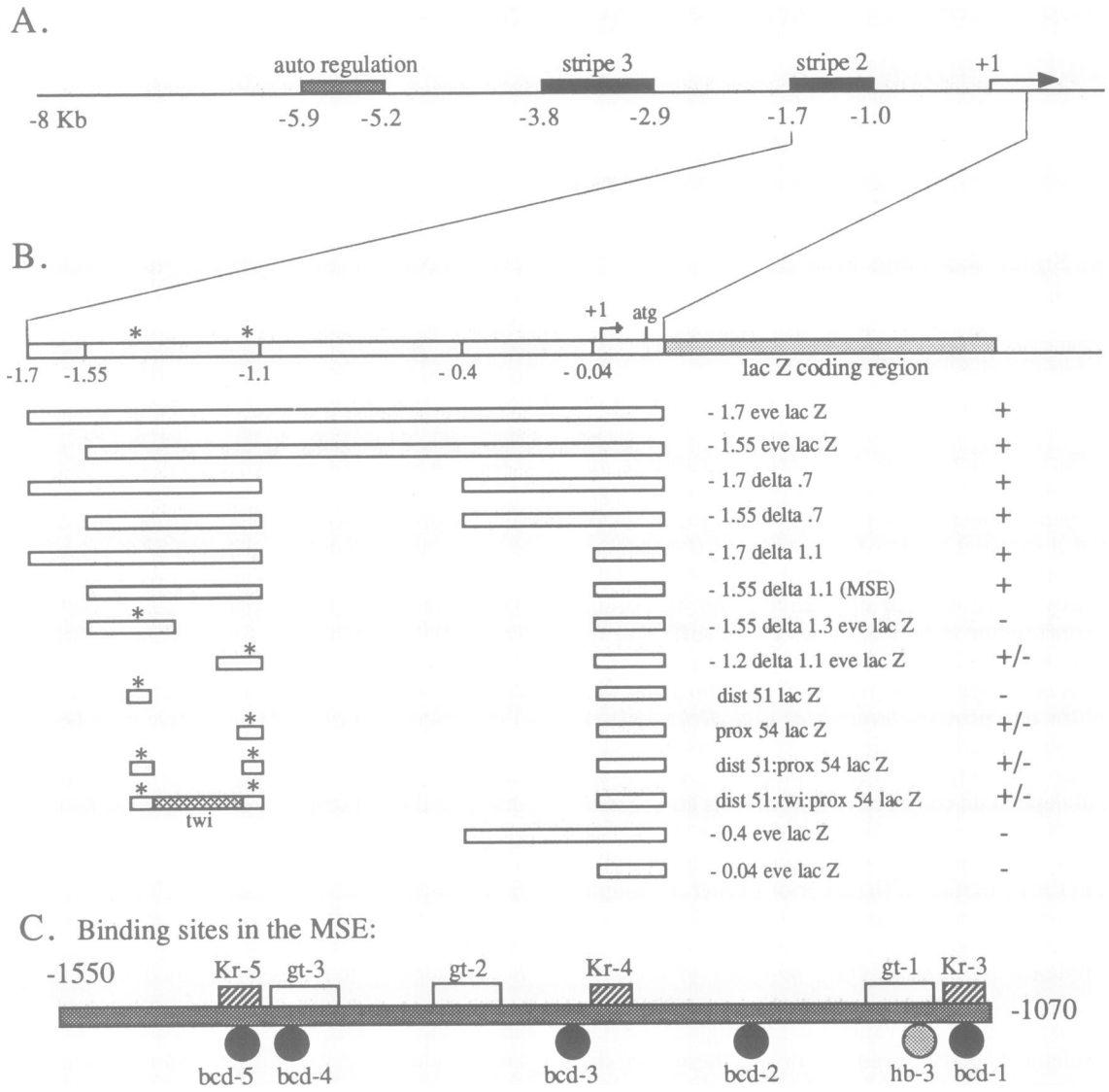

Fig. 1. Summary of *eve−LacZ* fusion genes. **A.** The horizontal line represents 8 kb of *eve* 5′ flanking sequences. This interval has been shown to contain discrete regulatory elements responsible for the initiation of stripes 2 and 3, as well as an autoregulatory element that controls the maintenance and refinement of all seven stripes during gastrulation (Goto *et al.*, 1989; Harding *et al.*, 1989). **B.** The rectangle represents the starting *eve−LacZ* fusion gene used for most of the P-transformation studies (Lawrence *et al.*, 1987). The unfilled portion of the rectangle corresponds to *eve* sequences, which includes the region from −1.7 kb upstream from the transcription start site and ends at codon #22 in the protein coding sequence at +160. The stippled region of the rectangle indicates the *LacZ* coding sequence. Asterisks indicate the locations of the two clusters of factor binding sites in the stripe 2 element (see Small *et al.*, 1991). The horizontal bars below the 1.7 kb *eve−LacZ* fusion gene represent the different truncated and deleted forms of the promoter that were tested in this study. The bars indicate sequences that were retained in each of the fusions. For example, the −1.55 delta 1.1 fusion gene contains the sequences from −1.55 kb to −1.1 kb and the basal promoter region up to −42 bp. It lacks sequences between −1.1 kb and −42 bp, as indicated by the gap between the horizontal bars. This construct is denoted 'MSE' because it represents the minimal stripe element that directs stripe 2 expression. The 'dist51:twi:prox54 *LacZ*' construct includes a 51 bp sequence that spans the distal cluster of factor binding sites and a 54 bp sequence containing the proximal cluster of sites. These are separated by a 400 bp region of the *twist* (*twi*) promoter that has no apparent function (Jiang *et al.*, 1991b). The + and − to the right of the figure indicate whether the corresponding *eve−LacZ* fusion gene specifies stripe 2 in P-transformed embryos. **C.** Summary of factor binding sites in the minimal stripe 2 element. The horizontal bar represents the 480 bp region that is sufficient to direct stripe 2 expression. It contains a total of five *bcd*, one *hb*, three *Kr* and three *gt* binding sites. The *bcd* and *hb* activator sites are shown below the map and the locations of the *Kr* and *gt* repressors are indicated above. Note the close linkage between activator and repressor binding sites.

Recent studies suggest that the four genetically defined regulators of stripe 2 expression act directly on the *eve* promoter and modulate its transcription. Proteins encoded by all four genes have been shown to bind with high affinity to sequences within the *eve* promoter that are essential for stripe 2 expression (Stanojevic *et al.*, 1989; Small *et al.*, 1991). Interestingly, virtually all of the *bcd* and *hb* binding sites overlap with, or are closely linked to, a *Kr* or *gt* recognition sequence. In preliminary experiments, mutations in some of these binding sites caused genetically predicted changes in the levels and limits of stripe 2 expression. In particular, mutations in two of the five *bcd* sites present in the stripe 2 element caused reduced levels of expression, while deletions of the three *gt* binding sites resulted in an anterior expansion of the stripe, similar to that observed in *gt−* mutants (Stanojevic *et al.*, 1991).

Here we define a 480 bp region of the *eve* promoter that is both necessary and sufficient to direct the expression of a *LacZ* reporter gene within the normal limits of the endogenous *eve* stripe 2. Expression driven by this minimal

