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# Role of CtBP in Transcriptional Repression by the *Drosophila* giant Protein

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**The giant protein is a short-range transcriptional repressor that refines the expression pattern of gap and pair-rule genes in the *Drosophila* blastoderm embryo. Short-range repressors including knirps, Krüppel, and snail utilize the CtBP cofactor for repression, but it is not known whether a functional interaction with CtBP is a general property of all short-range repressors. We studied giant repression activity in a *CtBP* mutant and find that this cofactor is required for giant repression of some, but not all, genes. While targets of giant such as the *even-skipped* stripe 2 enhancer and a synthetic *lacZ* reporter show clear derepression in the *CtBP* mutant, another giant target, the *hunchback* gene, is expressed normally. A more complex situation is seen with regulation of the *Krüppel* gene, in which one enhancer is repressed by giant in a CtBP-dependent manner, while another is repressed in a CtBP-independent manner. These results demonstrate that giant can repress both via CtBP-dependent and CtBP-independent pathways, and that promoter context is critical for determining giant-CtBP functional interaction. To initiate mechanistic studies of the giant repression activity, we have identified a minimal repression domain within giant that encompasses residues 89–205, including an evolutionarily conserved region bearing a putative CtBP binding motif.** © 2001 Academic Press

***Key Words:*** **giant; CtBP; transcriptional repression; Krüppel; even-skipped.**

## INTRODUCTION

The precise expression of developmentally regulated genes often reflects the coordinate activity of both transcriptional activators and repressors acting on complex regulatory elements (Arnone and Davidson, 1997; Ghazi and VijayRaghavan, 2000). Transcriptional repressors involved in early gene expression in *Drosophila* embryogenesis include the products of gap genes, pair-rule genes, and mesoderm-specific genes. A major advance in understanding the action of some of these proteins came in the recognition that some of these factors, including Krüppel, knirps, snail, and giant, are “short-range” repressors, able to act over distances of 100–150 bp to interfere with the activity of enhancers and basal promoter elements (Gray *et al.,* 1994). Other “long-range” repressor proteins such as hairy are able to interfere with enhancers and promoters over distances of >1 kb, and can block the activity of multiple enhancers simultaneously (Cai *et al.,* 1996).

The mechanisms by which short-range and long-range *Drosophila* repressors inhibit transcription are poorly understood, although a variety of potential pathways have been described, including competitive binding with activators or elements of the basal machinery, “quenching” of nearby activators, and chromatin remodeling (Stanojevic *et al.,* 1991; Hoch *et al.,* 1992; Gray *et al.,* 1994; Chen and Courey, 2000). Differences in cofactor requirement suggest that these proteins are likely to utilize distinct pathways to effect transcriptional repression. Long-range repression complexes involving Dorsal protein and the hairy protein have been shown to bind to the groucho corepressor, which is thought to act in turn through histone deacetylases (Jimenez *et al.,* 1997; Chen and Courey, 2000). Several short-range repressors have been shown to interact with the CtBP corepressor, although it is not known if this is a general characteristic of all short-range repressors (Nibu *et al.,* 1998a,b). It has been suggested that giant, in particular, does not require CtBP for repression of the *eve* stripe 2 enhancer (Nibu *et*

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*al.,* 1998b). In addition, CtBP has been shown to interact with hairy, although in this case the cofactor appears to inhibit, rather than potentiate, repression (Poortinga *et al.,* 1998; Zhang and Levine, 1999).

Previous work has established that the giant protein functions in a number of embryonic transcriptional circuits to regulate the expression of gap and pair rule genes, including *even-skipped* (*eve*), *hunchback* (*hb*), and *Krüppel* (*Kr*) (Stanojevic *et al.,* 1991; Kraut and Levine, 1991; Capovilla *et al.,* 1992; Wu *et al.,* 1998). Recent work has also identified the functional interaction of giant with the *iab-2* enhancer of the *abd-A* homeotic selector gene (Shimell *et al.,* 2000). Several lines of evidence suggest that *eve, Kr,* and *abd-A* are direct targets of giant: their expression is derepressed in a *giant* (*gt*) mutant background, and these genes' regulatory elements contain binding sites for giant protein. In the cases of *eve* and *abd-A,* the sites have been mutated to verify that giant repression is lost in vivo (Small *et al.,* 1992; Arnosti *et al.,* 1996; Shimell *et al.,* 2000). In addition, ectopic expression of giant, either via a heatshock inducible promoter or an ectopic *eve* stripe 2 enhancer, represses *Kr* and *eve* expression in the blastoderm embryo (Kraut and Levine, 1991; Capovilla *et al.,* 1992; Wu *et al.,* 1998). Acting within these regulatory regions, the short-range repression activity of giant prevents regulatory "crosstalk," so that giant repression of one enhancer does not interfere with the activity of another (Small *et al.,* 1993; Hewitt *et al.,* 1999). The short range of giant activity can be used to produce genetic switches which are finely "tuned" to respond to small differences in giant protein concentration (Hewitt *et al.,* 1999). Such fine adjustments in repression activity appear to have been used during the evolutionary modification of the *eve* stripe 2 enhancer, where a giant binding site has been repositioned to compensate for increased activation activity due to acquisition of a novel bicoid activator binding site (Ludwig *et al.,* 1998, 2000; Hewitt *et al.,* 1999).

While much is known about the action of giant in native regulatory circuits, we do not understand the molecular details of repression by the giant protein. In particular, it is not known whether giant functionally interacts with the CtBP cofactor. Furthermore, it is not known whether the ultimate target of giant is the transcriptional machinery, activator proteins, or chromatin, although giant, like other short-range repressors, is capable of repressing from within enhancers or when situated proximal to basal promoter elements (Small *et al.,* 1992; Hewitt *et al.,* 1999; Shimell *et al.,* 2000). To determine whether CtBP is required for giant's short-range repression activity, we have studied the activity of endogenous and chimeric repressors in wild-type and *CtBP* mutant embryos, and we have identified an evolutionarily conserved minimal repression region that is sufficient to mediate transcriptional repression in transgenic embryos.

