

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

Stephen Small*, David N. Arnosti and Michael Levine

Department of Biology, Center for Molecular Genetics, Bonner Hall, UCSD, 9500 Gilman Drive, La Jolla, CA 92093-0322, USA

*Present address: New York University, Department of Biology, 29 Washington Place, New York, New York 10003, USA

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 in 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., 1989; Small et al., 1991), 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 pE11 (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 *Bam*HI and *Pst*I. Construct 1 (see Fig. 1) contains a 500 bp *Bam*HI-*Sac*I fragment from the *eve* promoter (-3.8 to -3.3 kb) placed upstream of a 480 bp *Eco*RI-*Bss*HIII fragment (-1.6 to -1.1 kb) from the -1.55 -1.1 *eve-lacZ* pCaSpeR P-transposon (Small et al., 1992). Construct 2 contains an 800 bp *Bam*HI-*Afl*II fragment (-3.8 to -3.0 kb) joined 5' of a 480 bp *Eco*RI-*Bss*HIII (-1.6 to -1.1 kb) fragment of -1.55 -1.1 *eve-lacZ* Casper (Small et al., 1992). Construct 3 contains a 480 bp *Bst*EII-*Bss*HIII fragment (-1.6 to -1.1 kb) joined 5' of a 500 bp *Eco*RI-*Sac*I (-3.8 to -3.3 kb) from the -3.8 -3.3 *eve-lacZ* pCaSpeR transformation plasmid (Small et al., 1992). Construct 4 contains a 640 bp *Bst*EII-*Fsp*I 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 *Eco*RI-*Sac*I fragment (-3.8 to -3.0 kb) from the -3.8 -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 *bcd*I 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 Mutagenesis 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 *Kr*3, *Kr*4 and *Kr*5 oligonucleotides were identical to those described in Small et al. (1992), except for the *Kr*3 oligonucleotide, which was replaced by *Kr*3a containing the following sequence: GCGCGCCCC-TAATCCCACAAACATCATTATTATG-3'. The *Kr*3a oligonucleotide disrupts the *Kr*3-binding site without interfering with the adjacent *bcd*I 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).

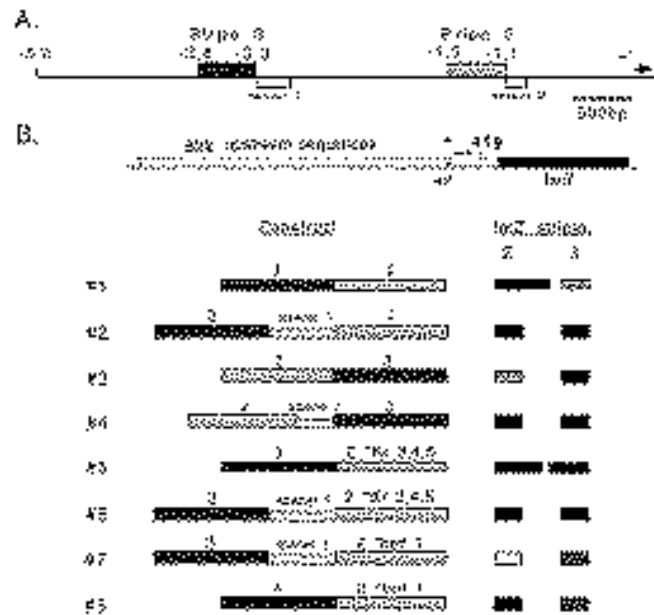


Fig. 1. Summary of *eve* stripe enhancers. (A) The first 5.2 kb of the *eve* promoter contains the stripe 2 and stripe 3 enhancers. The numbers above the line indicate distances from the transcription start site ('+1') in kilobase pairs (kb). The two enhancers are separated by 1.7 kb. A number of experiments involved the use of spacer sequences ('spacer 1' and 'spacer 2'). The spacer 1 sequence is 300 bp in length and is located between -3.3 and -3 kb upstream of the start site. Spacer 2 is 160 bp and is located between -1.1 kb and -900 bp. (B) Summary of *eve-lacZ* fusion genes. *eve* 5' sequences were attached to a minimal *eve* promoter-*lacZ* fusion gene, which contains the first 42 bp of the *eve* promoter as well as the 100 bp untranslated leader and first 66 bp of the protein coding sequence. The stripe 2 and stripe 3 enhancers were attached to this fusion gene in various orientations (constructs #1 through #8). All fusion genes involve the use of the 480 bp stripe 2 enhancer and the 500 bp stripe 3 enhancer (summarized in A). The expression patterns obtained with these fusion genes (in P-element transformed embryos) are summarized to the right of each construct. For example, placing the stripe 3 enhancer directly upstream of the stripe 2 enhancer (construct #1) causes overexpression of stripe 2 and diminished expression of stripe 3.