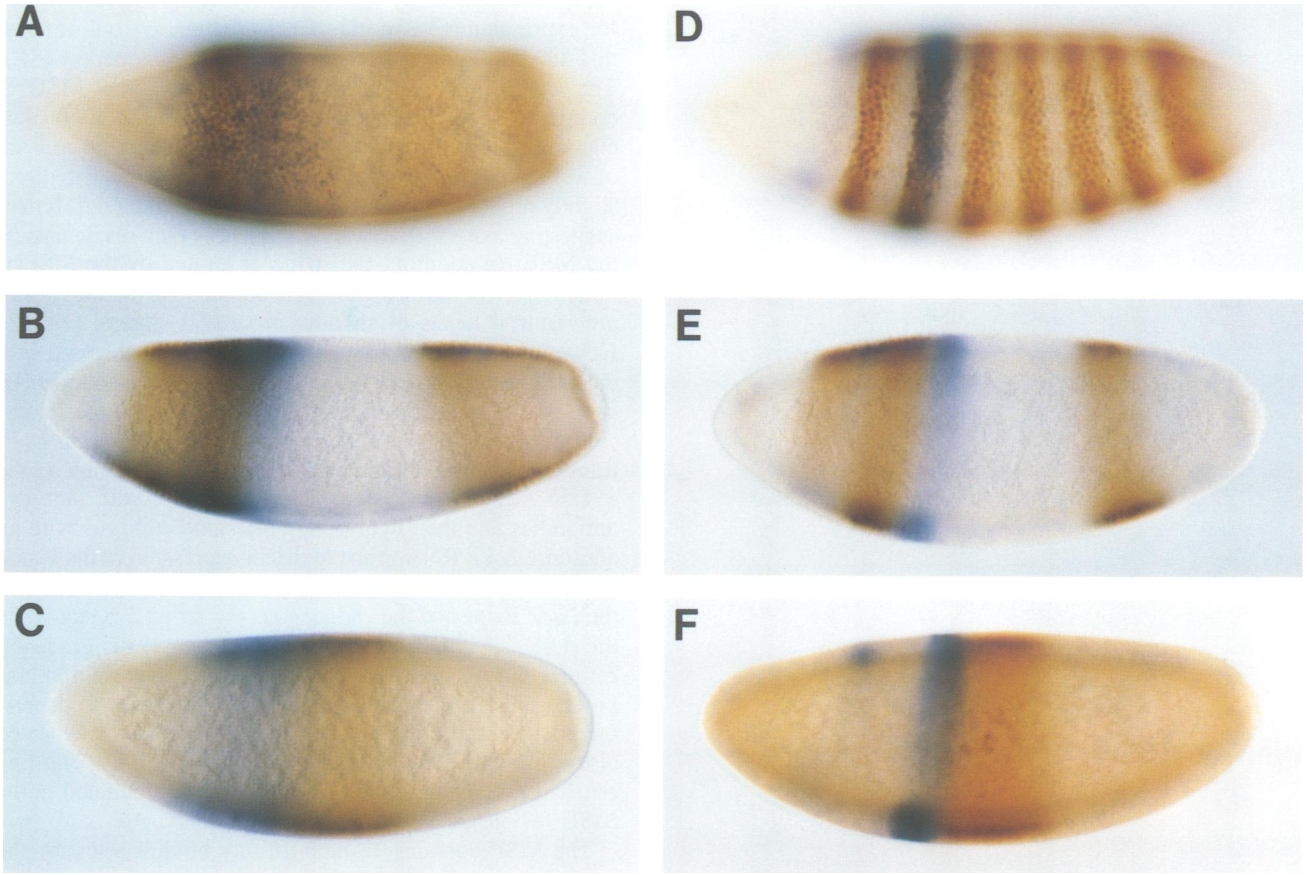

Fig. 2. Spatial expression of stripe 2 relative to the *Kr* and gt repressors. All embryos presented in this study are oriented with anterior to the left and dorsal up. Early cleavage cycle 14 (**A**, **B** and **C**) and late cleavage cycle 14 (**D**, **E** and **F**) embryos from a line transformed with the MSE−*LacZ* gene fusion (see Figure 1B). Embryos were stained with anti-*eve* (A and D), anti-*gt* (B and E), or anti-*Kr* (C and F) antibodies and then hybridized with a digoxigenin−UTP labelled *LacZ* antisense RNA probe. The MSE−*LacZ* fusion gene appears to direct expression within the normal limits of stripe 2 (A and D). The anterior domain of *gt* expression overlaps the prepattern expressed early by the MSE *LacZ* fusion (B) and abuts the anterior border of the mature stripe (E). The anterior border of the *Kr* domain abuts the posterior border of the MSE−*LacZ* stripe (C and F).

stripe element (MSE) is initially detected in a broad region that spans nearly the entire anterior half of the embryo, but after a short time the stripe borders are defined by selective repression. Activation of the MSE may depend on cooperative interactions among activator proteins; disrupting individual *bcd* or *hb* binding sites significantly reduces expression. The anterior border is established primarily by the *gt* repressor, although evidence is presented that additional repressors may participate in this process. The posterior border of the stripe generated by the MSE is probably formed by limiting levels of activators rather than by direct repression by *Kr*. The MSE has the properties of an integrating pattern element, which generates sharp limits of gene expression in response to overlapping gradients of transcriptional activators and repressors.

Results

Previous promoter truncation studies have shown that *eve* promoter sequences extending to 1.7 kb upstream of the transcription start site are sufficient to direct stripe 2 expression in the early *Drosophila* embryo (Harding *et al.*, 1989). Larger regions (>2.5 kb) are required to generate stripe 2 when fused to the heterologous HSP70 minimal promoter (Goto *et al.*, 1989; Small *et al.*, 1991). These results suggest that the *eve* basal promoter contributes to the overall levels of expression, but not necessarily to the quality (limits) of the stripe. Shorter upstream regions fused to the HSP70 basal promoter directed levels of expression that were undetectable with the relatively insensitive methods used. All of the gene fusions presented in this study include the basal *eve* promoter (to −42 bp), as well as 100 bp of untranslated leader sequence and the first 22 codons of the *eve* protein coding sequence fused to *LacZ*. Furthermore, we have used *in situ* hybridization with a digoxigenin−UTP labelled antisense RNA probe to detect *LacZ* expression (Tautz and Pfeifle, 1989; Kosman *et al.*, 1991), a method that is significantly more sensitive than antibodies or X-gal activity staining.

Identification of a minimal stripe 2 element

Fourteen different *eve*−*LacZ* fusion genes were tested by P-transformation to identify a minimal sequence that can direct expression of stripe 2 (Figure 1B). At least four independent transformed lines were obtained for each construct. Each of the *eve*−*LacZ* fusions containing the 480 bp region between −1.55 and −1.07 kb upstream from the *eve* transcription start site was found to direct the expression of stripe 2. This 480 bp interval corresponds to the region that was previously shown by deletion analysis to be required for the expression of the stripe (Goto *et al.*, 1989); however, this is the first demonstration that the region is also sufficient