## MATERIALS AND METHODS

### *Plasmids*

The following oligonucleotides were used in construction of Gal4-giant chimeric constructs: 5′-GATCCGCCGATTACAAGGATGACGATGACAAGTAGTAATTAGTTAGT-3′ (a), 5′-CTAGACTAACTAATTACTACTTGTCATCGTCATCCTTGTAATCGGCG-3′ (b), 5′-GATCCCGCCGATTACAAGGATGACGATGACAAGTAGTAATTAGTTAG T-3′ (c), 5′-CTAGACTAACTAATTACTACTTGTCATCGTCATCCTTGTAATCGGCGG-3′ (d), 5′-GGCCGCCGATTACAAGGATGACGATGACAAGTAGTAATTAGTT AGT-3′ (e), 5′-CTAGACTAACTAATTACTACTTGTCATCGTCATCCTTGTAATCGGC-3′ (f), 5′-ATGAAGCTACTGTCTTCTATC-3′ (g), 5′-GGGGTCTAGACTAACTA AT TACTACTTGTCATCGTCATCCTTGTAATCGGCGTAAAAAGCGGGATACAGGGAGGC-3′ (h), 5′-GGGGTCTAGACTAACTAATTACTACTTGTCATCGTCATCC TTGTAATCGGCTTGGGCGGCATACAGAAGATTGCT-3′ (i), 5′-GGCCGATTACAAGGATGACGATGACAAGTAGTAATTAGTTAGT-3′ (j), 5′-CTAGACTAACTA ATTACTACTTGTCATCGTCATCCTTGTAATCGGCCTGCA-3′ (k), 5′-CGCAGCT GCA-3′ (l), 5′-GCTGCGGTAC-3′ (m), 5′-GGGTCGGTAACCGCAGCCCAACAGCA GCAACATCAG-3′ (n), 5′-GGGTCGGTACCGCAGCCGCTGCCGCCTCTGCTGCG-3′ (o), 5′-CGCCGCAGC CG-3′ (p), 5′-GATCCGGCTGCGGCGGTAC-3′ (q), 5′-GGGGTACCGCCGCAGCGCAGCAGCAGCATACCTCCTCTGCA-3′ (r), 5′-GGGGTCTAGACTAACTAATTACTACTTGTCATCGTCATCCTTGTAATCGGC GTTAGCGGTTGGTGTGACCTTGGG-3′ (s), 5′-GGGGTCTAGACTAACTAATT ACTACTTGTCATCGTCATCCTTGTAATCGGCGTAAAAAGCGGGATACAGGGA GGC-3′ (t), 5′-GGGTCGGTACCGCCGCAGCGAGCGTAGAGACGCCCAGGAAGA CT-3′ (u), 5′-GGGTCGGTACCGCCGCAGCGAATCTTCTGTATGCCGCCCAA ATG-3′ (v), 5′-CTCTGTAGGTAGTTTGTCC-3′ (SV40 3′UTR) (w).

To generate construct 2, construct 1 (Gal4-giant1-389, described in Hewitt *et al.,* 1999) was digested first with *Xba*I and partially with *Hin*dIII. The digested plasmid was then ligated with oligonucleotides (a) and (b) to generate Gal4-giant (1–322). Construct 3 was made the same way, using the linearized vector containing *giant* codons 1–265 and ligating oligos (c) and (d). Construct 4 was made by digesting construct 1 with *Not*I and *Xba*I and ligating with oligos (e) and (f). Constructs 7, 8, and 11 were made in a similar fashion digesting construct 1 with *Pst*I and *Xba*I and ligating oligos (j) and (k) for construct 7, digesting construct 1 with *Kpn*I and *Pst*I and ligating oligos (l) and (m) for construct 8, and digesting construct 1 with *Kpn*I and *Bam*HI and ligating oligos (p) and (q) for construct 11. Constructs 5, 6, 9, and 10 were made by PCR amplifying the appropriate portion of the gene, digesting with *Kpn*I and *Xba*I, and ligating the fragment into pTwiggy (Hewitt *et al.,* 1999). Oligos (g) and (h) were used to generate construct 5, (g) and (i) were used for construct 6, (n) and (w) were used for construct 9, (o) and (w) were used for construct 10, (r) and (w) for construct 12, (r) and (t) for construct 14, (u) and (w) for construct 15, and (v) and (w) for construct 16, and (r) and (s) for construct 13.

### *Isolation of giant Homolog from Drosophila hydei*

*D. hydei,* originally derived from a parent stock collected in 1993 at the South Coast Agricultural Research Station, California, were obtained from the Scott Pitnick Laboratory, Syracuse University.

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Genomic DNA was prepared from adult flies by using the Promega Wizard Genomic Prep Kit (cat. #A1120). Degenerate oligos DA-190 5′-AAAAGAATTCATGCAYCAYCAYCARTAYCARC-3′ and DA-191 5′-AAAAGAATTCNGCNGCGAARTTNG-CNGCHAT-3′ were used to amplify a region of the gene corresponding to *D. melanogaster giant* codons 23–274 using 35 1-min cycles of 95, 50, and 72°C. Visible bands of approximately the correct size were isolated, digested with *Eco*RI, subcloned into pBluescript SK(+), and individual clones were sequenced. Sequence information was used to generate nondegenerate oligonucleotides DA-441 5′-AAAAGAATTCCAGCAGCAGCAAGCATCGCAT-3′, DA-443 5′-AAAAGAATTCCACGAGCGGATCACGC-GGAAAG-3′, and DA-444 5′-AAAAGAATTCGGAAAGGCCT-TAAACGGGCGCG-3′ corresponding to *D. melanogaster* codons 32 to 267. These oligonucleotides were used in genomic amplifications, and resulting products were directly sequenced without subcloning to reconfirm sequences. (GenBank Accession No. AF356543).

### *P*-Element Transformation, Whole-Mount in Situ Hybridization of Embryos, and Crosses to LacZ Reporter Lines

*P*-element mediated germline transformation and *in situ* hybridization was carried out as described, except that during the hybridization procedure, embryos were not fixed again with formaldehyde and not treated with proteinase K (Small *et al.*, 1992). Probes for *eve* and *Kr* staining were prepared by subcloning a 2.6-kbp *Eco*RI/*Xba*I *eve* fragment or 1.9-kbp *Eco*RI/*Xba*I *Kr* fragment from bacterial expression vector pAR3040 (S. Small) into pBluescript II KS(+) and performing *in vitro* transcription reactions of template linearized with *Xba*I with T3 RNA polymerase in the presence of digoxigenin UTP as described (Small *et al.*, 1992). Quantitative assays of percent repression by Gal4-giant fusions were performed as previously described, using *eve* stripe 2 *lacZ* and *eve* stripe 2/*eve* stripe 3 *lacZ* transgenes as reporters (Keller *et al.*, 2000). Levels of repression never exceed 50% because of heterozygosity of the Gal4-giant lines.