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

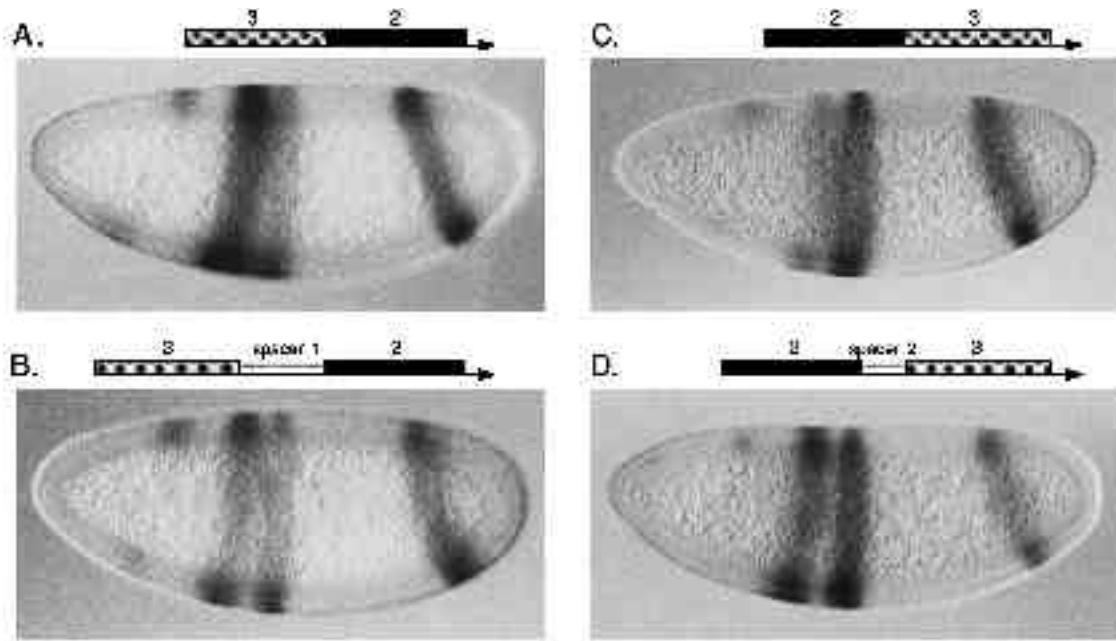


Fig. 2. Spacing is essential for enhancer autonomy. P-element transformed embryos containing different *eve-lacZ* fusion genes, as indicated at the top of each panel. Embryos are oriented with anterior to the left and dorsal up. (A) Expression pattern obtained when the stripe 3 enhancer is placed directly upstream of the stripe 2 enhancer. Stripe 2 is overexpressed and stripe 3 is reduced. In addition, stripe 2 expression extends into the interstripe region that normally separates the two stripes. Posterior stripe 7 expression is directed by sequences within the stripe 3 element. A weak 'head stripe' seen in all embryos is directed by sequences located in the vector. (B) Same as A, except that the 300 bp spacer 1 sequence was inserted between the stripe enhancers. An essentially normal expression pattern is observed, with the two stripes expressed at approximately equal levels. (C) Expression of a fusion gene containing the stripe 2 enhancer placed immediately upstream of the stripe 3 enhancer. There is a substantial reduction in stripe 2 expression (compare with D). (D) Same as C except that the 160 bp spacer 2 sequence was inserted between the enhancers. A normal expression pattern is observed, with equal expression of the two stripes.

