Development 126, 1201-1210 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
DEV5246

Transcriptional repression by the *Drosophila* Giant protein: *cis* element positioning provides an alternative means of interpreting an effector gradient

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Accepted 28 December 1998; published on WWW 15 February 1999

SUMMARY

Early developmental patterning of the *Drosophila* embryo is driven by the activities of a diverse set of maternally and zygotically derived transcription factors, including repressors encoded by gap genes such as *Krüppel*, *knirps*, *giant* and the mesoderm-specific *snail*. The mechanism of repression by gap transcription factors is not well understood at a molecular level. Initial characterization of these transcription factors suggests that they act as short-range repressors, interfering with the activity of enhancer or promoter elements 50 to 100 bp away. To better understand the molecular mechanism of short-range repression, we have investigated the properties of the Giant gap protein. We tested the ability of endogenous Giant to repress when bound close to the transcriptional initiation site and found that Giant effectively represses a heterologous promoter when binding sites are located at –55 bp with respect to the start of transcription. Consistent with its role as a short-range repressor, as the binding sites are moved to more distal locations, repression is diminished. Rather than exhibiting a sharp 'step-function' drop-off in activity, however, repression is progressively restricted to areas of highest Giant concentration. Less than a two-fold difference in Giant protein concentration is sufficient to determine a change in transcriptional status of a target gene. This effect demonstrates that Giant protein gradients can be differentially interpreted by target promoters, depending on the exact location of the Giant binding sites within the gene. Thus, in addition to binding site affinity and number, *cis* element positioning within a promoter can affect the response of a gene to a repressor gradient. We also demonstrate that a chimeric Gal4-Giant protein lacking the basic/zipper domain can specifically repress reporter genes, suggesting that the Giant effector domain is an autonomous repression domain.

Key words: *Drosophila*, Transcription, Repression, Gap gene, Morphogen gradient

INTRODUCTION

Important advances in understanding the molecular mechanisms of development have stemmed from genetic analysis of *Drosophila* embryogenesis. Regulation of gene expression in the early embryo is critical for determining specification of regional and segmental identity (Nüsslein-Volhard et al., 1987; Driever, 1993; Pankratz and Jäckle, 1993). Spatially localized expression of transcription factors sets in motion a host of interactions necessary for tissue differentiation (St. Johnston, 1993; Pankratz and Jäckle, 1993; Rivera-Pomar and Jäckle, 1996). Many of the embryonic segmentation genes identified in pioneering studies by Wieschaus and Nüsslein-Volhard, including gap genes and pair-rule genes, encode transcription factors (Nüsslein-Volhard and Wieschaus, 1980; Nüsslein-Volhard et al., 1985).

One of the most important concepts to emerge from genetic studies of development is that gradients of transcription factors can specify positional information by activating or repressing genes that have differential sensitivity to the effector protein (Driever, 1993; Chasen and Anderson, 1993). For transcriptional activators, the different threshold responses of genes have been traced to the affinity or number of the binding sites for that activator within promoters. Two well-studied transcription factor gradients provide important positional information in the early embryo: the anterior-to-posterior Bicoid gradient, and the ventral-to-dorsal Dorsal protein gradient. Bicoid protein binding to the *hunchback* (*hb*) promoter drives the expression of the *hb* gene, and increasing the number of Bicoid binding sites or enhancing their affinity is sufficient to activate a *hb* reporter gene in more posterior regions of the embryo where Bicoid protein levels are low (Driever and Nüsslein-Volhard, 1988; Driever et al., 1989; Struhl et al., 1989). The nuclear Dorsal protein gradient is established early in embryogenesis, and Dorsal defines three territories in the embryo by activating genes such as *twist* (*twi*) at high concentrations in the presumptive mesoderm, neuroectoderm genes such as *rho* at intermediate levels in the

presumptive neuroectoderm, and at lowest levels, defining the dorsal ectoderm through negative interactions with the *zerknullt*,(*zen*) *tolloid*, (*tld*) and *decapentaplegic* (*dpp*) promoters (Jiang et al., 1991, 1992; Jiang and Levine, 1993; Pan and Courey, 1992; Thisse and Thisse, 1992; Kirov, 1993; Cai et al., 1996). When low-affinity Dorsal binding sites within the *twi* promoter are converted to high affinity binding sites, the ventral band of expression expands from 12-14 cells to 20 cells wide (Jiang and Levine, 1993). High-affinity Dorsal binding sites within the *rho* element, together with binding sites for bHLH activators, ensure activation at lower levels of Dorsal (Jiang and Levine, 1993; Szymanski and Levine, 1995). In dorsal regions of the embryo, where Dorsal levels are lowest, the protein functions as part of a multiprotein complex in ventral repression elements of *zen*, *tld*, and *dpp* (Dubnikoff et al., 1997; Kirov et al., 1993; Cai et al., 1996).

While these experiments have suggested plausible molecular mechanisms for differential regulation of genes by gradients of transcriptional activators, the picture with transcriptional repressors is less complete. Different concentrations of the Knirps, Krüppel and Giant proteins are important for proper regulation of zygotic genes; for example, Knirps has been suggested to act as a morphogen based on the differential responses of the *even-skipped* (*eve*), *runt* (*run*) and *fushi tarazu* (*ftz*) promoters (Kosman and Small, 1997). In a similar vein, low concentrations of Giant are sufficient to repress the *Krüppel* (*Kr*) promoter, while greater amounts appear to be required to repress the *eve* stripe 2 element (Wu et al., 1998). Additionally, Krüppel can function as an activator at low concentrations and a repressor at high concentrations in cell culture, although these effects have not been demonstrated in embryos (Sauer and Jäckle, 1991). The molecular basis of differential responses to such repressors is not clear, although binding site affinity or binding site number within target genes might be important, as with activators.

To understand why different genes respond differently to gradients of repressor proteins in the developing embryo, we need to understand how these proteins repress transcription. Many repressors can be catagorized as 'short-range' or 'long-range' repressors (Gray et al., 1994; Gray and Levine, 1996b; Cai et al., 1996). Snail and Knirps typify short-range repressors; these proteins repress from within enhancer elements and from promoter proximal positions, but only when bound within 50-100 bp of transcriptional activators or basal promoter elements (Gray and Levine, 1996; Arnosti et al., 1996a). When located within an enhancer element, short-range repressors do not disturb the functioning of neighboring enhancer elements. This limited range of action allows proper functioning of independent, modular enhancers within complex promoters. In contrast, long-range repressors such as Hairy, or complexes of proteins including Dorsal, can repress a gene in a dominant fashion when bound as far as a kilobase away from the affected enhancers or promoter (Barolo and Levine, 1997; Cai et al., 1996).