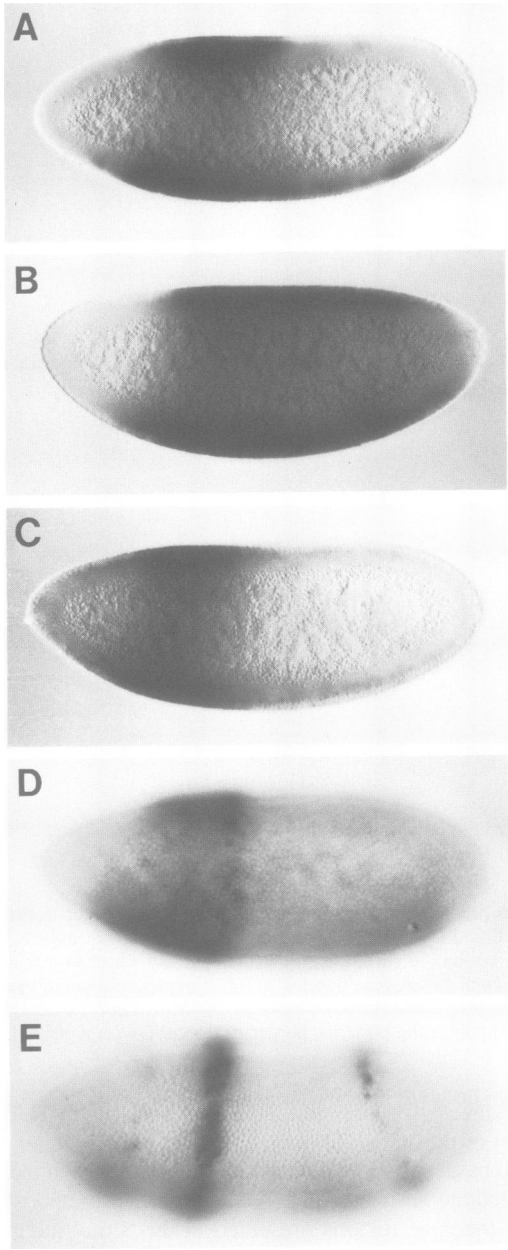

Fig. 3. Stripe 2−*LacZ* fusion genes direct broad prepatterns in early embryos. P-transformed embryos are oriented with anterior to the left and dorsal up. Expression of the *LacZ* reporter gene was visualized by histochemical staining after *in situ* hybridization. **A.** An early cleavage cycle 14 embryo that expresses a heterologous *eve* promoter fusion containing the region from −3 kb to −42 bp of the *eve* promoter attached to the hsp70 minimal promoter (see Small *et al.*, 1991). Staining is detected in a broad region, from ~80−40% egg length. **B.** An early cleavage cycle 14 embryo carrying the −1.7 kb *eve*−*LacZ* fusion gene. Staining is detected from ~80% egg length to the posterior pole, although expression is somewhat more intense in anterior regions. **C.** An early cleavage cycle 14 embryo carrying the MSE−*LacZ* fusion gene. Staining is similar to that observed for the embryo in (A), except that it does not extend quite as posteriorly (from ~80−55% egg length). **D.** A mid-cycle 14 embryo carrying the −3 kb to −42 bp heterologous fusion gene, as in (A). This embryo is ~10 min older than the one in (A) and shows a sharp posterior boundary of expression. Staining persists in regions anterior to the limits of the mature stripe 2. **E.** Late cycle 14 embryo carrying the MSE−*LacZ* fusion gene. By this time a mature stripe of staining is observed, as well as weaker, variable staining in posterior regions containing stripe 7. All of the stripe 2−*LacZ* fusion genes that were examined show a similar pattern of stripe 2 expression at this stage, although the 1.7 kb fusion gene generates a consistently stronger stripe 7.

for expression (Figure 2D−F). Hereafter, we will refer to this 480 bp sequence as the minimal stripe element (MSE). The quality of the stripe obtained with the MSE is comparable to that obtained with *eve*−*LacZ* fusions containing larger regions of the *eve* promoter, up to 8 kb of 5′ flanking sequence. The larger fusions appear to direct slightly higher levels of stripe 2 expression (data not shown) suggesting that some activation sequences may reside outside the MSE. In addition, the stripe generated by the MSE is not completely uniform along the dorsal−ventral axis; there are reduced levels of staining in ventral−lateral regions. However, the spatial limits of the *LacZ* stripe obtained with the MSE coincide with the initial borders of the endogenous *eve* stripe 2 as determined by double labelling experiments (Figure 2D). These experiments involved staining P-transformed embryos carrying MSE fusions (particularly the −1.55 delta 1.1 fusion; see Figure 1) with anti-*eve* antibodies and then hybridizing the same embryos with an antisense *LacZ* RNA probe to detect expression of the fusion gene. The only apparent discrepancy between the limits of the *LacZ* stripe and the endogenous *eve* stripe 2 is that while the posterior border of *eve* protein expression is gradually refined during cellularization, the *LacZ* pattern remains broad. This refinement process has been shown to depend on the *eve* autoregulatory element, which is located elsewhere in the *eve* promoter (Goto *et al.*, 1989; Harding *et al.*, 1989; Jiang *et al.*, 1991a) and is not included in the MSE fusion genes examined in this study.

The MSE contains 12 high affinity binding sites for the genetically defined regulators of stripe 2 expression (Figure 1C). Eight of these sites are contained in two clusters at opposite ends of the MSE. We tested several smaller DNA fragments centered around these clusters (Figure 1B) for their ability to specify stripe 2. None of the smaller fragments that were tested yielded a stripe 2 pattern comparable to that obtained with the intact MSE. However, several of the fusion genes containing the proximal cluster of binding sites directed variable expression patterns that included an extremely weak stripe (data not shown). These fusion genes included the 'prox 54' and 'dist 51/prox 54' fusions (Figure 1B), which correspond to the *eve* promoter fragments that were used in previous transient cotransfection assays (Small *et al.*, 1991).

Selective repression refines the initial MSE pattern

Localization studies using anti-*eve* antibodics showed that the protein is broadly expressed in early embryos, suggesting that stripes form through a process of selective repression (Frasch and Levine, 1987). However, subsequent promoter fusion studies demonstrated that discrete fragments of the *eve* (and *hairy*) promoter could activate specific subsets of stripes, implying that selective activation was the critical mechanism for the specification of stripe borders (Howard *et al.*, 1988; Goto *et al.*, 1989; Harding *et al.*, 1989; Howard and Struhl, 1991; Riddihough and Ish-Horowitz, 1991). In this study we have used the very sensitive method of *in situ* hybridization with an RNA probe to examine the patterns of expression generated by *eve* stripe 2 promoter elements earlier in development.

All stripe 2−*LacZ* fusion genes that were tested directed broad prepatterns of *LacZ* expression in the anterior half of the embryo. However, different *LacZ* fusions gave prepatterns with distinct posterior limits of expression

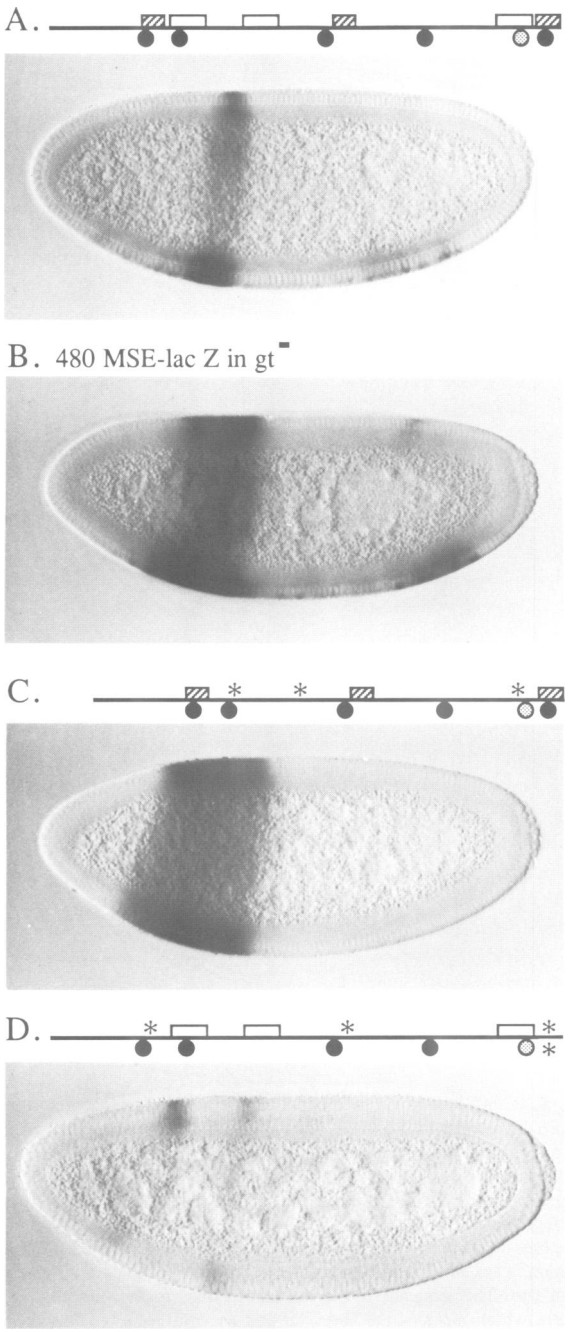

Fig. 4. Point mutations in individual repressor sites. P-transformed embryos are oriented with anterior to the left and dorsal up. *LacZ* reporter gene expression was detected by *in situ* hybridization. The embryos in A–C are at the midpoint of nuclear cleavage cycle 14, which is prior to the onset of the vector-driven dorsal head patch. **A.** Expression of the wild type MSE–*LacZ* fusion gene. The summary diagram above the embryo shows the locations of the activator and repressor sites within the MSE. **B.** Expression of the 1.7 kb *eve–LacZ* fusion gene in a *gt* mutant embryo (the *gt*YA82 allele; Wieschaus *et al.*, 1984). There is a substantial anterior expansion in the limits of stripe 2. **C.** Expression of a mutagenized MSE–*LacZ* fusion gene lacking the three *gt* binding sites (indicated by asterisks in the summary diagram). **D.** A cellularized embryo carrying a mutagenized MSE–*LacZ* fusion gene containing point mutations in all three *Kr* binding sites (indicated by asterisks). There is a substantial reduction in stripe 2 staining and expression is weaker than the dorsal hatch patch. Normally, at this stage in embryogenesis the stripe is at least 4–5 times more intense than the head patch.