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; Stanojevic et al., 1989; Fig. 3B). Stripe 3 appears to evade repression by *Kr* since the stripe 3 enhancer lacks high-affinity *Kr*-binding sites (Stanojevic 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

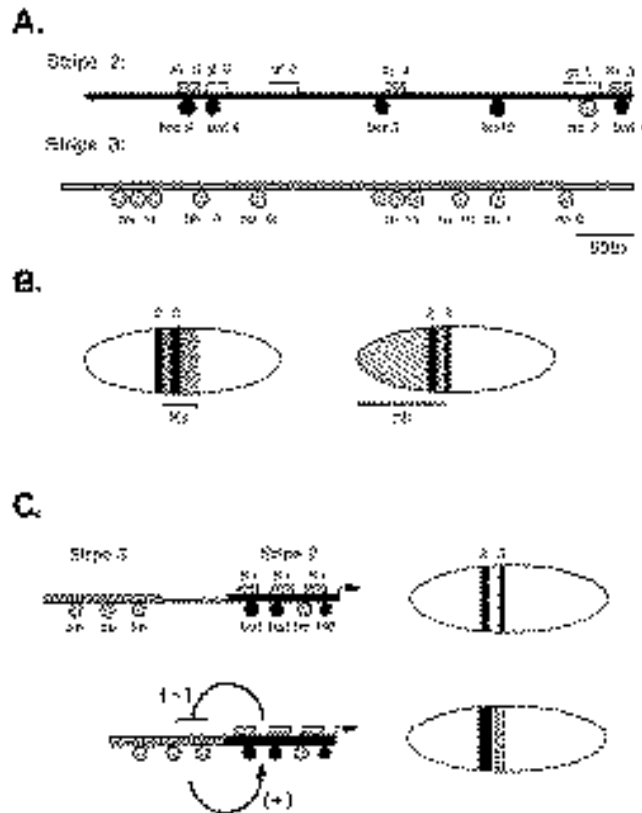


Fig. 3. Summary of enhancer interactions. (A) Diagrams of the minimal stripe 2 and stripe 3 enhancers. The stripe 2 enhancer is activated by *bcd* and *hb*, and repressed by *giant* (*gt*) and *Kr* to define the anterior and posterior stripe borders, respectively (Stanojevic et al., 1991; Small et al., 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.

enhanced 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 border of stripe 3 (Zuo et al., 1991; Struhl et al., 1992; Blair, Levine, and Small, unpublished data; Fig. 3A,B). Despite its role as a repressor of stripe 3 expression, several lines of evidence suggest that *bcd* and *hb* function multiplicatively to activate transcription (Small et al., 1991,

1992). Perhaps *hb* bound to the stripe 3 enhancer can interact with *bcd* bound to the stripe 2 enhancer to augment stripe 2 expression when the two enhancers are directly coupled (Fig. 3C).