Additional information on repressor function comes from recent studies indicating that repressor proteins interact with transcriptional cofactors to effect repression. The Groucho protein functions as part of Hairy, Runt, Engrailed and Dorsal repression complexes (Parkhurst, 1998). Groucho, and a structurally similar corepressor protein from yeast, TUP1, both feature WD-40 repeat domains, thought to be important for mediating protein-protein interactions (Keleher et al., 1992; Neer et al., 1994). Current models suggest that TUP1 represses promoters in yeast via chromatin-mediated effects or through direct interaction with RNA polymerase II holoenzyme subunits (Redd et al., 1996; Edmondson et al., 1996; Wahi and Johnson, 1995; Cooper et al., 1994). Knirps, Hairy, and Snail have been demonstrated to physically interact with the dCtBP protein, a *Drosophila* homolog of the human C-terminal Binding Protein (CtBP) (Nibu et al., 1998b). Furthermore, a mutation in the dCtBP gene causes a severe gap gene phenotype (Perrimon et al., 1996; Nibu et al., 1998a; Poortinga et al., 1998). The human CtBP protein binds the adenovirus E1a protein, and CtBP can interfere with the transforming activity of E1a (Schaeper et al., 1995). Murine CtBP2 functions as a cofactor for the BKLF repressor (Turner and Crossley, 1998).

The Giant gap protein has been characterized as a repressor of other gap genes, including *Krüppel* and *knirps*, as well as pair rule genes such as *eve* (Kraut and Levine, 1991; Eldon and Pirotta, 1991; Small et al., 1992). Analysis of Giant action on the stripe 2 enhancer of the *eve* gene suggests that Giant is a short-range repressor (Arnosti et al., 1996b; Small et al., 1993). The Giant protein contains a C-terminal dimerization/DNA binding domain of the basic/leucine zipper class (Capovilla et al., 1992; Vinson et al., 1989), and binds to DNA sequences in vitro which are similar to the elements bound by the CREB family of basic/leucine zipper proteins (Capovilla et al., 1992). *giant* (*gt*) mutants exhibit abdominal segment defects and loss of head structures, a pattern consistent with the regions in which the gene is expressed (Gergen and Wieschaus, 1986; Petschek et al., 1987). The protein appears to be phosphorylated in the embryo (Capovilla et al., 1992), although the significance of this modification is unknown.

To understand the molecular basis of transcriptional repression employed by short-range repressors, we have taken two complementary approaches to study the activity of the Giant protein in transgenic embryos. First, we have tested the ability of endogenous Giant to interact with promoter elements, to determine whether this protein can directly target basal promoter elements, as has been reported for other short-range repressors. We found that Giant can functionally interact with basal promoter elements. Strikingly, the repression activity of Giant depends both on the concentration of the repressor and on the exact placement of the binding site, suggesting that differential responses to repressor gradients can be determined not only by binding site affinity or number, but by the precise positioning of *cis* elements within genes. Secondly, we have assayed the activity of recombinant Giant protein in transgenic embryos. We show that the Giant effector domain is dissociable from the basic/zipper DNA binding region, and can function when fused to a Gal4 DNA binding domain. The activity of the chimeric gene demonstrates that no additional basic/leucine zipper partner protein is required to heterodimerize with Giant for repression to occur.

MATERIALS AND METHODS

Plasmid construction

Construction of the gt-55 gene. An oligonucleotide containing two Giant 'CD1' binding sites (Capovilla et al., 1992) separated by a *Bgl*II

restriction site and flanked by two *Sph*I sites (Giant binding sites in bold) 5′ GCA TGC **TAT GAC GCA AGA** AGA CCC AGA TCT TTT **TAT GAC GCA AGA** GCA TGC 3′, was annealed to a complementary oligonucleotide and inserted into the *Sph*I site of the C4PLZ vector 5′ of *lac*Z (Wharton and Crews, 1993). The 520 bp *twi* 2XPE enhancer (Jiang and Levine, 1993) and *rho* 330 NEE enhancer (Jiang and Levine, 1993) were excised from the $dw^3$29 vector (Arnosti et al., 1996a) with *Not*I and *Eco*RI respectively and inserted into the *Not*I and the *Eco*RI sites of C4PLZ 5′ of *lac*Z. Both of the enhancers retain their original 5′-3′ orientation.

Construction of the gt-80, gt-110, and gt-160 genes. The *Sph*I restriction site upstream of the Giant binding sites in gt-55 was eliminated by removing the original gt-55 *Sph*I oligonucleotide containing the twin Giant binding sites and replacing it with an oligonucleotide (Giant binding sites bold) 5′ T**TA TGA CGC AAG A**CC CAG ATC TTT T**TA TGA CGC AAG A**GC ATG 3′ identical to the original insert except for a point mutation that destroys the distal *Sph*I site. gt-80 and gt-110 were constructed from this modified gt-55 vector, designated Twi, to extend the distance between the reporter promoter and the twin Giant binding sites by 25 bp and 55 bp respectively. A 25 bp oligonucleotide 5′ ACG TGG ATA CGA TTA AGT ATG CAT G 3′ was inserted into the *Sph*I site of Twi to make gt-80 and a 55 bp oligonucleotide 5′ TCC ATG ATA AAC GCG TGC TAG ACT ATT GCA GGT ACT GAT CGA ATG CCT CTG CAT G 3′ was inserted into the *Sph*I site of Twi to make gt-110. Both gt-80 and gt-110 conserved only the *Sph*I restriction sequence proximal to the reporter promoter. gt-160 was constructed by inserting a 50 bp oligonucleotide 5′ TCG CTA GAC GTG AAT CTC GTA GCT TCC GTA CCA AAT GCG TAT CAG G CAT G 3′ into the *Sph*I site of gt-110, conserving only the *Sph*I restriction sequence distal to the reporter promoter. In gt-80, gt-110, and gt-160, the 3′ edge of the proximal Giant binding site is located at −80, −110, and −160 with respect to the transcriptional initiation site. The Uni vector was derived from gt-55 by excising the twin Giant site with *Sph*I and replacing it with a 24 bp oligonucleotide 5′ GCA TGA **TAT GAC GCA AGA** GCA TGC 3′, conserving only the *Sph*I restriction sequence proximal to the basal promoter.

