Spacing ensures autonomous expression of different stripe enhancers in the even-skipped promoter

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SUMMARY

The even-skipped (eve) promoter contains a series of enhancers that control the expression of different segmentation stripes in the Drosophila embryo. The stripe 3 enhancer is located 1.7 kb upstream of the stripe 2 enhancer. Here we demonstrate that these enhancers must be physically separated by a minimum distance for proper stripe expression. When they are directly coupled either orientation, the enhancers generate abnormal patterns of expression in the early embryo. For example, the levels of stripe 2 expression are augmented and there is a posterior expansion of the pattern when the stripe 3 enhancer is positioned immediately upstream of the stripe 2 enhancer. Despite this spacing requirement, the order of the enhancers within the eve promoter can be reversed without affecting the normal expression pattern. These results suggest that spacing maintains the autonomous activities of the stripe enhancers and that interactions between enhancers can generate novel patterns of gene expression.

Key words: Drosophila, even-skipped, spacing, expression, autonomous expression, promoter

INTRODUCTION

To determine how the bicoid (bcd) morphogen gradient directs sharp limits of gene expression in the early Drosophila embryo, we have conducted a detailed characterization of one of its target genes, even-skipped (eve). eve encodes a homeodomain protein that is essential for the segmentation process; mutant embryos lacking eve protein lack segment borders (Nusslein-Volhard et al., 1985; Macdonald et al., 1986; Harding et al., 1986; Lawrence et al., 1987; Frasch et al., 1988). The eve protein is expressed in a series of 7 transverse stripes that extend along the length of the embryo (Frasch and Levine, 1987). Previous promoter fusion studies have established that at least some of these stripes are regulated by separate enhancers present in the eve promoter (Goto et al., 1989; Harding et al., 1989). For example, a 500 bp enhancer located between −1.6 kb and −1.1 kb upstream of the eve transcription start site is essential for the regulation of stripe #2 (Small et al., 1992). A separate enhancer, located between −3.8 kb and −3.3 kb, controls the expression of stripe #3 (Goto et al., 1989; Harding et al., 1989; Blair, Levine and Small, unpublished data; see Fig. 1A).

Recent studies have provided considerable information regarding the cis and trans control of individual stripe enhancers. A combination of genetic studies (Frasch and Levine, 1987), in vitro DNA-binding assays (Stanojevic et al., 1991; Small et al., 1992), transient cotransfection assays (Small et al., 1991) and P-element transformation assays (Stanojevic et al., 1991; Small et al., 1992) have established that bcd is the primary activator of stripe 2 expression, and that cooperative interactions among bcd monomers may be important for filling the five bcd-binding sites present in the stripe 2 enhancer. Despite this information about individual enhancer elements, little is known about potential functional interactions between stripe enhancers of the eve promoter region. Thus, we have tested whether the orientation of the stripe enhancers is important for the regulated eve pattern; we also examine the role of 'spacer' sequences that separate individual enhancers.

The stripe 2 and 3 enhancers are separated by a 1.7 kb spacer sequence (Fig. 1A). In addition, a 1 kb spacer region separates the stripe 2 enhancer from the eve promoter. Neither of these spacers appear to make a significant contribution to the activities of individual stripe enhancers. For example, similar levels of stripe 2 expression are observed with an eve-lacZ fusion gene containing the entire 1.7 kb of 5′ flanking sequence and one that lacks the region from −1 kb to −42 bp (Small et al., 1992). Here, we investigate the consequences of removing all or parts of the spacer sequence that normally separates the stripe 2 and 3 enhancers. These studies suggest that individual stripe enhancers must be separated by minimal spacer sequences in order to function normally in the early embryo. We propose that interactions between enhancers might be an important mechanism for generating novel patterns of gene expression during development.
MATERIALS AND METHODS

