Gene expression stripes in Drosophila melanogaster embryos provide a model for how eukaryotic promoters are turned on and off in response to combinations of transcriptional regulators. Genetic studies suggested that even-skipped (eve) stripe 2 is controlled by three gap genes, hunchback (hb), Kruppel (Kr), and giant (gt), and by the maternal morphogen bicoid (bcd). A direct link is established between binding sites for these regulatory proteins in the stripe 2 promoter element and the expression of the stripe during early embryogenesis. The bcd and hb protein binding sites mediate activation, whereas neighboring gt and Kr protein sites repress expression and establish the stripe borders. The stripe 2 element has the properties of a genetic on-off switch.

The pair-rule gene eve encodes a homeobox protein important in the segmentation process. Mutations in eve produce severe segmentation defects, including the complete loss of segment borders in the middle body region (1). The eve protein is first detected 2 hours after fertilization, when it is uniformly distributed in all nuclei. This general expression gives way to a series of seven stripes along the length of the embryo before cellularization (2).

Individual stripes are regulated by separate cis elements contained within the eve promoter. For example, the first 1.7 kb of eve 5' flanking sequence drives the expression of a lacZ reporter gene only within the limits of stripes 2 and 7; a 480-bp deletion between -1.6 and -1.1 kb abolishes expression of stripe 2 (3, 4). Stripe 3 depends on sequences located between -3.8 and -3 kb. We focus here on the regulation of stripe 2.

Genetic studies suggest that a total of four segmentation genes are responsible for stripe 2 expression (Fig. 1A). There are anterior and posterior expansions of the stripe borders in gt or Kr embryos, respectively, whereas the stripe is abolished or reduced in bcd or hb embryos (5). The four genetic regulators of stripe 2 expression may act directly on the eve promoter and modulate its transcription because proteins encoded by all four genes bind with high affinity to sequences in the eve promoter that are essential for stripe 2 expression (5, 6) (Fig. 1B).

To test whether the bcd, hb, Kr, and gt protein binding sites in the eve promoter directly mediate the interactions predicted by the genetic studies, we used site-directed mutagenesis (7) to disrupt some of these sites in a fusion gene that contains 5.2 kb of eve 5' flanking sequence. This eve-lacZ fusion normally drives equally intense expression of stripes 2, 3, and 7 (3, 4). In this way, stripes 3 and 7 served as internal controls for perturbations of stripe 2 expression (8).
Decoyribonuclease (DNase I) protection assays indicated that the mutated sites failed to bind proteins within the detection limits of the method (9). Unfortunately, because the bcd1 and kr3 binding sites extensively overlap (Fig. 1B), it was not possible to disrupt one of the sites without affecting the other. The mutation in the bcd1 site also impairs the binding of Kr protein to the kr3 site (10). The bcd1-kr3 mutation alone did not cause a discernible change in stripe 2 expression. Also, the removal of the bcd2 site alone had no effect on stripe 2 expression. However, an eve-lacZ fusion gene containing mutations in both the bcd1-kr3 sequence and the bcd2 site resulted in reduced expression of stripe 2 relative to stripe 3 (Fig. 2, A and B) (11). Mutations in the hb3 binding site cause a similar but less severe reduction in stripe 2 expression (9, 10).

There are three gt protein binding sites present in the stripe 2 element (5) (Fig. 1B). These were disrupted by the creation of two small deletions; one deletion removes the gt2 and gt3 sites, whereas the other eliminates the gt1 site (12). This double mutation caused expansions of the anterior border of stripe 2 to a variable extent (Fig. 2C) (11). Despite this altered pattern, the level of stripe 2 expression is essentially normal. Transformants containing a single mutation lacking only the gt1 site or the gt2 and gt3 sites showed lesser expansions of the stripe (9, 13).

A series of mutations were made in each of the six high-affinity Kr protein binding sites present in the stripe 2 element. One of the eve-lacZ fusion genes that was examined contains point mutations in the kr1, kr2, kr4, and kr6 binding sites, none of which overlap any of the bcd protein sites (14) (Fig. 1B). This quadruple mutant causes a slight expansion of the stripe 2 posterior border that results in a narrower gap between stripes 2 and 3 (Fig. 3, A and B). Mutations in all six high-affinity Kr protein sites caused a greater posterior expansion of the border, so that stripes 2 and 3 were nearly contiguous (Fig. 3C).