(Figure 3A–C). For example, the prepattern obtained with a heterologous fusion gene containing *eve* 5′ flanking sequences from −3 kb to −42 bp attached to the minimal HSP70 promoter extends from the presumptive cephalic furrow to a region just beyond the midpoint of the embryo, at ~40% egg length (where 0% corresponds to the posterior pole; Figure 3A). An *eve–LacZ* fusion containing the first 1.7 kb of 5′ flanking sequence from the *eve* promoter directs a very striking prepattern that extends almost to the posterior pole (Figure 3B). The prepattern obtained with the MSE–*LacZ* construct (Figure 3C) only extends to ~60 to 55% egg-length, which is significantly less posterior than either prepattern mentioned above. This position is very close to the future posterior border of stripe 2. In all cases, the very broad prepattern is refined during cycle 14 to form a sharp stripe of *LacZ* expression within the limits of the endogenous *eve* stripe 2 (Figure 3E), suggesting that the borders are formed by selective repression.

For each of the stripe 2 fusion genes there is an intermediate stage when the posterior border is formed, but staining continues to extend anteriorly (Figure 3D). During the next 10 to 20 min this anterior expression is lost and the stripe is fully formed (Figure 3E). The kinetics of this refinement process are consistent with the timing of the known stripe 2 regulators. The initial prepattern may be due to broadly distributed *bcd* and *hb* proteins, which are maternally expressed and present prior to the appearance of the *Kr* and *gt* repressors (see Discussion).

The gt repressor forms the anterior stripe border

The spatial and temporal expression pattern of the *gt* protein is consistent with the possibility that it directly forms the anterior border of *eve* stripe 2 (Figure 2B and E). Previous studies with the 5.2 kb *eve–LacZ* fusion suggested that this *gt* repression involves direct binding to high affinity sites within the MSE (Stanojevic *et al.*, 1991). However, a potential limitation of the earlier work is that the relatively large deletions that were used to disrupt the three *gt* binding sites in the stripe 2 element also removed unknown activator sites. In addition, these mutations caused variable anterior expansions of the stripe, with some embryos showing nearly normal patterns of expression, while others displayed severe expansions. Here, we have used substantially smaller deletions (see Table I) to disrupt each *gt* binding site without affecting neighboring *bcd* and *hb* activator sites. And in order to circumvent potential problems with redundant elements, we have created these mutations in the context of the MSE.

Disruptions in the three *gt* sites cause a consistent and severe anterior expansion of the stripe, with expression detected in a broad band of ~16 cells (Figure 4C). However, this staining does not extend all the way to the anterior pole and is excluded from the anterior-most 20% of egg length. Since there are high levels of the *bcd* and *hb* activators in these anterior regions, it is possible that additional, unidentified repressor(s) also interact with the stripe 2 element (see Discussion).

The anterior expansion of stripe 2 expression caused by mutagenizing the three *gt* binding sites is somewhat more severe than that observed when stripe 2–*LacZ* fusion genes are expressed in *gt*– embryos (compare Figure 4B with C). There are normal levels of expression in *gt* mutants, but the anterior expansion only covers a band of ~12 cells. This observation suggests that there may be additional gene products that can recognize the *gt* repressor sites in the MSE to exclude expression from anterior regions (see Discussion).

```
bcd-5 -1450: overlaps Kr-5 site   bcd-4 -1430: overlaps gt-3 site
5'-TGTGTGTGCCGTGTTAATCCGTTTGCCATCAGCGAGATTATTAGTCAATTGCAGTTGCAG-3'
                ** *  *              *  ** *
from Kr-4 site: TGCAACCC              CAGCATC -from gt-2 site
3'-ACACACACGGCACACGTTGGGAAACGGTAGTCGGTCGTAGAATCAGTTAACGTCA-5' oligo mB4+5

bcd-3 -1285: no overlap
5'-GGGACCCTGGACTATAATCGCACAACGAGACCGG-3'
              *****
3'-CCCTGGGACCTGATGACGTCGTGTTGCTCTGGCC-5' oligo mB3

bcd-2 -1190: no overlap
5'-GTTTGTTTGTTTGCTGGGATTAGCCAAGGGCTTG
                 *   **
3'-CAAACAAACAAACGACTCTAGACGGTTCCCGAAC-5' oligo mB2

bcd-1 -1075: overlaps Kr-3 site
5'-TAATGATGTCGAAGGGATTAGGGGCGCGCAGGTC-3'
                 * **
from Kr-4 site: GGGTTGC
3'-ATTACTACAGCTTCCCAACGCCCCGCGCGTCCAG-5' oligo mB1

hb-3 -1110: overlaps gt-1 site
5'-TCATTAGGAAGTCATAAAAACACATAATAATGATG-3'
                ** ***
 from gt-2 site: GCATCT
3'-AGTAATCCTTCAGTCGTAGAGTGTATTATTACTAC-5' oligo mH3

Kr-5 -1450: overlaps bcd-5 site
5'-CTGTGTGTGCCGTGTTAATCCGTTTGCCATCAGCGAGA-3'
                **      ****
fr. bcd-3 site: TATAATCGCAC
3'-GACACACACGGCAATATTAGCGTGACGGTAGTCGCTCT-5' oligo mK5

Kr-4 -1270: no overlap
5'-CGCACAACGAGACCGGGTTGCGAAGTCAGGGC-3'
                * * *
3'-GCGTGTTGCTCTGGTCGACCGCTTCAGTCCCG-5' oligo mK4

Kr3 -1075: overlaps bcd-1 site
5'-CATAATAATGATGTCGAAGGGATTAGGGGCGCGC-3'
                **** *
from bcd-3 site: TTGTGCGATTA
3'-GTATTATTACTACAAACACGCTAATCCCCGCGCG-5' oligo mK3

gt-3 -1430: overlaps bcd-4 site
5'-TCAGCGAGATTATTAGTCAATTGCAGTTGCAGCGTTTCGCTTCC-3'
                  ----delete---
3'-AGTCGCTCTAATAAT              CGTCGCAAAGCGAAGG-5' oligo mG3

gt-2 -1350: no overlap
5'-CACTTTCGAGTTAGAC-43 bases-CTGTGCCATACTTTC-3'
                  --delete--
3'-GTGAAAGCTCAATCTG           GACACGGTATGAAAG-5' oligo mG2

gt-1 -1110: overlaps hb-3 site
5'-GGAAGTCATAAAAACACATAATAATGATGTCGAAGGGATTAGG-3'
                  ----delete----
3'-CCTTCAGTATTTTT               CAGCTTCCCTAATCC-5' oligo mG1
```

Table I. Mutagenesis of factor binding sites in the *eve* stripe 2 element. Binding sites that were mutagenized are underlined. Overlapping sites, if any, are overlined. Point mutations created by the mutagenesis are designated by asterisks and novel restriction sites generated by the mutagenesis are underlined in the oligo sequence shown on the bottom line of each set. Eight of the 12 individual sites overlap with another site. Each mutation was designed so that overlapping sites were not abolished. For example, the bcd-5 site overlaps the Kr-5 site. To specifically mutate bcd-5, the sequence was changed to another *Kr* site (Kr-4) that is unable to bind the *bcd* protein.