Construction of eve-lacZ P-element transposons

All eve-lacZ fusion genes were made by cloning various fragments from the eve promoter upstream of a PstI-XbaI fragment from pEl1 (Small et al., 1992), which contains the basal eve promoter (from −42 bp) and the intact 100 bp untranslated leader and the first 22 codons of the eve gene. Promoter fusions were cloned into the P-element transformation vector CaSpeR (Thummel et al., 1988) after digestion with BamHI and PstI. Construct 1 (see Fig. 1) contains a 500 bp BamHI-SacI fragment from the eve promoter (−3.8 to −3.3 kb) placed upstream of a 480 bp EcoRI-BssHII fragment (−1.6 to −1.1 kb) from the −1.55 Δ1.1 eve-lacZ pCaSpeR P-transposon (Small et al., 1992). Construct 2 contains an 800 bp BamHI-AfIII fragment (−3.8 to −3.0 kb) joined 5′ of a 480 bp EcoRI-BssHII (−1.6 to −1.1 kb) fragment of −1.55 Δ1.1 eve-lacZ Casper (Small et al., 1992). Construct 3 contains a 480 bp BstEI-BssHII fragment (−1.6 to −1.1 kb) joined 5′ of a 500 bp EcoRI-SacI (−3.8 to −3.3 kb) from the −3.8 Δ−3.3 eve-lacZ pCaSpeR transformation plasmid (Small et al., 1992). Construct 4 contains a 640 bp BstEI-FspI fragment (−1.6 to −1.0 kb) from the −1.55 eve-lacZ pCaSpeR plasmid (Small et al., 1992) joined 5′ of a 500 bp EcoRI-SacI fragment (−3.8 to −3.0 kb) from the −3.8 Δ−3.3 eve-lacZ pCaSpeR plasmid (Small et al., 1992). Constructs 5 and 6 were made in the same manner as constructs 1 and 2, respectively, using a stripe 2 fragment whose Kr-binding sites had been mutagenized as described below. Constructs 7 and 8 were made in the same manner as constructs 2 and 1, respectively, except that the stripe 2 element contained mutations in the bcd1 site which virtually inactivates the stripe element (Small et al., 1992).

In vitro mutagenesis

Individual Kr-binding sites were disrupted within the stripe 2 enhancer by oligonucleotide-directed mutagenesis using the Mutagene kit (Bio-Rad, Richmond, CA). Mutations were generated using a single-stranded DNA template generated from plasmids derived from the pBlueScript SK+ vector (Stratagene, La Jolla, CA.). The mutations were verified by dideoxy sequencing using Sequenase (US Biochemical, Cleveland, OH) and fragments containing the mutations were cloned into the pCaSpeR transformation vector (Thummel et al., 1988). The mutagenic Kr3, Kr4 and Kr5 oligonucleotides were identical to those described in Small et al. (1992), except for the Kr3 oligonucleotide, which was replaced by Kr3a containing the following sequence: GCCGCCCC-TAACCCCCAATACATATTATTG-3′. The Kr3a oligonucleotide disrupts the Kr3-binding site without interfering with the adjacent bcd1 site, thus allowing the stripe 2 element to retain activity (D. Arnosti, unpublished data), unlike the Kr− enhancer described in Fig. 4D of Small et al. (1992).

P-element transformation and whole-mount in situ hybridization

Vectors containing eve-lacZ fusion genes were introduced into the Drosophila germ line exactly as described in Small et al. (1992). At least four independent lines were examined for each construct; the results reported represent ‘consensus’ patterns generated by at least three of the lines. All of the embryos presented in Figs 2 and 4 were stained in parallel, so that differences in expression levels are directly comparable.

RESULTS

Importance of spacing
eve-lacZ fusion genes were expressed in early embryos via P-element transformation (summarized in Fig. 1B).