Genetic studies had failed to distinguish between direct and indirect regulatory interactions (2, 4). For example, the expansion of the posterior eve stripe 2 border observed in Kr− embryos could be due to direct repression by Kr protein or to an indirect effect of altering the expression pattern of hb (15). Stripe 2 expression is lost in bed− embryos (5), but because bed protein functions as a direct transcriptional activator of hb (16), it was unclear from the genetic studies whether bed protein is a direct or indirect regulator of stripe 2. We have presented evidence that at least some of the bed, hb, gt, and Kr protein binding sites present in the eve promoter are likely to directly regulate stripe 2 expression in the early embryo. Mutations in bed or hb protein binding sites cause reduced expression of stripe 2 but do not alter stripe 2's normal spatial limits. In contrast, mutations in either the gt or Kr protein sites change the positions of the stripe borders. Thus, the eve stripe 2 promoter element may act as a genetic on-off switch that integrates the activities of the bed and hb activators with overlapping gt and Kr repressors.

There is a potential discrepancy between the genetic model of stripe 2 expression and the results obtained with mutated eve promoters lacking Kr protein binding sites. Disruptions in all six high-affinity Kr protein sites lead to a relatively mild posterior expansion in the stripe border (Fig. 3C). A more dramatic expansion of the stripe is observed in Kr− embryos (2, 4, 5, 17). This could be explained by the fact that there are numerous low-affinity Kr protein binding sites present in the stripe 2 element (Fig. 1B). None of these sites were disrupted in the present study, and it is conceivable that

![Fig. 1. Summary of stripe 2 regulation.](image1)

(A) The limits of stripe 2 are shown relative to the expression domains of bcd, hb, Kr, and gt, which encode proteins distributed in broad, overlapping gradients (5, 6, 20). Both bcd and hb proteins function as transcriptional activators and define a broad domain in the anterior half of the embryo where the stripe 2 element can be activated. The borders of the stripe depend on selective repression by gt protein in anterior regions and Kr protein in posterior regions. (B) The nucleotide sequence of the stripe 2 promoter element. The sequence extends from −931 to −1601 bp upstream of the transcription start site. A 480-bp deletion from −1050 to −1530 bp completely abolishes the expression of the stripe (4). The locations of the bcd, hb, gt, and high-affinity Kr protein binding sites are indicated. The limits of these sites are based on DNase I protection assays and may extend beyond the core recognition sequences (5, 6). There is tight linkage of the bcd and hb activator sites (underlined sequences) and the gt and Kr repressor sites (indicated above the sequence). The broken lines indicate the locations of low-affinity Kr protein binding sites.

![Fig. 2. Mutations in bcd and gt protein binding sites disrupt stripe 2 expression in embryos at nuclear division cycle 14.](image2)
they can mediate repression by high concentrations of Kt protein. The g and Kr protein binding sites identified by in vitro assays correspond to authentic repressor sites in vivo. Because these sites overlap or are closely linked to the bcd and hb activator sites, it is likely that the g and Kr proteins define the stripe borders through a short-range mechanism of repression. This could involve competitive binding to DNA, whereby the binding of g or Kr protein precludes the binding of bcd and hb activators to overlapping sites. Alternatively, activators and repressors may bind to adjacent sites, but protein-protein interactions between the repressors and activators may "mask" the ability of activators to contact the transcription complex (18). Short-range repression could account for the autonomous action of individual stripe initiation elements. For example, the binding of Kr protein to stripe 2 sequences does not interfere with the activation of the stripe 3 element, which is activated in a region of the embryo containing high levels of the Kr repressor. Stripe 3 sequences do not contain any Kt protein binding sites (6) and map about 1.5 to 2.0 kb upstream of the stripe 2 element.

The interplay between activators and repressors observed here for the stripe 2 element may be a general component of eukaryotic enhancers. Although the early Drosophila embryo is a syncytium that permits the intermixing of regulatory factors by diffusion, there are numerous instances where small changes in the concentrations and combinations of factors might effect choices in cell fate. For example, in many cell lineages a common precursor gives rise to daughter cells that follow totally distinct differentiation pathways. Perhaps this involves a slight asymmetry in the concentrations or activities of regulatory factors inherited by daughter cells, which could be accomplished by the asymmetric partitioning of factors or distinct cell-cell interactions that cause slight differences in their activities. Once daughter cells have distinct combinations or concentrations of a common set of factors sharp on-off patterns in gene expression might be effected through enhancers analogous to the stripe 2 element.