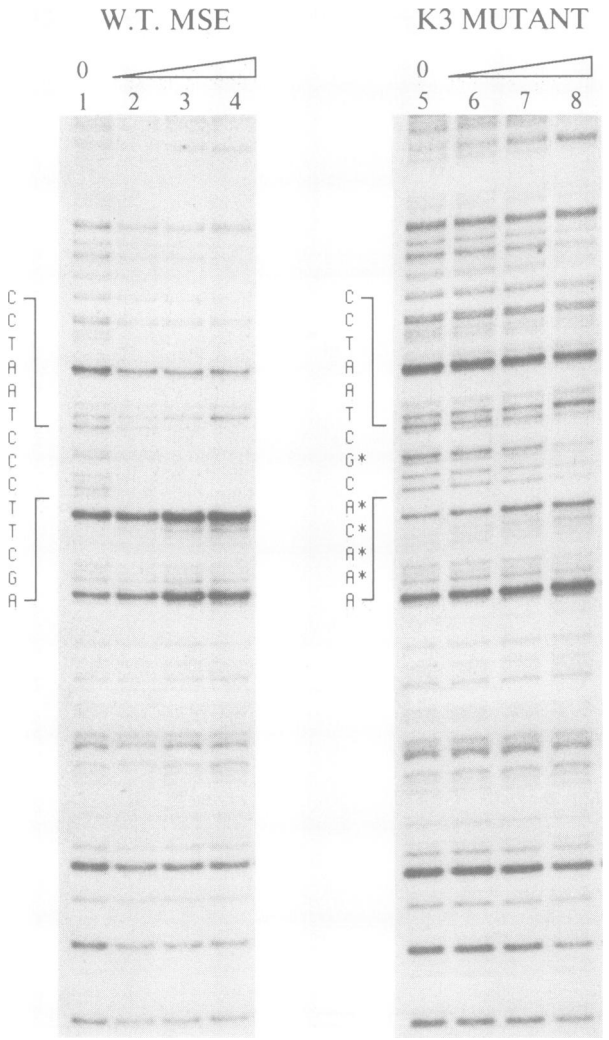

Fig. 5. Point mutations in the bcd-1 site reduce binding activity. DNase I footprint assays were done with wild type and mutagenized DNA templates from the stripe 2 region of the *eve* promoter. Lanes 1–4 show the wild type template and lanes 5–8 show the same DNA fragment containing point mutations in the Kr-3 site, which extensively overlaps the bcd-1 site. Lanes 2–4 and 6–8 show 3-fold incremental increases in the amount of affinity purified *bcd* protein used in each reaction. Peak amounts of the protein completely fill the wild type bcd-1 site. The sequence to the left corresponds to the protected region, which includes the core *bcd* and *Kr* consensus binding sites. Comparable amounts of *bcd* protein fail to fill the mutagenized template. Based on the appearance of hypersensitive bands that bracket the bcd-1/Kr-3 sequence, there is at least a 5-fold reduction in binding to the mutant template. The sequence to the left shows the protected region and the asterisks indicate the mutagenized nucleotides. Most of these are clustered around the core *Kr* site, only one nucleotide change was created in the *bcd* recognition sequence. The mutagenized bcd-1 site is identical to the normal bcd-3 site in the MSE (see Figure 1C summary).

The role of Kr in the formation of the posterior border

Previous studies with the 5.2 kb *eve–LacZ* fusion gene and the spatial and temporal distribution of the *Kr* protein (Figure 2C and F) suggested that *Kr* may function as a repressor to define the posterior border of stripe 2 (Stanojevic *et al.*, 1991). In this study, we have mutagenized the high affinity *Kr* sites in the context of the MSE to critically test the role of *Kr* in the formation of the posterior stripe border. Since two of the Kr sites overlap *bcd* activation sites (Figure 1C), the mutations were designed to abolish *Kr* binding without affecting nearby *bcd* sites (Table I). When tested in P-transformation experiments, a fusion gene containing mutations in all three *Kr* sites directed normal limits of expression, although there was a surprising reduction in the levels of staining (Figure 4D). The intensity of the stripe is roughly similar to the head stripe control, indicating a 4- to 5-fold reduction in the level of expression compared with the wild type MSE. This result suggested that *Kr* may play a positive as well as a negative role on stripe 2 expression, although previous studies implicated it solely as a repressor (Small *et al.*, 1991; Stanojevic *et al.*, 1991). However, positive regulation seems unlikely because the mutation in the Kr-3 site also reduces the binding affinity of the bcd-1 site as determined by DNase I footprint analysis (Figure 5). Thus, the reduced levels of expression probably

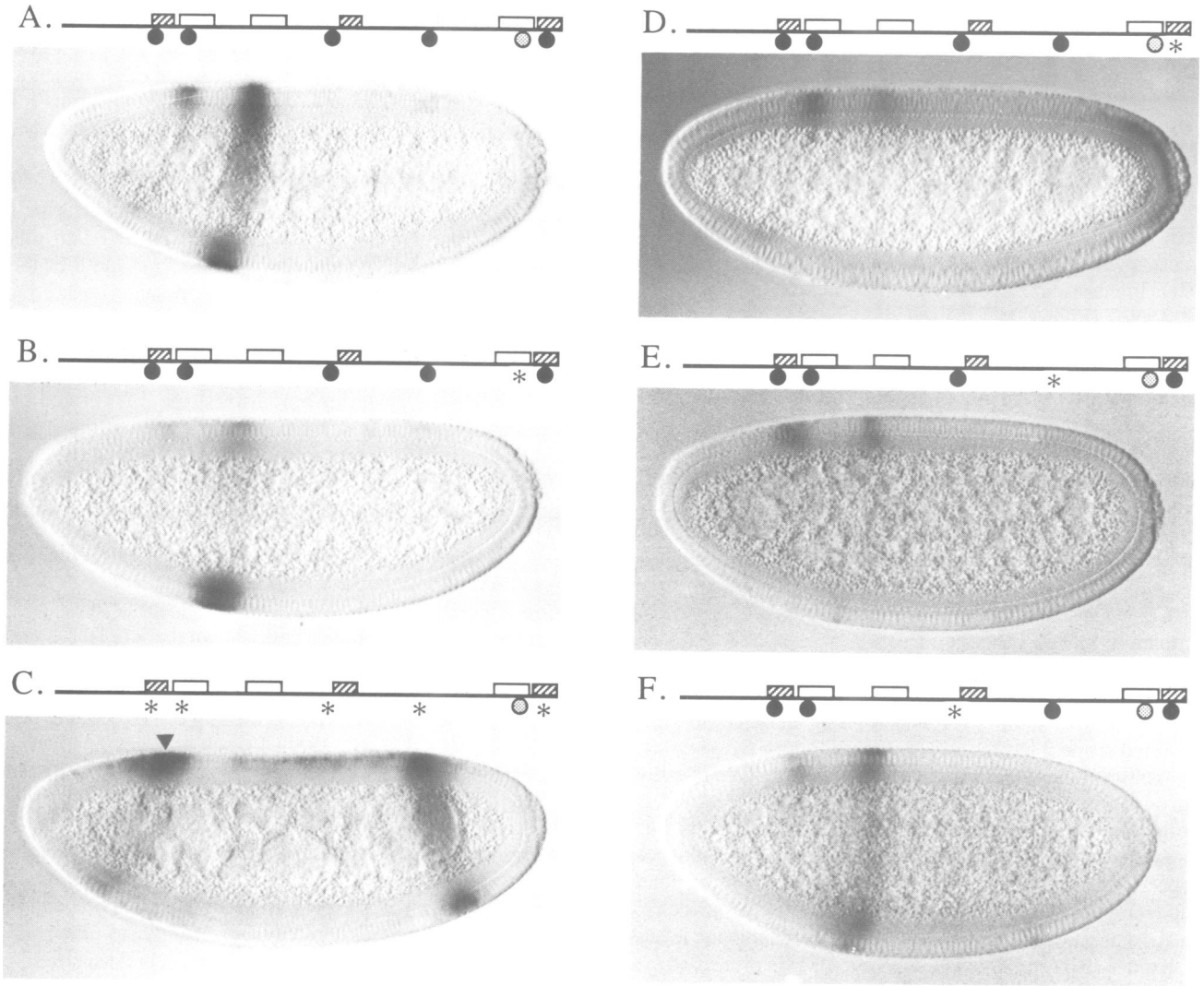

Fig. 6. Point mutations in individual activator sites. P-transformed embryos are at the cellular blasoterm stage and are oriented with anterior to the left and dorsal up. Expression of the *LacZ* reporter gene was visualized by histochemical staining after *in situ* hybridization. The horizontal lines above each embryo represents a summary of the activator and repressor sites in the MSE. **A.** Staining pattern obtained with the wild type MSE−*LacZ* fusion gene. A strong stripe 2 and weaker head patch of staining can be seen. **B.** Expression obtained with an MSE−*LacZ* fusion gene containing point mutations in the hb-3 site (indicated by an asterisk in the summary). There is a reduction in the levels of staining in dorsal regions, but expression appears normal in ventral regions. **C.** Expression obtained with an MSE−*LacZ* fusion gene containing point mutations in all five *bcd* binding sites (asterisks in summary). Stripe 2 expression is abolished, but there is a consistent increase in the levels of stripe 7 staining. The arrowhead indicates the dorsal 'head patch' that is due to vector sequences in the P-transposon. **D.** Expression obtained with an MSE−*LacZ* fusion gene containing mutations in the bcd-1 site (asterisk in summary). There is a severe reduction in stripe 2 staining. This embryo showed the strongest levels of staining among a population of several hundred transformants. Most embryos carrying this fusion gene show no stripe at all. Staining is also detected at the posterior pole. **E.** Expression obtained with an MSE−*LacZ* fusion gene containing mutations in the bcd-2 site. Expression is severely reduced, but the effect is not quite as dramatic as that seen with the bcd-1 mutation. **F.** Expression obtained with an MSE−*LacZ* fusion gene containing mutations in the bcd-3 site. The level of expression is significantly reduced but the effect is not as severe as that seen with the bcd-1 or bcd-2 mutations.

result from the decreased affinity of the bcd-1 site (see below).