### Analysis of Gene Expression in Embryos Lacking Maternal CtBP

*CtBP* germline clones were produced by using the autosomal FLP-DFS technique (Chou and Perrimon, 1996). Single reporter transgenes were assayed in the mutant embryo background by crossing males carrying the transgene to females producing *CtBP* embryos. To test the activity of Gal4-giant in a *CtBP* mutant background, the *eve* stripe 2 *lacZ* reporter gene was crossed into the *CtBP* mutant stock as described previously (Keller *et al.*, 2000).

## RESULTS

### *giant* Repression Is Compromised in a CtBP Mutant Background

To determine whether repression activity by the giant protein was affected in a *CtBP* mutant background, we studied the expression pattern of *eve* and synthetic *lacZ* reporter genes that are direct targets of giant, using *in situ* hybridization. giant protein helps to set the anterior border of *eve* stripe 2, and binding sites for the giant protein have been identified in the stripe 2 enhancer (Stanojevic *et al.,* 1991; Small *et al.,* 1992). Loss of *gt* activity or disruption of giant binding sites within the stripe 2 enhancer causes anterior expansion of expression of an *eve* stripe 2 *lacZ* reporter gene (Small *et al.,* 1992; Arnosti *et al.,* 1996). The expression pattern of endogenous *eve* shows complex changes in a *CtBP* mutant (Poortinga *et al.,* 1998; Nibu *et al.,* 1998b; Fig. 1B), including a possible anterior expansion of stripe 2, but the presence of multiple enhancers in the endogenous gene makes it difficult to determine specifically how the stripe 2 enhancer activity is affected. We therefore examined the expression pattern of an *eve* stripe 2 *lacZ* reporter gene and found, in contrast to an earlier report (Nibu *et al.,* 1998b) that in most embryos there is a significant anterior expansion of the *eve* stripe 2 expression pattern in the *CtBP* mutant background, as well as the posterior expansion previously noted. The expression pattern changes from the wild-type pattern of 56–62% egg length (S.D. 1.5%, $n = 25$) to 52–67% egg length (S.D. 3%, $n = 34$) in the mutant (Figs. 1C–1F). Posterior expansion results from loss of Krüppel activity (Nibu *et al.,* 1998b), while anterior expansion mimics that seen in a *gt* mutant (Stanojevic *et al.,* 1991; Small *et al.,* 1992; Wu *et al.,* 1998). This result is consistent with CtBP participating in establishment of the anterior border of expression of *eve* stripe 2, but does not prove that CtBP works through the giant protein. CtBP may interact with a putative heterodimeric partner of giant, or it may interact with other repressors that have been proposed to also play a role in setting the anterior border of *eve* stripe 2 (Vasisht, V., Theodosopoulou, K., Small, S., Abstract 452A; 40th Annual *Drosophila* Research Conference, Seattle, WA, 1999). Therefore, to study giant activity in the absence of other putative repressor sites, we employed a *lacZ* reporter gene that we showed previously is directly regulated by giant, containing two high-affinity giant binding sites 5′ of the *P* element basal promoter (Hewitt *et al.,* 1999). Expression is driven in lateral regions by an upstream *rhomboid* enhancer, and in ventral regions by the *twist* enhancer (Figs. 2A and 2C). The strong anterior and posterior repression of the *lacZ* transgene is almost completely abolished in the *CtBP* mutant background (Figs. 2B and 2D), leaving weakly attenuated expression in narrow anterior and posterior regions. This pattern is reminiscent of those obtained from *lacZ* reporter derivatives that have the giant binding sites moved to distal positions at −110 bp or −160 bp, at the limit of giant's range of activity (Hewitt *et al.,* 1999). The *gt* gene is still expressed in the *CtBP* mutant, indicating that the loss of repression is not simply due to loss of *gt* expression, although the area of posterior expression is expanded, as has been previously noted (Nibu *et al.,* 1998b; Figs. 2E and 2F).

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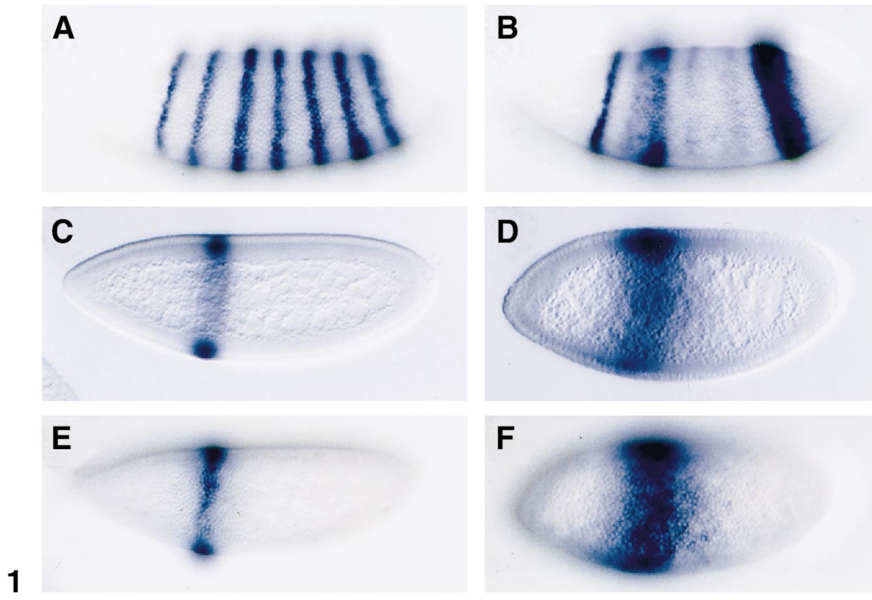