The *eve* stripe 3/ *rho*UAS*lac*Z reporter gene construction. A 660 bp *rho* element (Arnosti et al., 1996a) was modified by inserting twin UAS binding sites (approx. 50 bp 5′ of the Dorsal 1 and approx. 50 bp 3′ of the Dorsal 4 binding sites) using sequential mutagenesis of the *rho* element in pBluescript (Small et al., 1992; Arnosti et al., 1996a). The oligonucleotides used were 5′ AGC CAC CGT ACC AAC GGA GGA CAG TCC TCC GAG ATC TCT CGA GCT GGA AT 3′, 5′ AGT CCT CCG AGA TCT CGG ACG ACT CTC GTC CGG TAA CCG AGT GGG CGT GG 3′, 5′ TTT TTC CAT CCT GCC GGA GGA CAG TCC TCC GAG ATA TCC GAC AGG GGC GC 3′, and 5′ GAC AGT CCT CCG AGA TCT CGG ACG ACT CTC GTC CGT TCA AGC AGC TTC TG 3′. This 660 bp element was cloned into the *Bam*HI site of C4PLZ (Wharton and Crews, 1993). A 500 bp *eve* stripe 3 element with *Kpn*I sites at either end was generated by PCR amplification from *eve* promoter construct no. 1 (Small et al., 1993) using oligonucleotides 5′ GG GGT ACC GGA TCC TCG AAA TCG AGA GCG ACC 3′ and 5′ GGG GGT ACC GGA CCC GAG GAA CGA GCT CGT AAA 3′. This 500 bp *eve* stripe 3 element was inserted into the *Kpn*I site 5′ of the *rho* UAS element, preserving the original 5′ to 3′ orientation.

Construction of Gal4-Giant fusion gene. The *gal4-gt* gene was expressed using the pTwiggy vector, which drives Gal4 fusion genes using the *twi* 2XPE (Arnosti et al., 1996a). Two oligonucleotide sequences 5′ GGG TCG GTA CCG CAG CCA TGC TAA TGC ACG AGA AAC TCA TG 3′ and 5′ GGG GAA TCT AGA CTA ACT AAT TAC TAC TCG TAG TAT GCG GCA TCC TTA AC 3′ were used to generate *gt* cDNA by PCR from a bacterial *gt* expression vector (Small et al., 1991). The 5′ end of the PCR fragment contains a *Kpn*I site followed by *gt* codons 1-389, then a series of stop codons followed by an *Xba*I restriction site. The fragments were subsequently digested with *Kpn*I and *Xba*I, inserted in frame into the *Kpn*I-*Xba*I sites of the pTwiggy vector, yielding a fusion gene coding for the Gal(1-93)-Gly-Thr-Ala-Ala-*gt*(1-389). The entire *gt* open reading frame was sequenced before the gene was introduced into the *Drosophila* germline.

The *eve* stripe 2/3 reporter, a generous gift from S. Barolo, was constructed by cutting the *eve* stripe 2 2XUAS *lac*Z gene (Arnosti et al., 1996b) with *Pst*I and inserting a 339 bp spacer *Dra*I fragment of the CAT gene from pLysS (Novagen) and an *eve* stripe 3 element, such that the elements are arranged: stripe2/2xUAS/339 bp spacer/stripe3/*lac*Z.

The pBS-SK(+)gt1 riboprobe vector was constructed by cloning the *Kpn*I-*Xba*I fragment containing *gt* codons 1-389 from pTwiggy Gal4-Giant into pBluescript SK+(Strategene) at the *Eco*RI and *Xba*I sites using the following bridging oligonucleotides 5′ AATTCGCAGCTG-GTAC 3′ and 5′ CAGCTGCG 3′ to make pBSgt1.

P-element transformation, whole-mount in situ hybridization of embryos, cross to *gt*⁻ line

P-element transformation vectors were introduced into the *Drosophila* germline by injection of *y* w^{67} embryos as described (Small et al., 1992). For each gene construct, at least three separate lines were tested, and similar results were obtained with all lines. In situ hybridizations were performed as described by Small et al. (1992) using digoxigenin-UTP-labeled antisense RNA probes to *lac*Z, *white*, or *giant*. Antisense RNA probe for detection of *gt* gene expression was generated by transcription of pBSgt1 with T3 RNA polymerase as described (Small et al., 1992). Male transgenic reporter lines containing the gt-55 gene were crossed to female mutant gt^{A8}/FM7c (Bloomington Stock Center 1004.1) and embryos were stained with a *lac*Z antisense probe.

Antibody staining and quantitation of Giant protein in embryos

Embryos were fluorescently stained as described using FITC-conjugated goat anti-rat (Jackson Labs) together with rat anti-Giant, which was raised as described by Kosman et al. (1998). These embryos were also stained for Krüppel and Eve using Texas Red and Cy-5 second antibodies respectively. Confocal scanning was performed as described by Kosman et al. (1998). Images were automatically rotated and cropped by making a thresholded mask of the entire embryo, finding the principal moments, rotating the embryo to bring the principal moments in line with the x and y axes, and cropping the image to the edge of the mask. Correct D-V and A-P orientation was then obtained by visual inspection and manual flipping if needed. Segmentation of the rotated and cropped image was performed as described by Kosman et al. (1997) with the following modifications. First, masks were constructed from an image containing the maximum pixel from the three channels containing Giant, Eve, and Krüppel expression levels. Together these genes are expressed in virtually all nuclei, obviating the need for a nuclear counterstain. Second, the watershed method (instead of skeletonization) was used for the correction step following edge detection (Bowler, Reinitz and Kosman, unpublished data). These manipulations result in the transformation of a multichannel image of an embryo into a text file containing a numbered list of identified nuclei, one per line. The entry for each nucleus contains the coordinates of its centroid in percent egg length and percent dorsal-ventral length, together with the fluorescence values in each channel averaged over that nucleus. A single image gives about 2000 processed nuclei.

Imaging, measurement of repressed regions in embryos, and visual staging of embryos

Embryos were photographed with DIC imaging on a Leica DMLB microscope, and color slides were digitized and analyzed using Adobe Photoshop software. Blastoderm embryos were visually staged by

measuring the extent of cellularization around the embryo periphery and comparing to similar embryos that had been stained for *giant* mRNA. Pregastrula cellularizing embryos with cell width to height ratios of greater than one were assigned to 'early' embryos, and those with longer cells, i.e. width to height of less than one, were classified as 'late'. Anterior repression width was measured by visual inspection of early cellularizing mounted embryos; for gt-55, average width of anterior repression was 14±2 (s.d.) nuclei, *n*=108; gt-80, 11±2 nuclei, *n*=58; gt-110, 9±1 nuclei, *n*=53; gt-160, 5±1 nuclei, *n*=53.