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

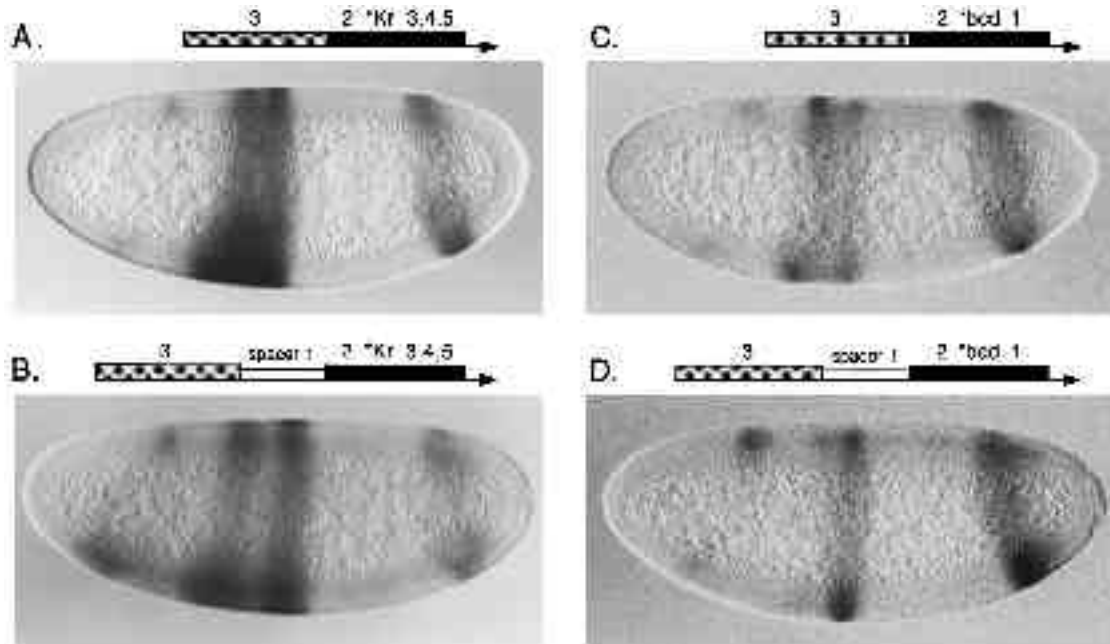


Fig. 4. Evidence for interactions between enhancers. P-element transformed embryos are presented as in Fig. 2. (A) Expression pattern obtained with an *eve-lacZ* fusion gene containing the stripe 3 enhancer placed immediately upstream of a defective stripe 2 enhancer lacking all three *Kr*-binding sites (construct #5, see Fig. 1B). The two stripes are expressed at approximately equal levels, but *lacZ* expression is also seen in the interstripe region, similar to the pattern in Fig. 2A. (B) Same as A except that the 300 bp spacer 1 sequence was inserted between the two enhancers. An essentially normal pattern is seen, with the two stripes expressed at similar levels. Unlike the *Kr3* mutation described in Small et al. (1992), the closely linked *bcd1* activator site was not affected in this experiment. (C) Expression obtained when the stripe 3 enhancer is placed immediately upstream of a defective stripe 2 enhancer completely lacking the *bcd-1* site (construct #8, Fig. 1C). Stripe 2 is somewhat stronger than stripe 3, although both are reduced as compared with *eve-lacZ* fusion genes containing intact enhancers separated by spacer sequences (see Fig. 2B,D). (D) Same as C except that the 300 bp spacer 1 was inserted between the two enhancers. Stripe 1 is virtually absent, while stripe 3 is expressed at normal levels. The loss of stripe 2 is due to mutations in the *bcd-1* activator site. This defective enhancer is augmented in C when directly coupled to the stripe 3 enhancer.

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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REFERENCES