There are several explanations for why the disruption of *Kr* binding sites in the MSE does not create a posterior expansion of the pattern. One possibility is that low affinity *Kr* binding sites contained within the MSE are able to form the posterior border (Stanojevic *et al.*, 1991). There are several such sites and none were altered in this study. However it is more likely that the posterior border of the stripe generated by the MSE may be formed by diminishing levels of the *bcd* and/or *hb* activators, making repression by *Kr* redundant in this context. There are declining levels of *bcd* posterior to the stripe 2 border (Driever and Nusslein-Volhard, 1988) and the six *bcd* and *hb* activator sites present in the MSE might not be sufficient to drive expression in these regions even in the absence of *Kr* binding sites. This explanation is supported by the observation that there is no posterior expansion when the wild type MSE *LacZ* fusion gene is crossed into *Kr*− embryos (D.Kosman, unpublished result). Furthermore, the careful inspection of the prepattern directed by the MSE (Figure 3C) shows that it extends only to about the position of the future posterior border of the mature stripe. The prepatterns directed by fusion genes containing larger fragments of the *eve* promoter extend to more posterior regions (Figure 3A and B) and in these cases repression by *Kr* is important for defining the posterior

border of the stripe (Stanojevic *et al.*, 1991). Thus, we suggest that *Kr* repression is important in defining the posterior border of the stripe directed by these larger promoter fragments, but in the case of the MSE, this border is formed by limiting amounts of the *bcd* and/or *hb* activators.

Activation of stripe 2 expression

Preliminary studies have suggested that *bcd* binding sites directly mediate stripe 2 activation *in vivo* (Stanojevic *et al.*, 1991). In these experiments, mutations in both the bcd-1 and bcd-2 sites (Figure 1C) caused reduced levels of stripe 2 expression in the context of an *eve*−*LacZ* fusion gene containing 5.2 kb of *eve* 5′ flanking sequence that directs equally intense expression of stripes 2, 3 and 7. This result suggested that these *bcd* binding sites are required for optimal stripe 2 expression. A limitation of these experiments is that point mutations in individual *bcd* binding sites produced variable results, probably due to redundancy of regulatory elements in the large *eve* promoter region that was tested. For example, mutations in the bcd-1 and bcd-2 sites caused variable reductions, with some embryos showing nearly normal levels of stripe 2 expression while others almost completely lacked the stripe.

To avoid problems with redundant elements, mutations in *bcd* binding sites were made in the context of the MSE. Point mutations in all five *bcd* binding sites completely abolished stripe 2 expression (compare Figure 6C with A). The embryo shown here was overstained to enhance the expression of the anterior 'head stripe' (arrow), which is due to vector sequences in the P-transposon and serves as an internal control. With the wild type MSE, staining of stripe 2 is at least 4- to 5-fold more intense than the head stripe (Figure 6A). In addition to the loss of stripe 2, mutation of all five sites leads to an enhancement in the levels of stripe 7 (Figure 6C). The wild type MSE directs stripe 7 expression to variable extents, but it is not as intense as the head stripe (Figure 6A). Perhaps the mutations in the *bcd* sites facilitate the binding of stripe 7 activators.

The next experiments were designed to test the effects of mutagenizing individual activator binding sites. First, we independently mutagenized the bcd-1, bcd-2 and bcd-3 sites (Figure 1C). Each of these mutagenized promoter fusions caused significant reductions in the level of expression (Figure 6D−F). Mutagenization of either the bcd-1 or bcd-2 sites nearly abolished the stripe (Figure 6D and E). Mutagenization of the bcd-3 site resulted in a consistent reduction in the levels of staining, but the reduction was not as severe as that seen with mutant bcd-1 or bcd-2 sites (Figure 6F). The difference in the response generated by these individual mutants may be due to differences in the *bcd*-binding affinity. Bacterially expressed *bcd* protein binds with 3- to 5-fold higher affinity to the bcd-1 and bcd-2 sites than to the bcd-3 site as determined by DNase I protection assays (data not shown). We also examined an MSE−*LacZ* fusion gene that contains point mutations in the hb-3 site (Figure 6B). There is a substantial reduction in stripe 2 expression in dorsal regions, but the staining in ventral regions is nearly normal. The basis for this asymmetric reduction in staining is not obvious since the *hb* activator is expressed uniformly along the dorsal−ventral axis (Tautz, 1988). A double mutation that disrupts both the bcd-1 and the hb-3 sites completely abolishes the stripe (data not shown).

Discussion

We have shown that a 480 bp region of the *eve* promoter, the MSE, is sufficient to direct the expression of an authentic stripe in the early embryo. The timing and limits of expression coincide with the endogenous stripe 2 pattern. The initial activation is mediated by the maternal morphogen *bcd* and the gap protein *hb* to form a broad prepattern of expression that encompasses almost the entire anterior half of the embryo. During a period of just 20−30 min this prepattern is refined to form first the posterior and then the anterior border of the stripe. *gt* defines the anterior border of the stripe, although we have obtained evidence that additional repressors are likely to participate in this process. Previous studies using larger pieces of the *eve* promoter indicated that the posterior border of stripe 2 is formed by *Kr* repression. However, the posterior border of the stripe directed by the MSE seems to be formed by limiting amounts of *bcd* activator, suggesting that the posterior border of the endogenous stripe may be formed by redundant mechanisms.

Interactions between activators and repressors define stripe 2 borders

Previous localization studies have shown that *eve* RNAs and proteins are ubiquitously expressed in all nuclei ~2 h after fertilization and there is a gradual refinement of this pattern to yield a series of seven transverse stripes (Harding *et al.*, 1986; Macdonald *et al.*, 1986; Frasch and Levine, 1987). Since these earlier localization studies involved the use of probes that simultaneously detect the activities of all stripe initiation elements in the *eve* promoter, it was difficult to determine the contributions of individual stripe elements to the initial ubiquitous pattern. Here we have shown that a single stripe element is expressed in a very broad prepattern, suggesting that it contributes significantly to the early ubiquitous pattern. The existence of the prepattern strongly suggests that the stripe repressors play a decisive role in specifying the position of the stripe borders.

Genetic studies suggest that *gt* functions as a repressor to establish the anterior border of the stripe. It is likely that additional factors also participate in this process. None of the stripe 2 prepatterns extend all the way to the anterior pole, even though there are high levels of the *bcd* and *hb* activators in this region (see Figure 3A−C). Several mechanisms may account for this exclusion from the anterior pole. First, *bcd* and *hb* may fail to activate stripe 2 expression in this region because one or both proteins are modified, possibly by the *torso* (*tor*) tyrosine kinase, which is activated at the poles (Casanova and Struhl, 1989; Sprenger *et al.*, 1989). According to this model, *bcd* and *hb* could activate transcription only outside the range of *tor* kinase activity. A second possibility is that *bcd* may activate additional unidentified repressors in the anterior-most regions. A potential candidate for such a repressor is *orthodenticle* (*otd*) (Finkelstein and Perrimon, 1990), which encodes a homeobox protein that binds the same sequences as *bcd* (C.Desplan, personal communication) and thus may compete for *bcd* activator sites in the MSE.

Deletions in the three *gt* binding sites present in the MSE cause a somewhat more severe anterior expansion of the stripe than that observed in *gt*− embryos, suggesting that *gt* may interact with another protein to effect repression. The *gt* protein contains a leucine zipper (bZIP) dimerization domain (Vinson *et al.*, 1989; Small *et al.*, 1991; Capovilla

et al., 1992) and thus could form a heterodimer with another bZIP protein to form the anterior border in wild type embryos. In *gt*− embryos this 'corepressor' would still be present and thus could provide partial function as a homodimer. Genetic studies have identified a novel gap gene, located on chromosome 2, which is a possible candidate for such a corepressor (Vavra and Carroll, 1989). Embryos that lack this genomic region exhibit a transient anterior expansion of *eve* stripe 2 which is similar to that observed in *gt*− mutants.