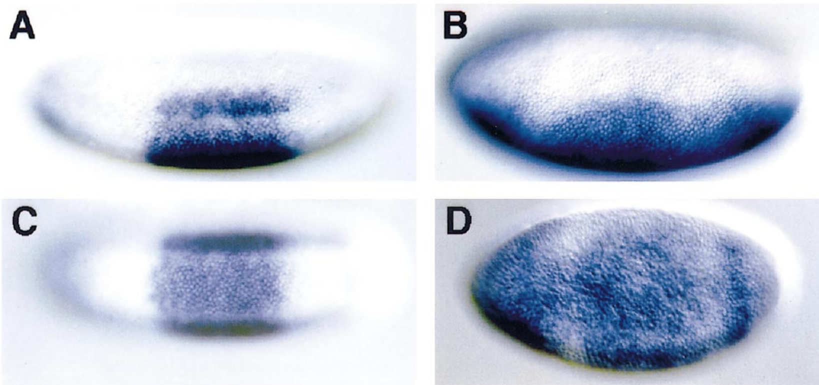

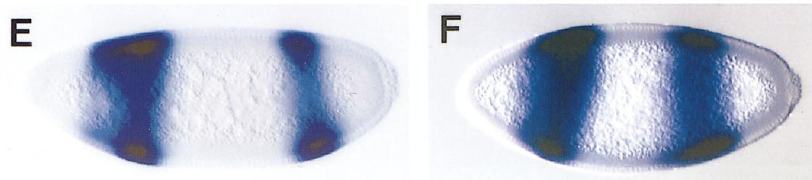


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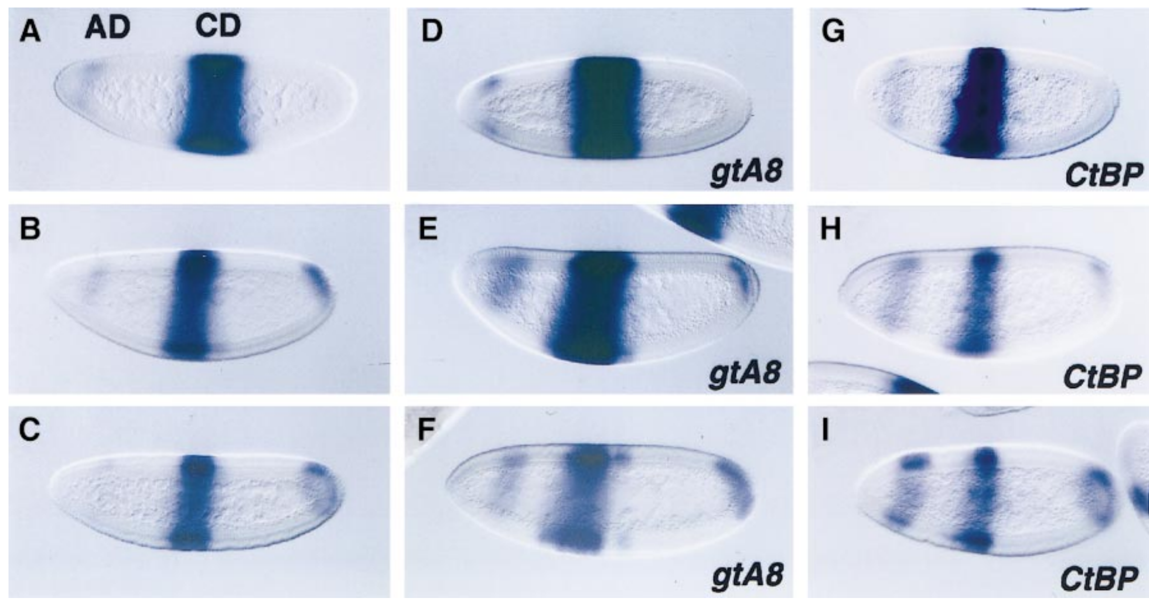


**FIG. 3.** Anterior domain (AD) of *Kr* expression regulated by CtBP and giant, while central domain (CD) is regulated by giant but not CtBP. *Kr* expression in staged embryos (youngest at top) was examined by *in situ* hybridization. Blastoderm expression is comprised of a small AD stripe, a prominent CD domain, and a posterior domain of expression. (A–C) Wild-type embryos, (D–F) $gt^{A8}$ mutant embryos, and (G–I) *CtBP* embryos. The expression of the AD is expanded in both *gt* and *CtBP* mutant embryos, while the CD is expanded only in *gt* mutant embryos. Abnormal ventral expression of AD in *CtBP* mutant embryos may represent loss of knirps activity. Embryos are oriented anterior to the left, dorsal side up.

### *Selective Requirement for CtBP in Regulation of Kr*

The loss of repression in the *CtBP* background exhibited by the two different *lacZ* reporter genes strongly suggests that giant repression can depend on CtBP; therefore, we carefully examined the patterns of *Kr* and *hb,* two endogenous targets of giant. The anterior border of *Kr* is highly sensitive to changes in levels of giant protein (Wu *et al.,* 1998), and two high-affinity binding sites for the giant protein have been identified within the upstream regulatory region that controls expression of *Kr* in the central domain (CD) of the embryo (Capovilla *et al.,* 1992). Previous studies indicated that the central domain of *Kr* expression is not grossly disrupted in a *CtBP* mutant (Nibu *et al.,* 1998b; Poortinga *et al.,* 1998), but as early blastoderm embryos were shown in these studies, it is unclear whether the later anterior shifts in *Kr* expression caused by loss of *gt* would have been noted. We compared the pattern of *Kr* expression in *wild-type, CtBP,* and *gt* embryos, and did not detect noticeable anterior expansion of the CD in the *CtBP* mu-

**FIG. 1.** Derepression of *eve* stripe 2 expression in a *CtBP* mutant background. The expression of the endogenous *eve* gene (A, B) or an *eve* stripe 2 *lacZ* reporter gene (C–F) was assayed in wild-type (A, C, E) or *CtBP* mutant embryos lacking maternal CtBP (B, D, F) by *in situ* hybridization. Anterior border expansion, consistent with loss of giant activity, and posterior border expansion, resulting from loss of Krüppel repression, can be seen in (D) and (F). The average position of the pattern generated by the *eve* stripe 2 *lacZ* transgene in wild-type embryos was 56–62% egg length ($n = 25$, standard deviation for each border 1.5%), while the average position of the pattern in *CtBP* mutant embryos was 52–67% egg length ($n = 34$, standard deviation for each border 3%). Embryos are shown anterior to the left, dorsal side up. (C, D) parasaggital views (to compare age of embryos); (E, F) surface views. *CtBP* embryos are typically shorter than wild-type embryos. *lacZ* mRNA and endogenous *eve* mRNA were visualized by *in situ* hybridization.

**FIG. 2.** Loss of giant repression activity in a *CtBP* mutant background. Wild-type (A, C) and *CtBP* mutant (B, D) embryos carrying a *lacZ* reporter gene with tandem giant binding sites at −55 bp were assayed by *in situ* hybridization. Ventral and ventrolateral expression is driven by *rhomboid* and *twist* enhancer elements, which in the absence of giant binding sites allow expression of the *lacZ* transgene from anterior to posterior (Hewitt *et al.,* 1999). The strong anterior and posterior repression mediated by giant (A, C) is greatly attenuated in the *CtBP* mutant embryos (B, D). Expression of *gt* in wild-type (E) and *CtBP* mutant (F) embryos indicates that *CtBP* mutant embryos express *gt* in an almost wild-type pattern. Embryos shown anterior to the left, dorsal side up (A, B, E, F), or ventral side toward viewer (C, D).