RESULTS

Giant protein dominantly represses a basal promoter element in a gene-specific manner

The expression pattern of *gt* is dynamic, and rapidly evolves from two broad stripes, one anterior and one posterior, in early nuclear division 14 embryos, to three anterior stripes and one posterior stripe in mid to late nuclear cycle 14 embryos (Fig. 1A-C). The posterior stripe begins to fade in late nuclear cycle 14 embryos (Fig. 1D). Giant protein patterns closely mirror the expression of *gt* mRNAs (Fig. 4; Kraut and Levine, 1991; Eldon and Pirrotta, 1991). Previous analysis of repression by the Giant protein in the *Drosophila* embryo has focused on the activity of this transcription factor in the context of endogenous target genes with complex regulatory elements, such as *eve* (Stanojevic et al., 1991; Arnosti et al., 1996b). Some of these regulatory elements may contain composite binding sites; for example, functional Giant elements identified within the *eve* stripe 2 enhancer by footprinting are 23-45 bp in length (Small et al., 1991), larger than the smaller binding sites found in the *Kr* promoter (Capovilla et al., 1992). To simplify the analysis of Giant repression, we targeted endogenous Giant to precisely identified sites in a well-characterized reporter gene complex previously used to analyze other short-range repressors. The analysis of Giant activity in transgenic embryos has the advantage that the reporter genes are inserted in a chromosomal context, and the expression levels of the repressors are similar or identical to those found in a normal embryo. Indeed, recent comparative work with the Engrailed repressor suggests that assays based on transient transfections may not measure important aspects of repressor function seen with integrated reporters (Tolkunova et al., 1998).

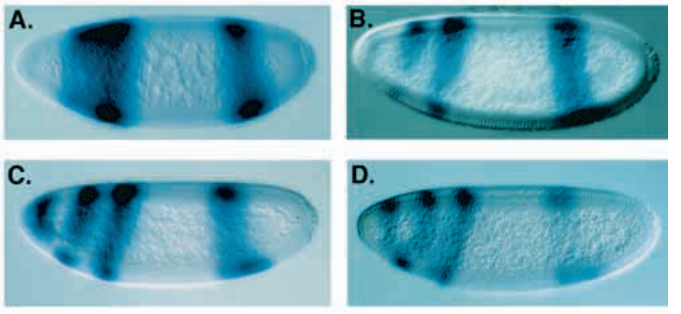

Fig. 1. Expression of the *giant* (*gt*) gene in the early embryo. Wild-type embryos were hybridized with *gt* antisense RNA probe and visualized by immunohistochemical staining. All embryos are oriented with anterior to the left and dorsal side up. (A) Expression pattern of *gt* gene in an early nuclear cycle 14 embryo. (B) In a slightly older cycle 14 embryo, the anterior band of expression divides into two domains. (C) A third anterior domain appears in mid cycle 14 embryos. (D) Posterior *gt* expression begins to disappear in mid to late cycle 14 embryos.

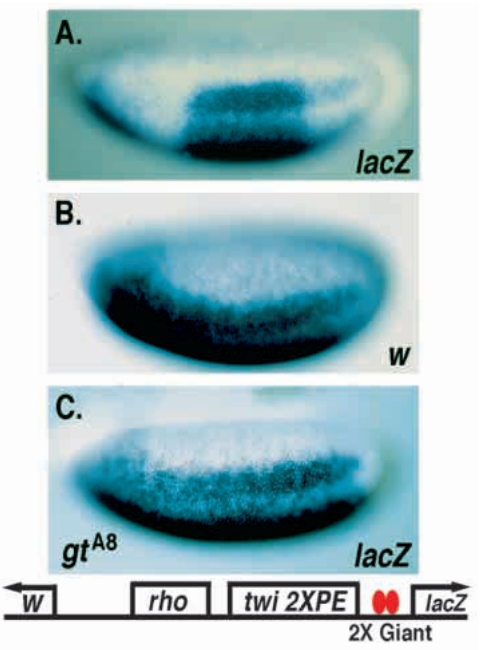

Fig. 2. Giant mediates repression of a gt-55 *lac*Z reporter gene from a promoter proximal position. Embryos were stained as described in Fig. 1 with an antisense *lac*Z or *w* probe. The diagram below illustrates the structure of the gene complex that was introduced into the genome via P-element mediated germline transformation. Embryos are oriented with anterior to the left and dorsal side up. (A) *lac*Z expression, showing strong repression of transcription in posterior and anterior regions. (B) *white* expression, showing normal *twi* and *rho* expression patterns, with no repression in regions of *gt* expression. (C) *lac*Z expression from the gt-55 transgene in an embryo carrying the gt^{A8} mutation. No repression is observed.

Tandem 'CD1' Giant binding sites derived from the *Krüppel* (*Kr*) promoter (Capovilla et al., 1992) were inserted 5′ of a transposase basal promoter (Wharton and Crews, 1993), 55 bp from the initiation of transcription of the *lac*Z reporter gene, and approximately 1.2 kbp from the divergently transcribed *white* (*w*) gene. The divergent *w* and *lac*Z promoters are both activated by a *twi* enhancer in ventral regions and a *rho* enhancer in ventrolateral regions. Strong repression of the *lac*Z gene is detected in embryos bearing this transgene; expression of the *lac*Z gene in both anterior and posterior Giant regions is strongly attenuated (Fig. 2A). *w* gene expression is not affected, indicating that Giant is capable of repressing the *lac*Z promoter in a gene-specific manner (Fig. 2B). This lack of *w* repression indicates that Giant is not completely blocking the function of the *twi* and *rho* enhancer elements. The repression of *lac*Z in anterior and posterior regions was relieved when the transgene was assayed in *gt* mutant embryos (Fig. 2C), confirming that the Giant protein is mediating the repression.

Binding site proximity to basal promoter element dictates response of *lacZ* transgene to Giant repression

To determine the distance over which Giant proteins can mediate repression of a basal promoter, small spacer oligonucleotides were inserted in the reporter gene to move the

proximal edge of the tandem Giant binding sites from −55 bp to −80 bp, −110 bp, or −160 bp. Embryos carrying the four transgene reporters were visually staged before analysis so that differences in repression activity were not confounded by the temporal changes in Giant protein pattern. In genes with Giant binding sites at successively greater distances, repression of the *lac*Z promoter was progressively attenuated. The anterior region of repression was narrower in early cycle 14 embryos with more distally positioned Giant sites, from 14 nuclei wide with construct gt-55, 11 nuclei wide with gt-80, 9 nuclei wide with gt-110 to 5 nuclei wide with gt-160 (Fig. 3A,C,E,G). In late cycle 14 embryos, posterior repression was weakened or abolished for the gt-110 and gt-160 genes, in which Giant binding sites are most distally located (Fig. 3F,H).

The progressive loss of repression observed in the gt-55, gt-80, gt-110, and gt-160 series indicates that the distance dependence of Giant repression is not an all-or-nothing effect. For gene constructs with Giant elements at more distal locations, higher levels of Giant protein are apparently required to mediate repression. In the embryo, genes are subject to repression in nuclei at the center of the anterior Giant expression domain (Fig. 3E,G), while genes in adjacent nuclei are not.