- Frasch, M. and Levine, M. (1987). Complementary patterns of *even-skipped* and *fushi tarazu* expression involve their differential regulation by a common set of segmentation genes in Drosophila. *Genes Dev.* **1**, 981-995.
- Frasch, M., Warrior, W., Tugwood, J. and Levine, M. (1988). Molecular analysis of *even-skipped* mutants in Drosophila development. *Genes Dev.* **2**, 1824-1838.
- Gaul, U. and Jackle, H. (1987). Pole region-dependent repression of the Drosophila gap gene *Kruppel* by maternal gene products. *Cell* **51**, 549-555.
- Geyer, P. K. and Corces, V. G. (1992). DNA position-specific repression of transcription by a Drosophila zinc finger protein. *Genes Dev.* **6**, 1865-1873.
- Goto, T., Macdonald, P. and Maniatis, T. (1989). Early and late periodic patterns of *even-skipped* expression are controlled by distinct regulatory elements that respond to different spatial cues. *Cell* **57**, 413-422.
- Harding, K., Rushlow, C., Doyle, H., Hoey, T. and Levine, M. (1986). Cross-regulatory interactions among pair-rule genes in Drosophila. *Science* **233**, 953-959.
- Harding, K., Hoey, T., Warrior, R. and Levine, M. (1989). Autoregulatory and gap response elements of the *even-skipped* promoter of Drosophila. *EMBO J.* **8**, 1205-1212.
- Hoey, T., Weinzierl, R. O. J., Gill, G., Chen, J. -L., Dynlacht, B. D. and Tjian, R. (1993). Molecular cloning and functional analysis of Drosophila TAF110 reveal properties expected of coactivators. *Cell* **72**, 247-260.
- Ip, Y. T., Park, R. E., Kosman, D., Bier, E. and Levine, M. (1992). The dorsal gradient morphogen regulates stripes of *rhomboid* expression in the presumptive neuroectoderm of the Drosophila embryo. *Genes Dev.* **6**, 1728-1739.
- Jiang, J., Kosman, D., Ip, Y. T. and Levine, M. (1991). The dorsal morphogen gradient regulates the mesoderm determinant *twist* in early Drosophila embryos. *Genes Dev.* **5**, 1881-1891.
- Jiang, J. and Levine, M. (1993). Binding affinities and cooperative interactions with bHLH activators delimit threshold responses to the dorsal gradient morphogen. *Cell* **72**, 741-752.
- Kellum, R. and Schedl, P. (1991). A position-effect assay for boundaries of higher order chromosomal domains. *Cell* **64**, 941-950.
- Lawrence, P., Johnston, P., Macdonald, P. and Struhl, G. (1987). Borders of parasegments in Drosophila are delimited by the *fushi tarazu* and *even-skipped* genes. *Nature* **328**, 440-442.
- Licht, J. D., Gossel, M. J., Figge, J. and Hansen, U. M. (1990). Drosophila *Kruppel* protein is a transcriptional repressor. *Nature* **346**, 76-79.
- Macdonald, P., Ingham, P. and Struhl, G. (1986). Isolation, structure, and expression of *even-skipped*: a second pair-rule gene of Drosophila containing a homeo box. *Cell* **47**, 721-734.
- McGinnis, W. and Krumlauf, R. (1992). Homeobox genes and axial patterning. *Cell* **68**, 283-302.
- Muller, J. and Bienz, M. (1991). Long range repression conferring boundaries of *Ultrabithorax* expression in the Drosophila embryo. *EMBO J.* **10**, 3147-3155.
- Nusslein-Volhard, C., Kluding, H. and Jurgens, G. (1985). Genes affecting the segmental subdivision of the Drosophila embryo. *Cold Spring Harbor Symp. Quant. Biol.* **50**, 145-154.
- Rosenberg, U. B., Schroder, C., Preiss, A., Kienlin, A., Cote, S., Riede, I. and Jackle, H. (1986). Structural homology of the Drosophila *Kruppel* gene with Xenopus transcription factor IIIa. *Nature* **319**, 336-339.
- Small, S., Kraut, R., Hoey, T., Warrior, R. and Levine, M. (1991). Transcriptional regulation of a pair-rule stripe in Drosophila. *Genes Dev.* **5**, 827-839.
- Small, S., Blair, A. and Levine, M. (1992). Regulation of *even-skipped* stripe 2 in the Drosophila embryo. *EMBO J.* **11**, 4047-4057.
- Stanojevic, D., Hoey, T. and Levine, M. (1989). Sequence-specific DNA-binding activities of the gap proteins encoded by *hunchback* and *Kruppel* in Drosophila. *Nature* **341**, 331-335.
- Stanojevic, D., Small, S. and Levine, M. (1991). Regulation of a segmentation stripe by overlapping activators and repressors in the Drosophila embryo. *Science* **254**, 1385-1387.
- Struhl, G., Johnston, P. and Lawrence, P. A. (1992). Control of Drosophila body pattern by the *hunchback* morphogen gradient. *Cell* **69**, 237-249.
- Tautz, D. and Pfeifle, C. (1989). A non-radioactive in situ hybridization method for the localization of specific RNAs in Drosophila embryos reveals translational control of the segmentation gene *hunchback*. *Chromosoma* **98**, 81-85.
- Thummel, C. S., Boulet, A. M. and Lipshitz, H. D. (1988). Vectors for Drosophila P-element-mediated transformation and tissue culture transformation. *Gene* **74**, 445-446.
- Zuo, P., Stanojevic, D., Colgan, J., Han, K., Levine, M. and Manley, J. L. (1991). Activation and repression of transcription by the Drosophila gap proteins *hunchback* and *Kruppel*. *Genes Dev.* **5**, 254-264.

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