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**TABLE 1**
Activity of Chimeric Gal4-giant Repressor Proteins in Transgenic Embryos

| Construct no. | Residues from giant protein | % Repressed[a] | No. of embryos scored | No. of lines analyzed |
|---|---|---|---|---|
| 1 | 1–389 | 26 ± 9 | 708 | 4 |
| 2 | 1–322 | 39 ± 5 | 579 | 3 |
| 3 | 1–265 | 18 ± 1 | 177 | 2 |
| 4 | 1–205 | 9 ± 1 | 199 | 3 |
| 5 | 1–169 | 3.5 ± 2 | 1202 | 5 |
| 6 | 1–142 | 2 ± 2 | 1010 | 5 |
| 7 | 1–89 | 0 | 471 | 4 |
| 8 | 96–389 | 13 ± 1 | 641 | 3 |
| 9 | 144–389 | 1–9[b] | 1107 | 3 |
| 10 | 198–389 | 1 | 338 | 1 |
| 11 | 321–389 | 0 | 936 | 3 |
| 12 | 89–205 | 9 ± 3 | 1674 | 7 |
| 13 | 89–192 | 1.5 ± 1 | 1514 | 4 |
| 14 | 89–169 | 3 | 262 | 1 |
| 15 | 107–205 | 0.6 ± 0.6 | 1522 | 3 |
| 16 | 136–204 | 0 | 475 | 3 |

[a] Embryos from individual lines were scored for repression. Average % repression and standard deviations were calculated for constructs where multiple lines were tested. No embryos showing repression were found for constructs 7, 11, and 16.
[b] Activities of the three lines tested were 1, 3, and 9%.

tant. However, a striking difference was noted in the *Kr* anterior domain (AD), which is wider and persists later in development in *CtBP* embryos than in wild-type embryos (Fig. 3). *gt* embryos show a similar pattern of altered expression in the AD (Figs. 3G–3I). The AD stripe is expressed in ventral regions in the *CtBP* mutant, while in the wild-type embryo and in the *gt* embryos, this stripe does not extend into ventral regions. This loss of ventral repression in a *CtBP* mutant is probably due to a loss of *knirps* activity, for *knirps* is expressed in ventral anterior regions, and knirps protein has been shown to bind to the *Kr* promoter (Hoch *et al.,* 1992). giant is also required for repression of *hb* expression in the region of the embryo anterior to the parasegment 4 stripe, and low levels of ectopic giant protein are sufficient to repress *hb* in this area, suggesting that the element is highly sensitive to giant (Wu *et al.,* 1998). We did not find any differences in the *hb* expression pattern between *wild-type* and *CtBP* mutant embryos (data not shown). These results indicate that CtBP is not required for giant-mediated repression of some endogenous genes and enhancers, consistent with earlier suggestions that giant may function by more than one mechanism (Wu *et al.,* 1998).

### Gal4-giant Repression Domain Can Function in a CtBP Mutant Embryo

It is not known whether giant normally acts as a homodimer or a heterodimer, thus assays of endogenous giant activity might reflect the contribution of a basic zipper partner protein rather than the giant protein itself (Vavra *et al.,* 1989). The non-DNA binding region of the giant protein is clearly a bona fide repressor; when tethered to the Gal4 DNA binding domain this protein can mediate repression in the embryo, indicating that another basic-zipper partner protein is not required for activity (Hewitt *et al.,* 1999). We tested whether the Gal4-giant fusion protein used in these assays was capable of repressing in a *CtBP* background. Females producing embryos that lacked maternal CtBP protein and containing the *eve* stripe 2 *lacZ* reporter gene were crossed to males carrying the Gal4-giant repressor gene (Fig. 4). A high percentage of embryos showed repression in ventral regions, where the Gal4-giant fusion protein is expressed under control of the *twist* promoter. These results indicate that the giant protein itself contains an activity that is capable of repressing under conditions where the CtBP protein is severely reduced or absent.

### Identification of a Minimal Repression Domain in giant

Repression by giant can be mediated by the N-terminal 389 residues of the protein, independent of its native basic-zipper DNA-binding domain (Hewitt *et al.,* 1999). We tested which residues are sufficient to mediate repression in transgenic embryo assays by preparing and testing transgenic lines expressing chimeric Gal4-giant proteins in ventral regions of the embryo (Fig. 5). These repressors were assayed on *eve* stripe 2 *lacZ* and *eve* stripe 2 + 3 *lacZ* reporter genes and the fraction of embryos showing repression was quantitated (Table 1). Fusion proteins containing most of the giant protein showed robust repression, com-

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parable to levels achieved with knirps fusion proteins (Keller *et al.,* 2000). C-terminal truncations to residue 205 retained significant, although somewhat reduced levels of repression activity, as did N-terminal deletions to residue 96. A minimal Gal4-giant (89–205) chimeric protein was also active for repression in these assays (Fig. 5 and Table 1); however, a further N-terminal deletion of this minimal repressor, removing residues 89–106, produced an inactive construct. Within this short deletion is a sequence (residues 98–104, V-DLS-R) that is similar to a high-affinity CtBP binding motif (P-DLS-K/R) (Nibu *et al.,* 1998b; Poortinga *et al.,* 1998); therefore, it is possible that the N-terminal deletion removes a CtBP interacting site. The first residue of the canonical CtBP-binding motif, a proline, is required for high-affinity *in vitro* binding (Molloy *et al.,* 1998), consistent with the lack of measurable direct *in vitro* interaction between giant and GST-CtBP (data not shown, see Discussion). Constructs truncated after residue 169 (numbers 5 and 14) had detectable, but significantly reduced activity, while the activity of lines containing a construct truncated after residue 143 (number 6) was close to background levels (Table 1). Some lines expressing a Gal4-giant fusion including residues 144–389 showed activity, suggesting that the putative CtBP binding motif is apparently not strictly required for repression activity. The transgenes were not expressed at high enough levels to for us to quantitate expression by antibody staining (data not shown); therefore, it is possible that the inactive constructs are simply not well expressed or are unstable.