Quantitation of Giant protein levels in embryos

Two aspects of differential Giant activity are evident from the expression of the transgenes shown in Fig. 3; first, repression by Giant appears to be more effective in anterior regions of the embryo compared to posterior regions (Fig. 3D,F,H), and second, within the anterior domain, on reporter genes weakly repressed by Giant, repression is only seen in an area corresponding to *giant* stripe 3, rather than the entire anterior region (Fig. 3C,E,G). Consistent with these observations, previous experiments indicate that what appear to be peak levels of Giant protein are present in anterior regions (Small et al., 1992; Kraut and Levine, 1991; Eldon and Pirrotta, 1991), but no efforts to quantitate relative protein levels have been reported.

The lower levels of Giant repression in the stripe 2 ventral regions are clearly linked to loss of expression of the *giant* gene in ventral regions in older embryos (Fig. 1B,C). We therefore focused on the more subtle differences between expression levels in the anterior Giant stripe at 66-70% egg length and the posterior stripe at 25-30% egg length, regions of the blastoderm where Giant protein is present, but repression is dependent on target gene construction. To determine whether the peak levels of Giant protein indeed correspond to those areas of the embryo with maximal Giant activity, we stained embryos with Giant-specific antibodies (Fig. 4A,C) and quantitated the fluorescence signals using confocal scanning microscopy, followed by a step in which the signal over each individual nucleus is averaged by computer. Nucleus by

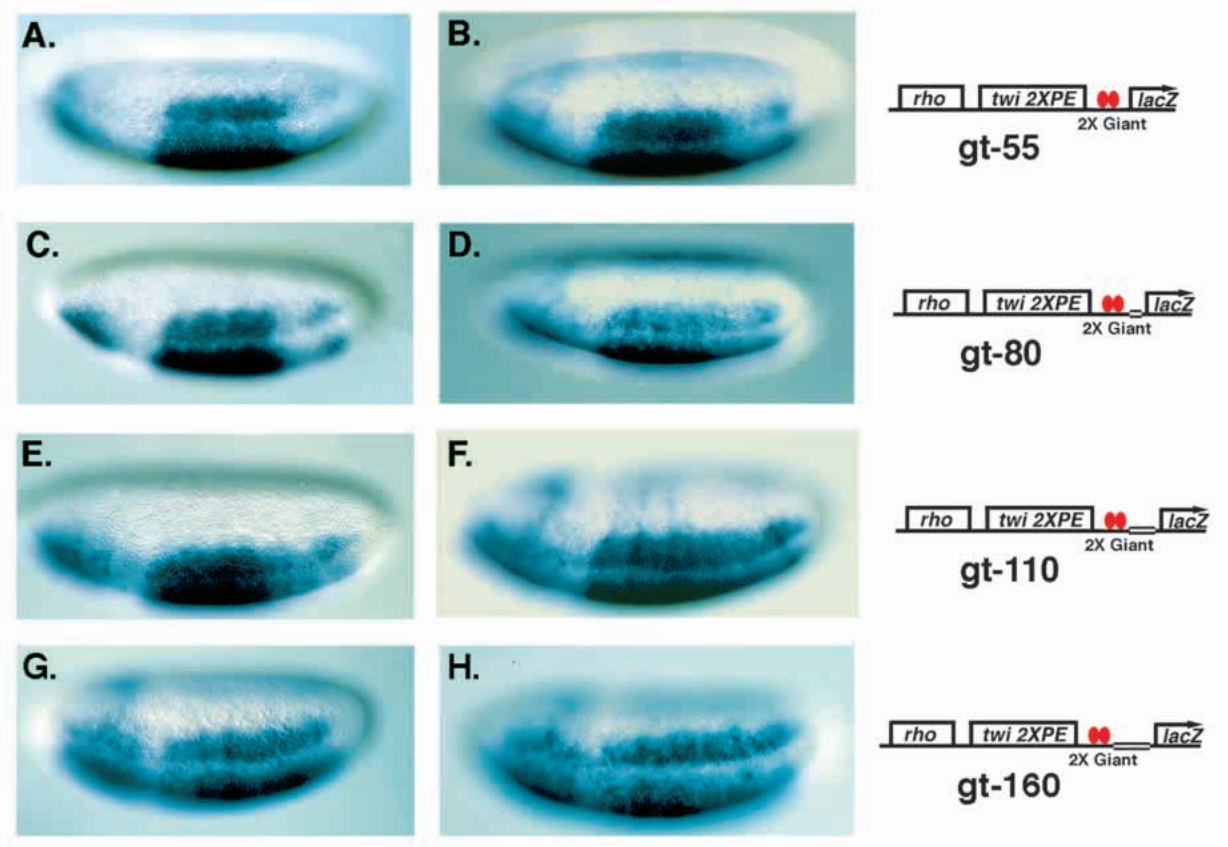

Fig. 3. Giant repression of *lac*Z reporter transgenes with successively greater spacing between the Giant binding sites and the initiation site. Embryos are oriented with anterior to the left and dorsal side up. The structure of each transgene is illustrated at the right of each pair of embryos. (A,C,E,G) Early cycle 14 embryos; (B,D,F,H) mid-late cycle 14 embryos. Anterior and posterior regions show less effective repression in constructs with more distally located Giant binding sites. Loss of Giant repression is especially evident in posterior regions of older embryos.

nucleus expression levels are thereby measured without the noise introduced by reduced expression levels between nuclei or punctate staining within them. The signals were digitized and plotted relative to percent egg length (Fig. 4B,D).

In early cycle 14 embryos, a broad band of anterior *giant* expression has a peak at approximately 70% egg length (Fig. 4A,B), where peak repression is also noted (Fig. 3C,E,G). Giant levels measured in the anterior peak are 1.49±0.27 (n=27) times greater than levels in the posterior stripe of the embryo (4A,B). In slightly older embryos (Fig. 4C,D), in which anterior expression is resolving into three separate stripes, the ratio of stripe 3 to the posterior stripe 4 is 1.41±0.21 (n=15); this ratio increases as stripe 4 expression weakens in older embryos (not shown). The less than two-fold differences between anterior and posterior domains appear to be responsible for the differential repression activity observed on the gt-110 and gt-160 transgenes in anterior and posterior regions (Fig. 3D,F,H).

Repression from a single Giant binding motif

Giant protein can interact with three distinct binding sites within the 480 bp *eve* stripe 2 enhancer element. Partial repression can be mediated by a single, albeit lengthy, gt-2 site (Small et al., 1991, 1992; Arnosti et al., 1996b). The genes tested in Figs 2 and 3 contain two repressor binding sites, similar to genes used to assay Knirps, Snail and Krüppel activity (Gray and Levine, 1996a; Arnosti et al., 1996b). While additivity or synergy is commonly observed for multimerized activator binding sites near a promoter (Tanaka, 1996; Chi et al., 1995; Vashee and Kodadek, 1995), it is not known whether repressor binding sites also exhibit such activity. To determine whether both sites in the gt-55 construct contribute to repression activity, we tested the activity of a single Giant site in the *lac*Z/*w* gene complex. A single Giant binding element inserted at −55 bp was sufficient to repress *lac*Z expression (Fig. 5C,D). However, the region of repression of this reporter gene is noticeably smaller than the repression seen with the gt-55 gene containing two Giant binding sites (Fig. 5A,B), indicating that the second binding site for Giant is contributing to function at the basal promoter location.

