Regulation of even-skipped stripe 2 in the Drosophila embryo

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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., 1985; 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-Horowiz, 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., 1983; Hiromi and Gehring, 1987; Dearolf et al., 1989). For the most part, disruptions of 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 bed- 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 gr in anterior regions and Kr in posterior regions.
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
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 hh 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...
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 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/dist 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 antibodies 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 Stuhl, 1991; Riddihough and Ish−Horovitz, 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.
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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 prepatter that extends almost to the posterior pole (Figure 3B). The prepatter obtained with the MSE-LacZ construct (Figure 3C) only extends to -60 to 55% egg-length, which is significantly less posterior than either prepatter mentioned above. This position is very close to the future posterior border of stripe 2. In all cases, the very broad prepatter 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 prepatter 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 normally 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 mutating 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)
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 levels 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 blastoelem 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 suppressor 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 h5-3 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 bed 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 bed-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 bed-2 site. Expression is severely reduced, but the effect is not quite as dramatic as that seen with the bed-1 mutation. F. Expression obtained with an MSE- LacZ fusion gene containing mutations in the bed-3 site. The level of expression is significantly reduced but the effect is not as severe as that seen with the bed-1 or bed-2 mutations.

result from the decreased affinity of the bed-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 bed and/or hb activators, making repression by Kr redundant in this context. There are declining levels of bed 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 mutants (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
Discussion

We have shown that a 480 bp region of the eve promoter, the MGE, 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 MGE 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 MGE.

Deletions in the three gt binding sites present in the MGE 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; Stanoev 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 (Stanoev 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 prepatter 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 mutating 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 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 two 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 3′ ends [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; Riddiford 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 synergistically 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-β-galactosidase P-transposons

All eve-β-galactosidase fusion genes were made by cloning various fragments from the eve promoter upstream of the unique PvuII site of pE111 (kindly provided by Paul Macdonald). pE111 contains the basal eve promoter (from –42), the intact 100 bp untranslatable leader and the coding sequence for the first 22 amino acids of the eve protein fused to ccdodon 5 of the LacZ coding sequence (Lawrence et al., 1987). These promoter LacZ fusions were then cloned into the P-element transposition vector CsSp7 (Thummel et al., 1988) using the unique BamHI or XbaI sites or both by conventional cloning methods. The CsSp7 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, XbaI; –1.55, BstEII; –1.13, DraI; –1.2, SphI; –1.1, BstHII; –0.4, Apot; –0.04, PstI. 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 pB531, pB534 and pB531:54 (Small et al., 1991) and fused to the basal eve promoter as described above. The dist51:prox54-β-galactosidase construct contains 400 bp EcoRI-EcoNI fragment from the twi promoter placed between the two-chains of binding sites (see Jiang et al., 1991b).

In vitro mutagenesis

Individual binding sites in the MSe were displaced by oligonucleotide directed mutagenesis using the Mutagen kit (Bio-Rad, Richmond, CA). All mutations and deletions were generated using a single-stranded DNA template containing a 460 bp BstEII-Aspl fragment from the eve promoter in the pBlueScript SK− vector (Stratagene, La Jolla, CA). The mutations and deletions were verified by direct sequencing using Sequenase (US Biochemical, Cleveland, OH) and fragments containing the mutations were cloned into the CsSp7 transposition 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 1.

P-transformation and whole-mount in situ hybridization

P-transformation vectors containing eve-β-galactosidase 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 conjected 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-β-galactosidase 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 KH2PO4 pH 6.8, 4.5 mM KCl, 15 mM NaCl, 2 mM MgCl2) containing 3.7% formaldehyde and 50% heptane, and then shaken for 12 min. After removal of the bottom phase, embryos were then deproteinized 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% PB (1×PBS plus 0.1% Tween 80). The embryos in PB were then rinsed in 10% SBA in PBS and blocked in the same buffer for 1 h at room temperature, and incubated in the primary antibody (rabbit anti-eve, a gift from Manfred Frasch; rabbit anti-anti, a gift from Christine Ruhl; guinea pig anti-gt; a gift from Rachel Kran; overnight at 4°C). After washing in PNB (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 PB (15 min changes), the anti-eve antibody staining was visualized using the Elite Vectorstar 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 dried with a digest of Simulcumin-UTP labelled antisense LacZ RNA probe exactly as described in Kosman et al. (1991) starting with the 50:50 EDTA–xylene step. Alkaline phosphatase activity staining was done with the Genius kit (Boehringer Mannheim) as described by the manufacturer.

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References