### *giant Homolog from Drosophila hydei Contains Regions of Conserved Residues*

To identify regions of the giant protein that have been evolutionarily conserved, and hence of possible functional importance, we sought the homologous gene from a related *Drosophila* species, *Drosophila hydei,* which is thought to have shared a last common ancestor with *D. melanogaster* approximately 60–80 million years ago (Beverley and Wilson, 1984). No close homolog to *giant* has yet been reported, aside from genes that encode similar basic-leucine zipper dimerization/DNA-binding domains; therefore, we designed degenerate oligonucleotides corresponding to several regions of the repression domain and carried out PCRs under conditions of low stringency. Southern blotting was used to analyze PCR products, and primer pairs that yielded products of similar size to the *D. melanogaster* clone were used in further rounds of PCR. A degenerate primer pair that amplified a region corresponding to codons 30 to 268 of *D. melanogaster* was found to give optimal results, and several clones of the corresponding PCR products were sequenced. A 774-bp fragment encompassing 258 codons was recovered, including the entire minimal repression domain (Fig. 6). This portion of the gene had 66% identity (70% similarity) at the amino acid level and 65% identity at the nucleic acid level. The minimal repression domain was overall somewhat more conserved, including three large blocks of identical residues, with overall 73% identity (78% similarity) at the amino acid level (Fig. 6). These levels of identity with *D. melanogaster* genes are similar to those of the *D. hydei fushi tarazu* (68%) and *Hairless* genes (69%) (Jost *et al.,* 1995; Marquart *et al.,* 1999). The putative CtBP binding motif spanning residues 98–104 is absolutely conserved, as are other blocks of residues throughout the predicted protein sequence.

## DISCUSSION

### *giant Represses through CtBP-Dependent and -Independent Mechanisms*

Previous work suggested that giant, in contrast to other short-range repressors, does not require CtBP activity to mediate repression, based on the expression pattern of an *eve* stripe 2 *lacZ* transgene (Nibu *et al.,* 1998). Our studies of the same enhancer element in a *CtBP* mutant background indicate that, although there is a range of phenotypes, with a small percentage of embryos showing a sharp anterior border, the border of the stripe is clearly expanded in most embryos, consistent with loss of giant function (Fig. 1). Additional evidence for CtBP-dependence comes from analysis of a reporter gene that is directly repressed through tandem giant binding sites at −55 bp. This gene shows strong, but not complete, derepression in a *CtBP* mutant background, indicating that CtBP is required for full activity of giant on this gene (Fig. 2). Repression by giant of the *eve* stripe 5 enhancer is also reportedly compromised in a *CtBP* mutant background (M. Levine and Y. Nibu, personal communication). Clear evidence for giant- and CtBP-dependent repression of an endogenous target gene comes from expression in the anterior domain (AD) of the *Krüppel* gene, where marked derepression is observed in both *giant* and *CtBP* mutant backgrounds (Fig. 3). Although the roles of individual giant binding sites in the *Kr* promoter are less well characterized than with *eve* stripe 2 and the synthetic *lacZ* reporters shown in Figs. 1 and 2, giant and CtBP most likely work through common DNA elements on the *Kr* promoter because giant protein is present in the region of AD expression and there are identified high-affinity binding sites for giant within the AD enhancer region (Capovilla *et al.,* 1992).

Our results clearly indicate that giant repression can be CtBP-dependent, but giant also appears to act through CtBP-independent pathways. A direct indication of such activity is that the Gal4-giant repressor is still active in a CtBP mutant background (Fig. 4). In addition, giant repression of the *Kr* CD enhancer elements is unaffected by loss of CtBP activity (Fig. 3). Another endogenous target of the giant repressor, *hb,* is not derepressed in a *CtBP* mutant, suggesting that CtBP dependence of giant activity can vary on a gene-to-gene as well as enhancer-to-enhancer basis. Ironically, while this study consolidates the view that a characteristic property of short-range repressors is functional interaction with CtBP, our results also indicate that

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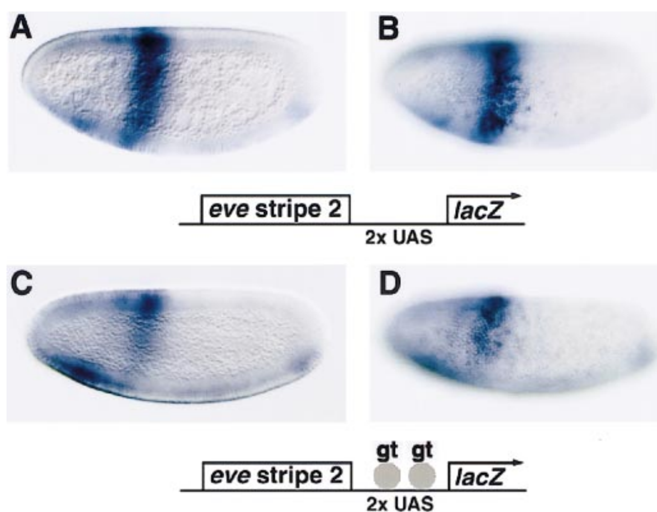


**FIG. 4.** Gal4-giant protein is an active repressor in a *CtBP* mutant background. Expression of an *eve* stripe 2 *lacZ* reporter gene is shown in wild-type (A, B) and a *CtBP* mutant background (C, D). Embryo shown in (C, D) contains Gal4-giant chimeric protein expressed in ventral regions under control of the *twist* promoter. Embryos are oriented anterior to the left, dorsal side up. Parasagittal views (A, C) shown to compare stage of embryos, and surface views (B, D) to illustrate ventral interruption of stripe. In *CtBP* embryos expressing Gal4-giant, 32 of 81 (40%) embryos scored showed loss of ventral activation, compared with 4 of 132 (3%) in *CtBP* embryos without Gal4-giant. (In wild-type embryos, less than 0.5% of embryos show abnormal stripes.)

providing a binding platform for CtBP is not the only activity of short-range repressors. A growing body of evidence demonstrates that many, or perhaps all, short-range repressor proteins also exhibit CtBP-independent activity: knirps can repress the *eve* stripe 3 enhancer in a *CtBP* mutant background (Keller *et al.,* 2000), Krüppel can repress the *hairy* stripe 7 enhancer in the absence of CtBP (La Rosée-Borggreve *et al.,* 1999), and this study indicates that giant likewise possesses CtBP-independent repression activity.

The CtBP-independent activity of short-range repressors is still poorly characterized, although this activity must by definition be limited to a short-range of action. The CtBP-independent activity may be mediated in part through direct competition with transcriptional activators. The tight linkage of activators and repressors on the *eve* stripe 2 enhancer has been suggested to be an example of this competitive situation, and experimentally, competition between the bicoid activator and the knirps repressor has been demonstrated on the *Kr* promoter (Hoch *et al.,* 1992). Competitive binding between repressors and activators cannot explain all CtBP-independent repression, however; the N-terminus of knirps contains a CtBP-independent repression activity that can inhibit activators binding to non-overlapping sites (Keller *et al.,* 2000).