Giant repression domain is independent of the basic/zipper DNA binding domain

The Giant protein contains a basic/leucine zipper DNA binding/dimerization domain, suggesting that Giant forms homo- or heterodimers. Giant protein produced in bacteria is capable of binding in vitro to functional DNA motifs, suggesting that Giant can form homodimers (Capovilla et al., 1992). However, genetic evidence suggests that Giant may interact with other factors to repress specific target genes, and it is possible that in vivo, Giant heterodimerizes with an as-yet unidentified basic/leucine zipper partner. To test whether Giant protein can function as an autonomous repressor, and to define the region of the Giant protein needed to effect transcriptional repression in vivo, a fusion gene encoding a Gal4-Giant chimera was created in which the DNA binding domain was removed from Giant and replaced with a heterologous Gal4 DNA binding domain. This gene was expressed in the ventral region of transgenic embryos under the control of a *twi* promoter element. The activity of the Gal4-Giant protein was tested on a reporter gene bearing two stripe elements from the *eve* upstream regulatory region. Strong repression of the *eve* stripe 2 enhancer was noted in the ventral region of embryos where the Gal4-giant chimera is expressed (Fig. 6). The Giant basic/leucine zipper domain deleted from the Gal4-Giant chimera therefore appears to be dispensible for Giant function. The repression by Gal4-Giant was limited to the *eve* stripe 2 enhancer, and did not extend to the stripe 3 enhancer, which is located >300 bp from the closest Gal4-Giant binding sites, most likely because Gal4-Giant is repressing only over a short range in this gene complex, although it is possible that stripe 3 is resistant to the activity of Giant.

Repression of a Dorsal and Twist activated reporter gene by Gal4-Giant

The *eve* stripe 2 enhancer utilized in the reporter construct in Fig. 6 is activated by the Bicoid and Hunchback transcriptional activators, proteins whose activity is known to be blocked by the Giant protein (Stanojevic, 1991; Small et al., 1991, 1992). Therefore, it was not unexpected that the Gal4-Giant chimera was also able to block the *eve* stripe 2 element. We wished to test whether Giant repression is specific to certain activators such as Bicoid or Hunchback, or

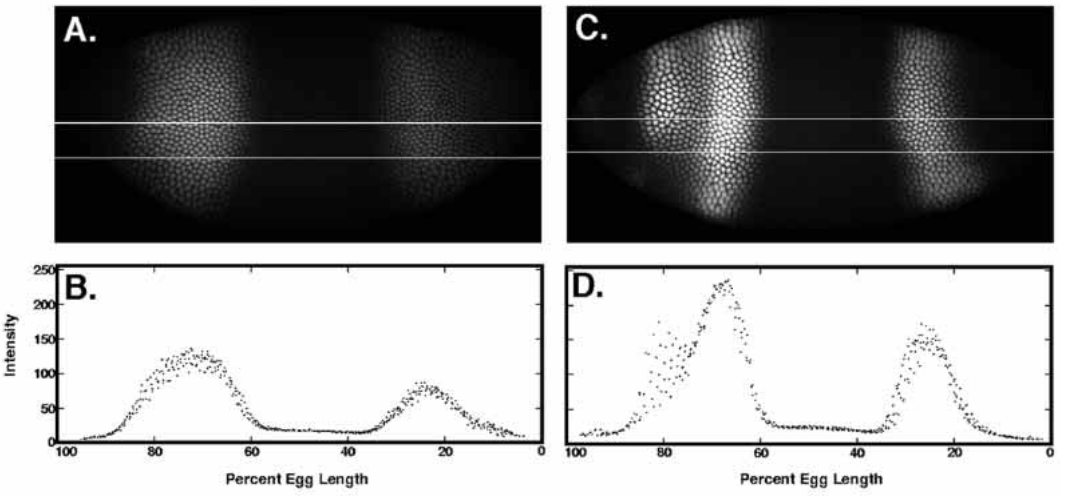

Fig. 4. Quantitation of Giant protein levels in embryos. (A,C) Giant protein in fixed embryos was visualized with FITC-conjugated goat anti-rat and rat anti-Giant antibodies, and embryos were scanned with a confocal microscope as described in Material and Methods. (B,D) Relative levels of Giant protein displayed as fluorescent signal intensity versus percent egg length. Ventrolateral optical sections of embryos (indicated by region between white lines) were digitized and normalized as described in Materials and Methods. *giant* expression is symmetrically distributed in the dorsal/ventral axis in stripe 3 (anterior) and stripe 4 (posterior) regions (data not shown). Of more than 100 imaged embryos, representative data of two are shown.

whether Giant is effective in blocking the activity of a range of different transcriptional activators, as has been noted for the Snail and Knirps short-range repressors (Gray et al., 1994; Arnosti et al., 1996a; Gray and Levine, 1996). Indeed, in the embryos shown in Figs 2 and 3, endogenous Giant protein was found to functionally repress genes activated by *rho* and *twi* enhancers, elements which bind to the Dorsal and Twist activators. However, due to the basal promoter proximity of the Giant binding sites in these constructs, it is possible that Giant is directly repressing the basal promoter complex. Thus, these constructs may not directly test repressor-activator specificity. Consistent with this idea, the activity of the enhancer elements on the divergent *w* gene is not inhibited (Fig. 2B), demonstrating that the activators are still able to function. We therefore constructed an alternative reporter gene in which the repressor binding sites are located within the *rho* enhancer, within 50 bp of key Dorsal activator binding sites and more than 200 bp from the initiation site of *lac*Z. A distal *eve* stripe 3 element serves as an internal control to test the range of repression activity (Fig. 7A). The Gal4-Giant protein was able to repress the *rho* enhancer (Fig. 7B), abolishing staining in the ventralmost regions of the embryos where the repressor is expressed. The band of expression driven by the control *eve* stripe 3 enhancer is still visible in ventral regions, again suggesting that the Gal4-Giant fusion protein most probably functions only over a short range, as does the endogenous Giant protein.