Expression patterns were visualized by hybridizing P-transformed embryos with a digoxigenin-labeled lacZ antisense RNA probe (Tautz and Pfeifle, 1989; Jiang et al., 1991). An eve-lacZ fusion gene that completely lacks the 1.7 kb spacer separating the stripe 2 and stripe 3 enhancers directs a severely abnormal pattern of expression (Fig. 2A). Stripe 2 expression is enhanced, stripe 3 is reduced, and there is a posterior expansion of stripe 2 such that it extends into the interstripe region that normally separates the two stripes. An essentially normal pattern is restored when the 300 bp spacer 1 sequence (Fig. 1A) is placed between the two stripe enhancers (Fig. 2B). The patterns shown in panels A and B are directly comparable since all stainings presented in this study were done in parallel.

Similar results were obtained when the orientation of the stripe enhancers was reversed. When the stripe 2 enhancer is placed directly upstream of the stripe 3 enhancer, there is
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A severe reduction in stripe 2 expression (Fig. 2C). However, an essentially normal pattern is obtained when the 160 bp spacer 2 sequence is inserted between the enhancers (Fig. 2D). This sequence is distinct from the spacer 1 sequence, which was used in the previous experiment (see Fig. 1 and legend). These results suggest that the stripe 2 and 3 enhancers must be separated by spacer sequences in order to function autonomously. It is conceivable that the spacers employed here are not neutral, but contain ‘insulating’ sequences (see Discussion).

When the stripe 2 and 3 enhancers are joined together in different configurations, the upstream enhancer is weakened (Fig. 2A versus C). This effect is clearly not solely due to the distance from the basal promoter since it is not seen when the enhancers are separated (Fig. 2B versus D). As described below, Kr repressor sites in the stripe 2 enhancer can attenuate stripe 3 expression when the two enhancers are coupled.

**Interactions between stripe enhancers**

To determine the basis for the abnormal expression patterns obtained when the stripe enhancers are directly coupled, we examined a series of eve-lacZ fusion genes containing defective stripe 2 enhancers (Fig. 1B). The first experiments were designed to examine the role of the gap gene Kruppel (Kr) (Rosenberg et al., 1986) in attenuating stripe 3 expression. Kr encodes a repressor that defines the posterior border of stripe 2 (Licht et al., 1990; Small et al., 1991, 1992). There are high concentrations of Kr in central regions of the embryo where stripe 3 is expressed (Gaul and Jackle, 1987; Stanoevic et al., 1989; Fig. 3B). Stripe 3 appears to evade repression by Kr since the stripe 3 enhancer lacks high-affinity Kr-binding sites (Stanoevic et al., 1989; Fig. 3A). Perhaps Kr bound to the stripe 2 enhancer does not interfere with stripe 3 expression when the two enhancers are separated by spacer sequences. However, it might be able to act over short distances to repress stripe 3 when the two enhancers are coupled (Fig. 3C).

A defective stripe 2 enhancer lacking all three high affinity Kr-binding sites was placed immediately downstream of the stripe 3 enhancer (construct #5, Fig. 1B). This eve-lacZ fusion gene directs an extremely abnormal pattern of expression, whereby stripes 2 and 3 are nearly fused (Fig. 4A). Stripes 2 and 3 are quite intense, and expressed at comparable levels. This pattern is similar to that observed when the two normal enhancers are fused (Fig. 2A), except that there is no reduction in the levels of stripe 3 expression when the Kr-binding sites are removed from the stripe 2 enhancer. In contrast, the intact stripe 2 enhancer causes reduced expression of stripe 3 expression. This result suggests that Kr can act over short distances to repress stripe 3 activators (Fig. 3C).