### *Identification of an Evolutionarily Conserved Minimal Repression Domain*

The deletional analysis of Gal4-giant chimeras indicates that giant repression function can be localized to residues 89–205, an area of the protein that contains several tracts of highly conserved residues (construct 12; Fig. 5 and Fig. 6). Chimeras containing other portions of the giant protein (constructs 7, 10, 11) did not exhibit significant repression activity, suggesting that these regions cannot act autonomously to mediate repression, and might instead contribute to protein stability or expression. In particular, residues 266–322, present in constructs 1 and 2, appear to correlate with significantly higher repression activity of these proteins. The low levels of chimeric protein expression in the embryo precluded direct quantitation of each protein, thus our analysis is based primarily on those that did show significant activity.

We have not detected a significant physical interaction between giant and CtBP *in vitro* (A. Kumar, unpublished results), and the giant protein lacks a perfect match to the consensus CtBP binding motif P-DLS-K/R/H found in the knirps, Krüppel, and snail proteins. However, a partial match is present: VLDLSRR (residues 98–104). The motif is evolutionarily conserved and is found within the minimal repression domain we have defined (Figs. 5 and 6), consistent with a possible role in repression. Indeed, deletion of residues 89–107 inactivates the chimeric repressor (Fig. 5 and Table 1). This region is clearly not sufficient for high-level repression, however, (demonstrated by the weak activity of constructs 5, 6, 13, and 14), suggesting that other portions of the protein play important structural or functional roles. If CtBP directly contacts giant *in vivo,* the lack of strong interaction *in vitro* may indicate that giant must be posttranscriptionally modified to facilitate interaction with CtBP, perhaps via phosphorylation (Capovilla *et al.,* 1992). Posttranslational modifications are known to play a role in CtBP binding in some instances; E1A-CtBP interactions have been shown to be regulated by acetylation of a conserved lysine residue in the CtBP binding motif (Zhang *et al.,* 2000). Alternatively, giant may bind CtBP indirectly through a cofactor, much as BRCA1 has been suggested to bind CtBP through CtIP (Li *et al.,* 1999), or CtBP might be recruited via a heterodimeric basic-zipper partner of giant. To determine whether CtBP-dependent and CtBP-independent repression activities are mediated by the same or distinct portions of the giant protein, future studies will need to focus on identifying mutant proteins that are deficient in each of these activities.

### *What Characteristics of a Regulatory Region Dictate CtBP-Dependent or CtBP-Independent Repression?*

In considering which features of a gene determine CtBP-dependence or -independence, the structure of the basal promoter cannot be the deciding factor, for the same *Kr* promoter is regulated by distinct elements, some that

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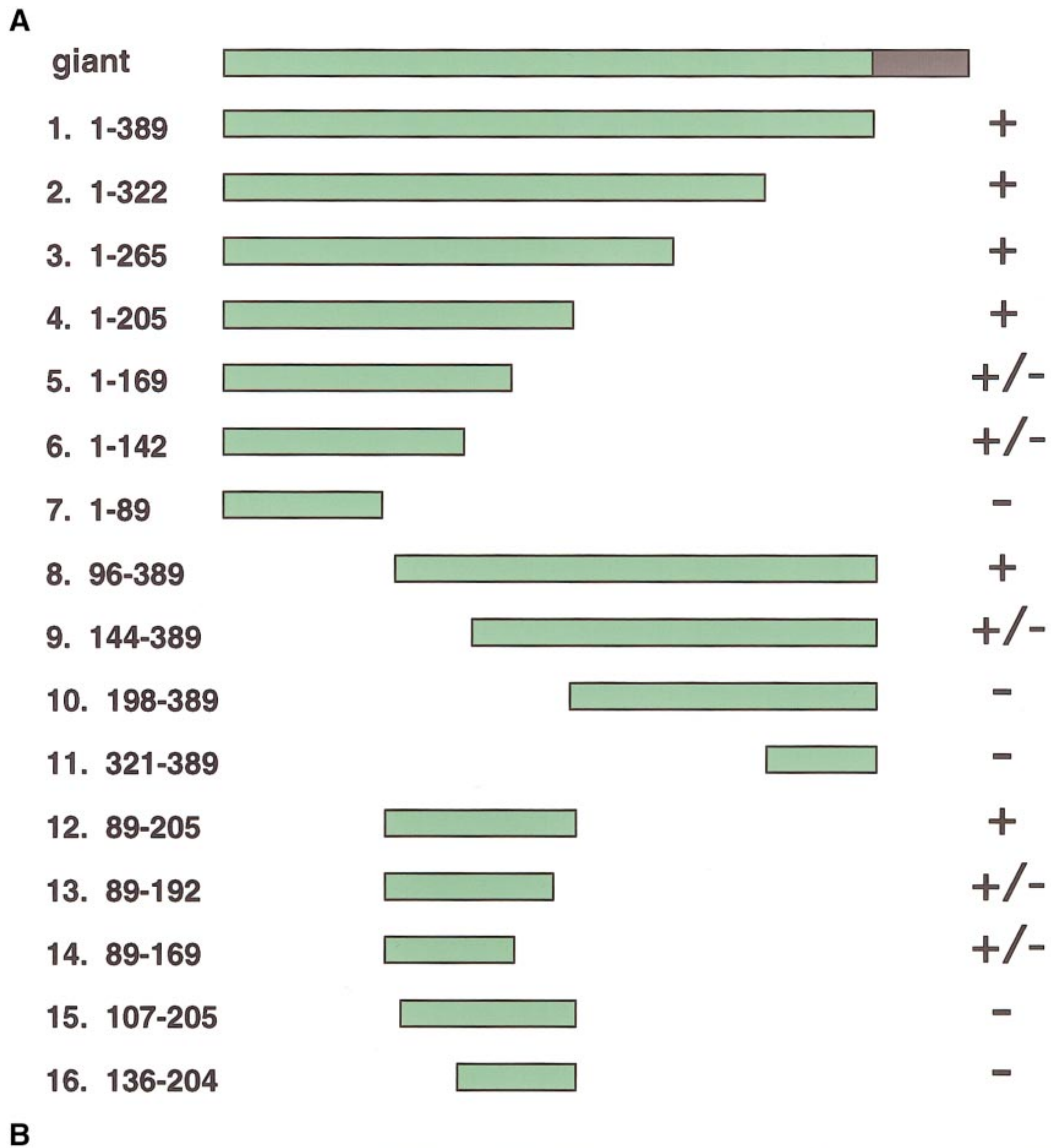