DISCUSSION

Setting promoter thresholds in the embryo

Gradients of morphogens have been proposed to play a central role in determining developmental fates in embryos. In this model, a diffusible molecule, or morphogen, sets up a concentration gradient within a field of cells (or nuclei), triggering alternative developmental fates depending on how a cell 'interprets' the given concentration of the morphogen (Wolpert, 1989). One mechanism by which such a gradient is interpreted is through differential gene activation, and consistent with this notion, molecular gradients of transcription factors have been found to act as morphogens (Ip et al., 1992). In the developing *Drosophila* embryo, these proteins transactivate different promoters possessing inherently different sensitivities, allowing a gradient of a single protein to define multiple territories. At a molecular level, differential responses to activator gradients have been linked to binding site affinity and number in promoters.

Concentration gradients of repressors are likewise thought to influence promoters with different sensitivities. Based on cell culture studies, different levels of the Krüppel protein have been suggested to mediate either activation at low concentrations or repression at high levels (Sauer and Jäckle, 1991). Recent work has directly tested the effects of repressor concentration gradients on endogenous target genes. Misexpression of Knirps has a differential effect on the *run*, *ftz* and *eve* promoters, and these effects have been proposed to represent the action of a morphogen gradient (Kosman and Small, 1997). Giant also appears to be able to act differentially in a concentration-dependent manner; ectopic expression of Giant protein in the *Drosophila* embryo leads to differential effects on *eve*, *hb* and *Kr* target genes (Wu et al., 1998).

It is probable that more distally located repressor sites (gt-110, gt-160, Fig. 3) may be effective only at higher concentrations of Giant because the binding sites may not be filled effectively. A partially filled site may still be effective at close range because when Giant is close to the promoter, chance interactions between Giant and its target will occur more frequently, obviating the need for saturation of the binding site by Giant. At a greater distance, these chance interactions would be less frequent, leading to weak repression. Partial filling of binding sites may simply be an indication that the levels of Giant protein are below the K_d for the binding site, so the site is empty for a fraction of the time. Alternatively, even identical sites may not bind Giant protein equally well; Giant may bind cooperatively with a 'target' protein to its cognate sites, so that moving the sites farther from the target may break repressor-target cooperative interactions. Raising the level of Giant protein ('moving up' the gradient) would suffice to give greater occupancy of Giant sites, and reestablish repression. Quantitation of relative Giant protein levels in embryos (Fig. 4) suggests that differences in repressor protein levels of less than two fold is sufficient to switch a gene from an active to an inactive state. Similar cooperative effects have been reported for activators, including the Bicoid activator (Tanaka, 1996; Hanes et al., 1994) In contrast, if Giant is located close to the promoter, chance interactions between Giant and its target will occur more frequently because of the short diffusion distance, obviating the need for saturation of the binding site by Giant.

The mechanism whereby endogenous promoters might differentially respond to different concentrations of repressors is not known, but the results obtained from this study suggest that exact placement of short-range repressors with respect to other promoter elements (Fig. 3) and number of binding sites (Fig. 5) might suffice to endow a promoter with high or low sensitivity. Additional factors, such as binding site affinity, may also contribute to differential promoter sensitivity toward repression. For example, a binding site located within the *eve* stripe 2 enhancer was found to bind Giant protein less well than a site in the *Kr* promoter (Capovilla et al., 1992), and consistent with this finding, *eve* has been found to be less sensitive to Giant repression than *Kr* (Wu et al., 1998).

Enhancer flexibility

Some promoters have been demonstrated to possess rigid constraints on factor placement, such as the 'enhanceosome' proposed for the interferon beta promoter (Thanos and Maniatis, 1995). While exact distances between short-range repressors and other functional elements of a gene can have important consequences, it is clear that not all genetic regulatory regions are 'locked' into tight geometrical constraints. Careful examination of the *eve* promoter has shown examples of the role of Giant and the flexibility of this element. First, experimental manipulation of regulatory regions demonstrates the flexibility within which activators and repressors are able to act. Removal of a critical Hunchback-3 binding site within the *eve* stripe 2 enhancer reduces expression of the stripe. However, a slightly larger deletion that removes this hb-3 and the adjacent gt-1 binding site restores stripe expression (Stanojevic et al., 1991).

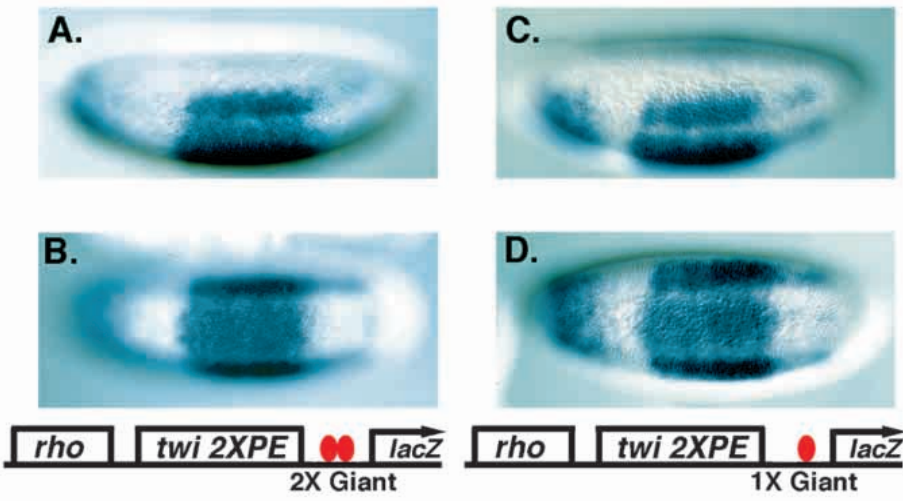

Fig. 5. Repression of *lac*Z transgenes by single or tandem Giant binding sites located at −55. The structure of each of the genes is illustrated below each pair of embryos. Early cycle 14 embryos are oriented with anterior to the left. (A,B) tandem Giant binding sites (gt-55 gene); (C,D) single Giant binding site (Uni gene). A single binding site is sufficient to mediate repression in regions with high levels of Giant protein. Tandem Giant binding sites mediate more effective repression, especially in extreme anterior regions where Giant protein is limiting. (A), (C), lateral views, (B), (D), ventral views.