REFERENCES AND NOTES
7. A 785-bp Xho I-Bal I enhancer fragment extending from 1.7 to 0.9 kb upstream of the start site was subcloned into the plasmid vector (Strategene) and used for mutagenesis. Site-directed mutations were made with mutagenic oligonucleotides and the Bio-Rad mutagenesis kit. Single-stranded DNA templates were derived from the plasmid vector. Oligonucleotides contained 25 to 40 bases with at least ten nucleotides of homology on either side of the mutated region. We made multiple mutants by simultaneously annealing two or more mutagenic oligonucleotides to the same DNA template. We exchanged all mutagenized fragments for wild-type sequences in the 5.2-kb eve-lacZ fusion gene (3, 4) by replacing wild-type fragments with the corresponding mutant templates in the pSS17 vector (19), which contains a unique Not I restriction site. The eve promoter sequences were inserted into the pDM30 P-element vector at the unique Not I site (19). All eve-lacZ fusions were inserted into the pDM30 vector in the same orientation as the ray marker gene. All mutations created within stripe 2 sequences were confirmed by restriction digestions, DNA sequencing, and DNase I footprint analyses.
8. The ray-lacZ fusion gene was cloned into the pDM30 P-transformation vector and injected into rayembryos along with the ∆2,3 helper plasmid (19). For each construct, the following numbers of independent P-transformation lines were examined: Ahb3, two lines; abd-1kr-3, one line; abd-1 and bcd2, four lines; Agt1, two lines; Agt2 and gft5, five lines; Agt1, gft5, and gft3, six lines; skk1, kr2, kr3, and kr6, five lines; and skk1, kr2, kr3, kn5, and kr6, seven lines. For most experiments, expression of the ray reporter gene was detected in P-transformed embryos by immunohistochemical staining with an antibody against β-galactosidase and visualized with a horseradish peroxidase-conjugated secondary antibody with the Elite kit (Vector Laboratories).
10. We used the following mutagenic oligonucleotides to create mutations in bcd and Kt protein binding sites (the altered bases are in italics): bcd-1kr-2, CATTAGACGAGGCGGAGCCGAGGCGG; bcd-3, GCGGAGGCGGAGGCGG; bcd-4, ATGCTTTGACGAGGCGG; and kn5, CATTAGACGAGGCGGAGGCGG; GATTAGAAGACGCGGAGGCGG; and kn5, CATTAGACGAGGCGGAGGCGG.
12. A 19-bp sequence that includes most of the g6 recognition sequence was deleted and replaced by a 6-bp Sph I restriction site. A 100-bp sequence was deleted from the g6 to g6 region and also replaced with an Sph I site. This deletion nearly coincides with the sequences that are protected in DNase I footprint assays with high concentrations of g6 protein. The neighboring bcd binding site (Fig. 1B) was not affected. The following mutagenic oligonucleotides were used to delete this site: g6, GTCTTTTCTATGCGGAGG; and g6 and g3, GGCATCACGGATAGCCGCTTGCCGATCTTCC.
13. The intensity of stripe 2 is normal even though the g6 deletion also removes the bcd site. Disrupting the bcd site in another normal eve-lacZ fusion results in reduced expression of the stripe. Thus, it would appear that the simultaneous loss of the g6 site somehow compensates for the removal of bcd.
14. We used six oligonucleotides to mutate the Kt protein binding sites. Each of the oligonucleotides (except kb) precisely removes the Kt-binding consensus sequences and replaces it with a restriction site (in italics). The following oligonucleotides were used: kr1, CTGTATCCCGGAGCTGAGGAGCGGAGCAGC; kr2, CGGAGCGAGTCCCGGCTGGC; kr3, TGCTTTTCTATGCGGAGGAGCTG; kb, GGCAGAGAAA, and kn5 oligonucleotide used for bcd-1kr-3 (in parentheses). kr4, GGCCGCGGAGACGCTGGC; kr5, CGGAGCGAGTCCCGGCTGGCAGC; kr6, GGCCGCGGAGACGCTGGCAGC; and kr6, GGCCGCGGAGACGCTGGCAGC.
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