The coupling of the normal stripe 2 and 3 enhancers not only causes reduced expression of stripe 3, but leads to...
rapidly diminishes in the vicinity of the stripe 3 anterior border. Perhaps $hb$ bound to the stripe 3 enhancer can facilitate the expression of stripe 2 (Fig. 2A). This enhancement might be caused by linking $hb$-binding sites in the stripe 3 enhancer near $bcd$ sites in the stripe 2 enhancer. $bcd$ is the primary activator of stripe 2 expression (Small et al., 1991, 1992), while $hb$ functions as a repressor that defines the anterior and posterior stripe borders, respectively (Stanojevic et al., 1991; Small et al., 1991, 1992). The stripe 3 enhancer is activated by one or more ubiquitously distributed activators, and the anterior border is formed through repression by $hb$. (B) Diagrams of embryos showing the relationship between the expression of stripes 2 and 3, and the $hb$ and $Kr$ repressors. $Kr$ is expressed in central regions of the embryo; its anterior limit coincides with the posterior border of stripe 2. $hb$ is expressed in a broad gradient emanating from anterior regions. This gradient rapidly diminishes in the vicinity of the stripe 3 anterior border. (C) Model for enhancer interactions. A normal stripe/interstripe/stripe pattern is obtained when the two enhancers are separated by spacer sequences. However, when the stripe 3 enhancer is placed directly upstream of the stripe 2 enhancer, there is reduced expression of stripe 3 and overexpression of stripe 2. Perhaps $Kr$ bound to the stripe 2 enhancer can interfere with stripe 3 activators. Moreover, $hb$ bound to the stripe 3 enhancer might be able to interact over short distances with $bcd$ bound to the stripe 2 enhancer to augment expression.

Fig. 3. Summary of enhancer interactions. (A) Diagrams of the minimal stripe 2 and stripe 3 enhancers. The stripe 2 enhancer is activated by $bcd$ and $hb$, and repressed by giant ($gt$) and $Kr$ to define the anterior and posterior stripe borders, respectively (Stanojevic et al., 1991; Small et al., 1991, 1992). The stripe 3 enhancer is activated by one or more ubiquitously distributed activators, and the anterior border is formed through repression by $hb$. (B) Diagrams of embryos showing the relationship between the expression of stripes 2 and 3, and the $hb$ and $Kr$ repressors. $Kr$ is expressed in central regions of the embryo; its anterior limit coincides with the posterior border of stripe 2. $hb$ is expressed in a broad gradient emanating from anterior regions. This gradient rapidly diminishes in the vicinity of the stripe 3 anterior border. (C) Model for enhancer interactions. A normal stripe/interstripe/stripe pattern is obtained when the two enhancers are separated by spacer sequences. However, when the stripe 3 enhancer is placed directly upstream of the stripe 2 enhancer, there is reduced expression of stripe 3 and overexpression of stripe 2. Perhaps $Kr$ bound to the stripe 2 enhancer can interfere with stripe 3 activators. Moreover, $hb$ bound to the stripe 3 enhancer might be able to interact over short distances with $bcd$ bound to the stripe 2 enhancer to augment expression.

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To test this model, we used a defective stripe 2 enhancer that lacks the $bcd1$ activator site (constructs 7 and 8, Fig. 1B). Elimination of $bcd1$ causes a nearly complete loss in stripe 2 expression (Small et al., 1992). This is apparent when the defective enhancer is separated from the stripe 3 enhancer by the spacer 1 sequence (Fig. 4D). Stripe 3 staining is considerably more intense and there is only a vestige of stripe 2. However, removal of the spacer results in a dramatic restoration of stripe 2 expression (Fig. 4C). This result strongly suggests that the stripe 3 enhancer can augment stripe 2 expression when the two enhancers are coupled. In addition, there is a slight reduction in the levels of stripe 3 (Fig. 4C; compare with D), consistent with the previous demonstration that stripe 2 enhancers containing intact $Kr$ sites can repress stripe 3 expression when the enhancers are coupled (Figs 2A, 4A).

**DISCUSSION**

The results presented in this study suggest that spacing is required for the autonomous expression of the stripe 2 and 3 enhancers. The disruptions caused by coupling the enhancers can be divided into two classes. First, there is reduced expression of the stripe directed by the upstream enhancer. Second, stripe 2 is overexpressed when the stripe 3 enhancer is placed directly upstream of the stripe 2 enhancer. Both of these effects are eliminated when the two enhancers are separated either by the 300 bp spacer 1 sequence or the 160 bp spacer 2. Both spacers restore a normal pattern of expression.