Second, evolutionary drift serves as a larger laboratory for testing functional consequences of factor rearrangements, including Giant, on a promoter. Comparison of functionally equivalent *eve* stripe 2 enhancers from divergent Drosophilids has revealed that transcription factor organization on these elements is largely, but not absolutely conserved (Ludwig et al., 1998). Most notably, a binding site for the Bicoid protein, bcd-3, that is critical for activity of the *D. melanogaster* minimal stripe 2 enhancer (Small et al., 1992), is not present in the homologous elements from four other related species, and is thought to represent a relatively recent addition to the stripe 2 element (Ludwig et al., 1998). Perhaps to compensate for increased Bicoid activity on the stripe 2 element in *D. melanogaster* compared with enhancers from *D. yakuba* and *D. erecta*, deletions of intervening DNA have moved the adjacent gt-2 Giant binding site closer to the bcd-3 site. In *D. melanogaster*, the gt-2 site is 22-27 basepairs closer to the bcd-

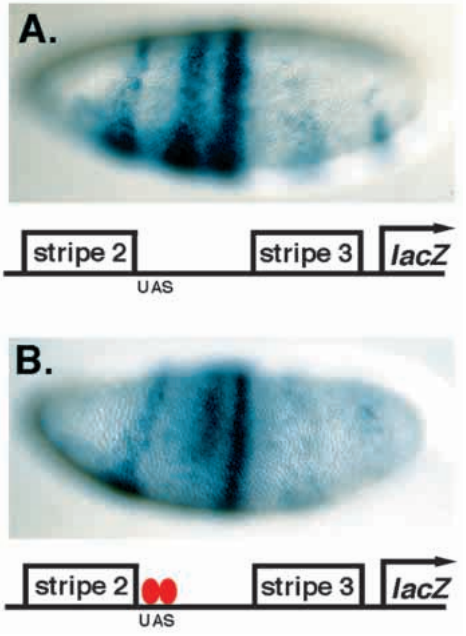

Fig. 6. A Gal4-Giant chimeric protein represses a *lac*Z reporter gene activated by stripe enhancers from the *even-skipped* gene. The structure of the reporter transgene is shown beneath each embryo. Embryos are oriented with anterior to the left and dorsal side up. (A) Reporter gene *lac*Z expression in a wild-type embryo, showing expression of *eve* stripe 2 and 3. A more anterior stripe in the head region is due to cryptic activation sequences in the vector. (B) Reporter gene *lac*Z expression in an embryo expressing the Gal4-Giant chimera in ventral regions. Strong repression of the *eve* stripe 2, but not stripe 3 enhancer activity, is evident in ventral regions.

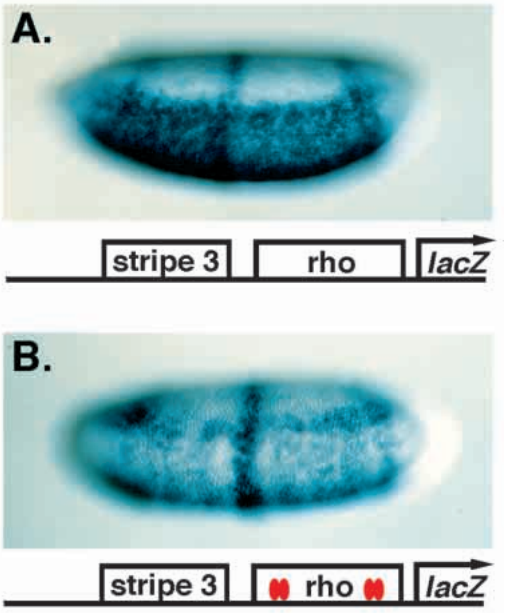

Fig. 7. Gal4-Giant protein repression of an enhancer activated by Dorsal and Twist proteins. The structure of the reporter transgene is shown beneath each embryo; Gal4-binding UAS sites have been introduced into the *rho* element, 50 bp from the Dorsal 1 and Dorsal 4 activator sites. Embryos are oriented with anterior to the left; ventrolateral views are shown. (A) Expression of a *lac*Z transgene activated by the *eve* stripe 3 and *rho* enhancers. The stripe 3 enhancer also directs expression of a stripe 7 domain in these embryos. Strong ventral staining directed by the *rho* element is seen. (B) Ventral *rho* activity is abolished when the transgene is tested in an embryo expressing Gal4-Giant protein in ventral regions. Stripe 3 expression is not repressed.

3 site than in other species, resulting in a spacing of 50 basepairs between activator and repressor (Ludwig et al, 1998). Small changes such as this in the location of Giant binding sites can have measureable impacts on gene expression (Fig. 3). These data suggest that there is flexibility within enhancer organization, and that 'drift' in position of binding sites is possible within the overall context of the enhancer, with fine tuning represented by addition of new activator sites, or closer apposition of repressors to activators.

Giant basic/leucine zipper dimers

Giant binding sites in the *Kr* and *eve* promoters have been identified by DNA footprinting experiments. The three Giant sites in the *eve* promoter cover large regions and do not closely resemble the compact sites from the *Kr* promoter (Small et al., 1991; Capovilla et al., 1992). It was previously suggested that Giant may interact as a homodimer with the *Kr* promoter, and as a heterodimer with the complex *eve* stripe 2 enhancer sites, using an as-yet unidentified partner basic/leucine zipper protein (Capovilla et al., 1992). Such a gene product may be encoded by a locus identified on the left arm of chromosome 2, mutations in which can cause a *gt*-like phenotype (Vavra and Carroll, 1989). Consistent with the possibility that Giant protein repression may involve other factors, recent experiments suggest that *eve* might be co-regulated by a Giant partner protein localized to the anterior portion of the embryo (Wu et al., 1998). However, the activity of the Gal4-Giant protein on a variety of activators (Figs 5 and 6) shows Giant can act as a homodimer on reporter genes (the Gal4 DNA binding domain binds to its cognate site as a dimer). If Giant binds to DNA as a heterodimer in the embryo, the partner protein may serve to modulate DNA binding rather than to effect transcriptional repression.

How do short-range repressors work?

The analysis of transgenes with successively greater repressor-promoter spacing demonstrates that short-range repression can act on more than an 'all-or-nothing' basis (Fig. 3), however, the activity of these repressors is sharply attenuated over relatively short distances. It has been suggested that short-range repression may directly target adjacent transcription factors through 'quenching'; in this case, Giant would alternately 'quench' transcription activators or the basal machinery depending on the location of the binding site (Arnosti et al., 1996a; Gray and Levine, 1996b). Such lack of specificity may be consistent with quenching acting through chromatin modification on an extremely local level, as has been reported for Ume6-mediated repression in yeast (Rundlett et al., 1998). Alternatively, repression by direct targeting of the basal transcription machinery is possible, as has been reported for the Krüppel and Even-Skipped proteins (Sauer et al., 1995; Li and Manley, 1998). Cofactors such as dCtBP are implicated in the activity of some short-range repressors, such as Knirps, Krüppel and Snail, but apparently not Giant (Nibu et al., 1998b). Further molecular characterization of Giant repression activity will be necessary to distinguish between these alternatives.

We thank E. Barnafo and C. Browning for technical assistance and K. Foley, L. Kroos, Z. Burton, R. Wagner, and S. Triezenberg for critical reading of the manuscript. This research was supported by an All University Research Initiation Grant from Michigan State University and grant GM56976 from the National Institutes of Health to D. N. A., and grant NIH RR-07801-07 to J. R. G. F. H. was supported by an MSU Biochemistry Department undergraduate research award.

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