Previous studies suggested that the posterior border of stripe 2 is defined by the *Kr* repressor (Small *et al.*, 1991; Stanojevic *et al.*, 1991). For example, there is a posterior expansion when large regions of the *eve* promoter containing the stripe 2 element are expressed in *Kr*− embryos. In addition, the anterior limit of the *Kr* pattern coincides with the posterior border of stripe 2 and there is a weak posterior expansion of the stripe when *Kr* binding sites in the stripe 2 element are disrupted in the context of larger fragments of the promoter (Stanojevic *et al.*, 1991). These promoter fusions direct stronger expression than the MSE and generate early patterns that extend posterior to the border of the mature stripe, presumably due to activation sites that lie outside the MSE. We propose that it is this more extended expression which is responsible for the posterior expansion observed in *Kr*− embryos. Since the prepattern generated by the MSE does not extend to as posterior a position, expansion of the pattern is not observed in *Kr*−.

It is difficult to assess the extent to which the endogenous stripe border is formed by limiting amounts of *bcd* as opposed to repression by *Kr*. Mutations in *Kr* binding sites cause only a slight expansion of large *eve*−*LacZ* fusion genes and the more severe expansion observed in *Kr*− mutants might result from altered expression of the *hb* activator (Gaul and Jäckle, 1987). The large fusion genes include additional *hb* binding sites that map outside the limits of the MSE and perhaps these mediate expression in response to the expanded *hb* pattern. Whether or not limiting amounts of *bcd* proves to be the primary mechanism for forming the border of the endogenous stripe, the concentration-dependent activation of the MSE may be analogous to the interaction of *bcd* with the *hb* promoter (Driever *et al.*, 1989; Struhl *et al.*, 1989).

Cooperativity among bicoid activators

This study provides strong evidence that the *bcd* morphogen is the primary activator of stripe 2 expression. Mutations that inactivate individual *bcd* sites virtually abolish expression. However, since these mutations alter the core TAAT recognition sequence common to all homeodomain proteins, it is conceivable that *bcd* acts indirectly by regulating the expression of one or more intermediates, which in turn bind to the stripe 2 activator sites. This possibility is unlikely in light of the results obtained by mutagenizing the Kr-3/bcd-1 sequence. The nucleotide substitutions did not alter the core TAAT recognition sequence in the bcd-1 site, but nonetheless reduced *bcd* binding based on *in vitro* assays (Figure 5). There is a concomitant reduction in the levels of stripe 2 expression *in vivo*. The close correspondence between *in vitro* affinity and *in vivo* expression provides strong evidence that *bcd* is the *bona fide* activator. Consistent with this conclusion is the previous demonstration that *bcd* and *hb* function multiplicatively to activate transcription via MSE sequences in transient cotransfection assays (Small *et al.*, 1991).

The MSE contains a total of six known activator sites and the results presented here suggest that they must all be intact for optimal expression. Furthermore, the severe reduction observed with point mutations in three separate *bcd* binding sites and the *hb* site suggest that the initiation of stripe 2 might depend on cooperative interactions between activator proteins. Such cooperative interactions could occur by several different mechanisms. Perhaps the binding of *bcd* monomers to the highest affinity sites (bcd-1 and bcd-2 both contain eight out of nine matches with the consensus) facilitates binding to the lower affinity bcd-3, bcd-4 and bcd-5 sites (which contain only seven out of nine or six out of nine matches with the consensus). A nonexclusive alternative is that efficient occupancy of the five *bcd* binding sites contained in the MSE might depend on interactions with the neighboring *hb* binding site, since mutating this site also caused a significant reduction in expression. Future *in vitro* binding assays will determine whether the binding of *hb* to the MSE facilitates the binding of *bcd*. Once bound, protein−protein interactions among *bcd* monomers may be important for synergistic contact or activation of the basal transcription complex, as has been proposed previously (Driever *et al.*, 1989; Struhl *et al.*, 1989). Such *bcd* cooperativity would have important implications for the mechanism of repression that defines the stripe borders. Our data suggest that the disruption of the ability of *bcd* to bind to a single site could have a dramatic effect on expression. Therefore, the binding of *Kr* and *gt* repressors could effectively shut off the promoter by interfering with just one or two of the activator sites. Such a mechanism might also govern the regulation of the endogenous stripe. Sequences that flank the MSE within the *eve* promoter contain additional activator and repressor sites. The formation of the stripe borders generated by the MSE may involve interfering with just one or two activators out of a total of six. The endogenous stripe may be regulated by as many as 12 activators and interfering with three or four of these could be sufficient to form the borders.

At first glance, the catastrophic loss of stripe 2 expression obtained by mutagenizing individual *bcd* binding sites is not in agreement with previous studies on the interaction between *bcd* protein and *hb* promoter sequences. In the latter case, the removal of individual binding sites led to relatively minor effects, such as slightly reduced levels of expression or more restricted expression in more anterior regions (Driever *et al.*, 1989; Struhl *et al.*, 1989). Activation by *bcd* was found to depend on a minimum of two binding sites. In contrast, we have shown that as many as four *bcd* binding sites are not necessarily sufficient to mediate substantial stripe 2 activation. We believe that the basis for this apparent discrepancy is that activation of stripe 2 occurs in a relatively posterior position of the embryo where there are diminishing amounts of *bcd* activator. The removal of a single high affinity site might permit only higher levels of *bcd* in more anterior regions to activate expression, but this would be obscured by the *gt* repressor. In the case of the *bcd*−*hb* interaction, there were no anterior repressors to mask activation by peak levels of *bcd*. We would expect the simultaneous mutation of the bcd-1 (or bcd-2) site and the three *gt* repressor sites to shift the entire stripe to a more anterior position.

Integrating pattern element

The *eve* MSE has the properties of an integrating pattern element, which generates sharp limits of gene expression

in response to overlapping gradients of transcriptional activators and repressors. It is striking that the regulators of stripe 2 expression are all restricted to a small, discrete region of the *eve* promoter. The promoter is quite large and 8 kb of 5′ flanking sequence directs only three of the seven stripes (2, 3 and 7; Goto *et al.*, 1989; Harding *et al.*, 1989). By analogy to *hairy*, it is conceivable that the intact *eve* promoter is as large as 15–20 kb (Howard *et al.*, 1988; Howard and Struhl, 1990; Ridddihough and Ish-Horowicz, 1991). An implication of our studies on stripe 2 is that the periodic, 7-stripe *eve* pattern does not depend on interactions between regulatory factors bound to distant regions of the promoter. Instead, the binding sites for the regulators of a particular stripe are tightly linked within a small interval. We propose that the reason for this linkage is to bring weakly interacting regulatory proteins into close apposition so that they can function both cooperatively and synergisitically to control transcription. The region of the *eve* promoter that is important for the regulation of stripe 2 maps ~1.5 kb from the stripe 3 element (Goto *et al.*, 1989; Harding *et al.*, 1989). Perhaps this spacing between elements is required to ensure their autonomous action in specifying different stripes.

Materials and methods

Construction of eve–LacZ P-transposons

All *eve–LacZ* fusion genes were made by cloning various fragments from the *eve* promoter upstream of the unique *Pst*I site of pEl1 (kindly provided by Paul Macdonald). pEl1 contains the basal *eve* promoter (from −42), the intact 100 bp untranslated leader and the coding sequence for the first 22 amino acids of the *eve* protein fused to codon #5 of the *LacZ* coding sequence (Lawrence *et al.*, 1987). These promoter *LacZ* fusions were then cloned into the P-element transformation vector CaSpeR (Thummel *et al.*, 1988) using the unique *Bam*HI or *Xba*I sites or both by conventional cloning methods. The CaSpeR vector contains the *white* gene as a marker. The restriction sites in the *eve* promoter that were used in these constructions are: −1.7 kb, *Xho*I; −1.55, *Bst*EII; −1.3, *Dra*III; −1.2, *Sty*I; −1.1, *Bss*HII; −0.4, *Apa*I; −0.04, *Pst*I. Four of the vectors were constructed using oligonucleotides (dist 51 and prox 54) that contain minimal clusters of four binding sites (see Figure 1). The fragments used for these clones were cut out of intermediate subclones pBS51, pBS54 and pBS51/54 (Small *et al.*, 1991) and fused to the basal *eve* promoter as described above. The dist51:twi:prox54–*LacZ* construct contains a 400 bp *Eco*RI–*Eco*NI fragment from the *twi* promoter placed between the two clusters of binding sites (see Jiang *et al.*, 1991b).