**Chromatin insulating sequences**

The spacer 1 and spacer 2 sequences used in this study were equally effective in preventing the stripe 2 and 3 enhancers from interfering with each other. Since these spacers are unrelated and contain distinct sequences, we believe that mere distance is sufficient to prevent enhancer interactions. However, we have not exhaustively mutagenized the spacer DNAs, and therefore it is conceivable that they are more than simple neutral spacing elements. Perhaps they contain sequences that actively isolate the enhancers into separate chromatin domains. Chromatin insulating sequences, $scs$, have been identified that can function over long distances to block the expression of distal regulatory elements (Kellum and Schedl, 1991).

**Short-range interactions between stripe enhancers**

When the stripe 2 enhancer is located immediately downstream of the stripe 3 enhancer, stripe 2 expression is dramatically increased and expanded into the interstripe region. Perhaps $hb$ bound to the stripe 3 enhancer can facilitate the binding of $bcd$ protein to the distal $bcd4$ and $bcd5$ sites in the stripe 2 enhancer when these sites are brought into close proximity by directly linking the two enhancers (see Fig. 3). Recent studies support the idea that cooperative interactions
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are important for the efficient filling of activator sites in the stripe 2 enhancer. For example, mutations in individual stripe 2 activator sites cause substantial reductions in stripe 2 expression (Small et al., 1992). One interpretation of this observation is that the naturally occurring bcd-binding sites have mediocre affinities, and that efficient filling of the sites depends on cooperative interactions. Perhaps stripe2/stripe 3 enhancer interactions augment the effective affinities of the stripe 2 activator sites. This interaction occurs only over short distances and is blocked by separating the two enhancers.

Another example of short-range enhancer interactions involves the repression of stripe 3 expression by the stripe 2 enhancer. Evidence was presented that Kr bound to the stripe 2 enhancer can attenuate stripe 3 expression when the two enhancers are directly coupled. A defective stripe 2 enhancer lacking Kr-binding sites cannot repress stripe 3 expression. The mechanism of repression by Kr might be similar to that employed by the Su(Hw) zinc-finger protein, which blocks the expression of distal, but not proximal, enhancers (Geyer and Corces, 1992). Repression by Kr requires close linkage of the upstream enhancer, while Su(Hw) can act over considerable distances. Nonetheless, it is conceivable that a DNA fragment containing numerous, closely linked Kr-binding sites might mediate robust, directional repression as is observed for Su(Hw).

Enhancers as integrating pattern elements

Swapping the order of the stripe enhancers has no effect on their activities, even though the organization of the eve promoter appears to be conserved in the distantly related Drosophila grimshawi species (B. Sackerson and R. Warrior, personal communication). This observation is consistent with studies suggesting that the order of enhancers associated with Ultrabithorax is not important for normal patterns of homeotic gene expression (Muller and Bienz, 1991), despite the striking evolutionary conservation in the organization of the Bithorax complex and mammalian HOX clusters (reviewed by McGinnis and Krumlauf, 1992).

There is mounting evidence that enhancers function as templates to bring weakly interacting regulatory factors into close proximity so that they can function combinatorially to activate and repress transcription (Ip et al., 1992; Small et al., 1992; Jiang and Levine, 1993). The results presented here support the idea that these interactions require close linkage of regulatory factors; enhancers function autonomously as long as they are separated by minimal distances. The coupling of normal stripe 2 and stripe 3 enhancers leads to a novel pattern of expression.

It is striking that eve enhancers can interact only when directly coupled, yet these same enhancers can communicate over very long distances with the basal machinery to activate transcription. This disparity suggests that enhancer-
enhancer interactions are fundamentally different from enhancer-promoter interactions. It has been proposed that there are proteins responsible for allowing enhancers to contact the transcription complex (e.g., TAFs, see Hoey et al., 1993). It is conceivable that other regulatory proteins permit distantly linked enhancers to interact to generate novel patterns of gene expression.

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