In vitro mutagenesis

Individual binding sites in the MSE were disrupted by oligonucleotide directed mutagenesis using the Mutagene kit (Bio-Rad, Richmond, CA). All mutations and deletions were generated using a single-stranded DNA template containing a 640 bp *Bst*EII–*Fsp*I fragment from the *eve* promoter in the pBluescript SK+ vector (Stratagene, La Jolla, CA). The mutations and/or deletions were verified by dideoxy sequencing using Sequenase (US Biochemical, Cleveland, OH) and fragments containing the mutations were cloned into the CaSpeR transformation vector as described above. The *eve* promoter sequence surrounding each of the twelve binding sites in the MSE and the oligonucleotide used for mutagenesis of each individual site is shown in Table I.

P-transformation and whole-mount in situ hybridization

P-transformation vectors containing *eve–LacZ* fusion genes were introduced into the *Drosophila* germ line by injection (Rubin and Spradling, 1982). The w67 *white*− strain was used for all injections. P-transposons were coinjected with the delta 2,3 helper (kindly provided by Frank Laski). Between four and 10 independent transformed lines were generated for each construct and at least three independent lines were tested for *LacZ* expression by *in situ* hybridization. Hybridizations using an anti-sense *LacZ* RNA probe were performed exactly as reported previously (Jiang *et al.*, 1991b).

Embryos that were double stained for *LacZ* RNA and *eve* protein were dechorionated for 2–3 min in 100% bleach and fixed in 50% buffer B (10 mM KH_2PO_4 pH 6.8, 45 mM KCl, 15 mM NaCl, 2 mM $MgCl_2$) containing 3.7% formaldehyde and 50% heptane, and then shaken for 12 min. After removal of the bottom phase, embryos were then devitellinized by adding 7 ml of methanol and then shaking vigorously for 1 min. Embryos were then washed several times in methanol, rocked for 10 min in 50% methanol–50% PBT (1×PBS plus 0.1% Tween 80). The embryos in PBT were then rinsed in 10% BSA in PBS and blocked in the same buffer for 2 h at room temperature, and incubated in the primary antibody (rabbit anti-*eve*, a gift from Manfred Frasch; rabbit anti-Kr, a gift from Christine Rushlow; guinea pig anti-gt, a gift from Rachel Kraut) overnight at 4°C. After washing in PNBT (six changes during a period of 1.5 h), the embryos were incubated with appropriate biotinylated secondary antibodies (Vector Labs, Burlingame, CA) for 3 h at room temperature. After washing for a further 2 h in PBT (15 min changes), the *eve* antibody staining was visualized using the Elite Vectorstain kit (Vector Labs) as directed by the manufacturer. The reaction was stopped in 33 mM Tris pH 7.5, dehydrated by washing six times in 100% Ethanol and then hybridized with a digoxigenin–UTP labelled antisense *LacZ* RNA probe exactly as described in Kosman *et al.* (1991) starting with the 50:50 EtOH–xylene step. Alkaline phosphatase activity staining was done with the Genius kit (Boehringer Mannheim) as described by the manufacturer.

Acknowledgements

We thank Susan Gray for advice on the double staining experiments, Tony Ip for help with embryo injections, Dave Kosman for help with *in situ* hybridizations and Tim Hoey for *bcd* protein and advice on footprint assays. We also thank Eric Davidson for critically reviewing the manuscript. S.S. is supported by an NIH postdoctoral fellowship, and this work was funded by a grant from the NIH (GM 34431).

References

Capovilla,M., Eldon,E. and Pirotta,V. (1992) *Development*, **114**, 99–112.
Carroll,S.B. (1990) *Cell*, **60**, 9–16.
Casanova,J. and Struhl,G. (1989) *Genes Dev.*, **3**, 2025–2038.
Dearolf,C., Topol,J. and Parker,C. (1989) *Genes Dev.*, **3**, 384–398.
Driever,W. and Nusslein-Volhard,C. (1988) *Cell*, **54**, 83–93.
Driever,W., Thoma,G. and Nusslein-Volhard,C. (1989) *Nature*, **340**, 363–367.
Finkelstein,R. and Perrimon,N. (1990) *Nature*, **346**, 485–488.
Frasch,M. and Levine,M. (1987) *Genes Dev.*, **1**, 981–995.
Frasch,M., Hoey,T., Rushlow,C., Doyle,H. and Levine,M. (1987) *EMBO J.*, **6**, 749–759.
Gaul,U. and Jäckle,H. (1987) *Cell*, **51**, 549–555.
Goto,T., Macdonald,P. and Maniatis,T. (1989) *Cell*, **57**, 413–422.
Harding,K., Rushlow,C., Doyle,H., Hoey,T. and Levine,M. (1986) *Science*, **233**, 953–959.
Harding,K., Hoey,T., Warrior,R. and Levine,M. (1989) *EMBO J.*, **8**, 1205–1212.
Hiromi,Y. and Gehring,W.J. (1987) *Cell*, **50**, 963–971.
Hiromi,Y., Kuroiwa,A. and Gehring,W.J. (1985) *Cell*, **43**, 603–613.
Howard,K.R. and Struhl,G. (1990) *Development*, **110**, 1223–1231.
Howard,K., Ingham,P. and Rushlow,C. (1988) *Genes Dev.*, **2**, 1037–1046.
Ingham,P. (1988) *Nature*, **335**, 25–34.
Jiang,J., Hoey,T. and Levine,M. (1991a) *Genes Dev.*, **5**, 265–277.
Jiang,J., Kosman,D., Ip,Y.T. and Levine,M. (1991b) *Genes Dev.*, **5**, 1881–1891.
Kosman,D., Ip,Y.T., Levine,M. and Arora,K. (1991) *Science*, **254**, 118–122.
Lawrence,P., Johnston,P., Macdonald,P. and Struhl,G. (1987) *Nature*, **328**, 440–442.
Macdonald,P., Ingham,P. and Struhl,G. (1986) *Cell*, **47**, 721–734.
Pankratz,M. and Jackle,H. (1990) *Trends Genet.*, **6**, 287–292.
Pankratz,M., Seifert,E., Gerwin,N., Billi,B., Nauber,U. and Jackle,H. (1990) *Cell*, **61**, 309–317.
Pignoni,F., Baldarelli,R., Steingrimsson,E., Diaz,R., Patapoutian,A., Merriam,J. and Lengyel,J. (1990) *Cell*, **62**, 151–163.
Riddihough,G. and Ish-Horowicz,D. (1991) *Genes Dev.*, **5**, 840–854.
Rubin,G.M. and Spradling,A.C. (1982) *Science*, **218**, 348–353.

Small,S. and Levine,M. (1991) *Curr. Opin. Genet. Dev.*, **1**, 255–260.
Small,S., Kraut,R., Hoey,T., Warrior,R. and Levine,M. (1991) *Genes Dev.*, **5**, 827–839.
Sprenger,F., Stevens,L.M. and Nusslein-Volhard,C. (1989) *Nature*, **338**, 478–483.
Stanojevic,D., Hoey,T. and Levine,M. (1989) *Nature*, **341**, 331–335.
Stanojevic,D., Small,S. and Levine,M. (1991) *Science*, **254**, 1385–1387.
Struhl,G., Struhl,K. and Macdonald,P. (1989) *Cell*, **57**, 1259–1273.
Tautz,D. (1988) *Nature*, **332**, 281–284.
Tautz,D. and Pfeifle,C. (1989) *Chromosoma*, **98**, 81–85.
Thummel,C.S., Boulet,A.M. and Lipshitz,H.D. (1988) *Gene*, **74**, 445–456.
Vavra,S.H. and Carroll,S.B. (1989) *Development*, **107**, 663–672.
Vinson,C., Sigler,P. and McKnight,S.L. (1989) *Science*, **246**, 911–916.
Wieschaus,E., Nusslein-Volhard,C. and Jurgens,G. (1984) *Roux's Arch. Dev. Biol.*, **193**, 296–307.

Received on 29 May, 1992; revised on 20 July, 1992