**FIG. 5.** Structure and activity of Gal4-giant chimeras assayed in transgenic embryos. (A) Genes encoding Gal4 fusions including giant residues indicated in constructs 1–16 were introduced into *Drosophila* by *P*-element mediated germline transformation. Repression activity of the chimeric proteins was assayed by crossing Gal4-giant lines to reporter lines containing *eve* stripe 2 *lacZ* and *eve* stripe 2 + stripe 3 *lacZ* reporter genes. Activities are shown as "+" (≥9% repressed), "+/−" (1.5–4% repressed), and "−" (<1% repressed). (B) Representative embryos showing pattern of the unrepressed reporter gene, and embryos from Gal4-giant (1–389), Gal4-giant (1–322), and Gal4-giant (89–205) crosses, showing ventral interruption of stripe 2 pattern. In comparing embryos showing ventral repression, no significant differences in extent of repression of stripe patterns were noted.

exhibit CtBP-dependence and some that show CtBP-independence. Similarly, the *eve* gene is repressed by knirps via CtBP-dependent and CtBP-independent regulatory elements (Keller *et al.,* 2000). While the *eve* enhancers in question are kilobases apart, the *Kr* regulatory elements driving AD and CD expression are closely intertwined, and

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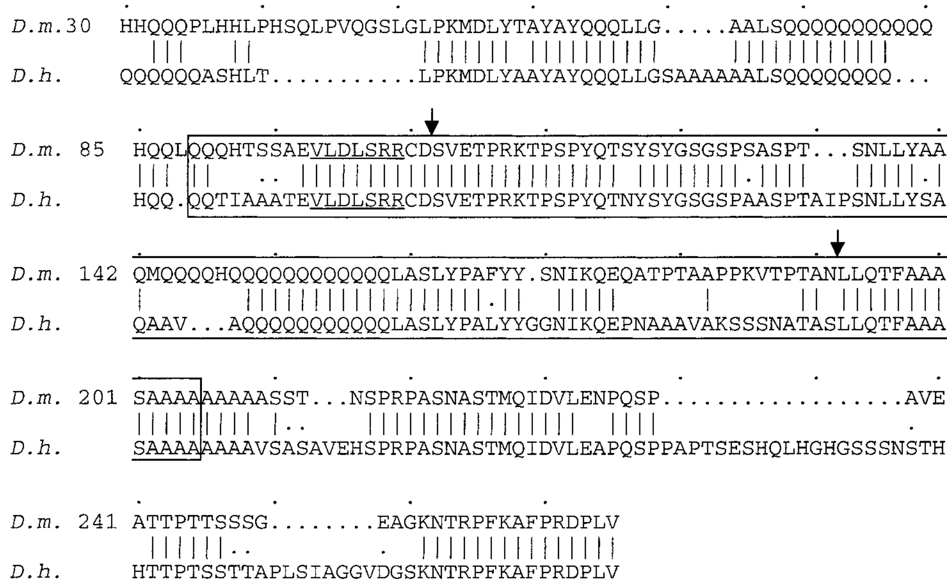


**FIG. 6.** Peptide sequence of *giant* homolog isolated from *D. hydei* aligned with *D. melanogaster* sequence. Minimal repression domain spanning residues 89–205 in *D. melanogaster* shown in box, conserved putative CtBP binding motif underlined. Vertical arrows indicate N- and C-terminal deletions in minimal repression region that abolish repression activity. A *D. hydei* sequence corresponding to *D. melanogaster* residues 30–268 was isolated from genomic DNA by degenerate PCR. Sequences were obtained from multiple isolates of independently generated PCR products.

appear to share at least some of the same activator binding sites, suggesting that subtle differences in enhancer architecture or differences in levels of regulatory proteins interacting with those elements may dictate CtBP dependence (Hoch *et al.,* 1990, 1991; Jacob *et al.,* 1991). The giant binding site in the *Kr* CD2 enhancer site was shown to be of higher affinity than the gt1 site in the *eve* stripe 2 enhancer (Capovilla *et al.,* 1992). Thus, there may be a correlation between giant binding site affinity and the requirement for CtBP, with elements containing giant sites of lower affinity showing CtBP-dependence. We derived a consensus for the giant protein by aligning binding sites for giant from *eve, Kr,* and the recently identified *abdA iab-2* enhancer site (Fig. 7; Shimell *et al.,* 2000). The consensus features an extended half-site inverted repeat TNTTAC, consistent with the dimeric nature of basic zipper proteins, and a central ACGT core common to recognition motifs for many basic zipper proteins (Capovilla *et al.,* 1992; Dlakic *et al.,* 2001). The higher affinity sequences from the CtBP-independent *Kr* CD element are closer to the consensus than those of the CtBP-dependent *eve* stripe 2 enhancer. Weaker sites may only be partially occupied, resulting in an overall lower level of giant mediated repression. A loss of CtBP might further depress repression activity below a critical threshold, leading to the derepression we observe in Figs. 1 and 3. Repression of the *lacZ* reporter containing the giant CD1 site from *Kr* was CtBP-dependent, a result that contrasts with the CtBP independence of the CD itself (Fig. 2), but this particular site may not be optimal, as it contains

| | |
|---|---|
| *eve* gt 1 | AAAC**ACA**T**AA**T**A** |
| *eve* gt 1 | **T**AGA**A**A**GT**C**A**T**A** |
| *eve* gt 2 | AG**TT**TG**GTAA**C**A** |
| *eve* gt 3 | **T**A**TTA**GTC**AA**TT |
| *Kr* CD1 | **T**C**TT**G**CGT**C**A**T**A** |
| *Kr* CD2 | **T**T**TTACGTAA**C**A** |
| a*bdA* iab-2 | **T**A**TTACGTAA**A**A** |
| | gtcg |
| consensus | **T**n**TTACGTAA**n**A** |

**FIG. 7.** Alignment of giant binding sites and consensus. Footprinted sites from the *eve* stripe 2 enhancer (Stanojevic *et al.,* 1991), the *Kr* CD1 and CD2 enhancers (Capovilla *et al.,* 1992), and the *abdA iab-2* enhancer (Shimell *et al.,* 2000) were aligned with a sequence derived from the center of the small footprinted regions (13–16 nt) found in the *Kr* and *abd-A* genes. Residues that match the consensus are indicated in bold. The central ACGT cluster is identical to that found in motifs recognized by other basic zipper DNA binding proteins (Dlakic *et al.,* 2001). The sequence from the *eve* gt3 site is located in the center of a 26-nt footprinted region, and the two sequences from the gt1 site are adjacent to one another within the 22-nt footprinted region. The sequence from the gt2 site is in the 3′ region of the large 44-nt footprinted site (Stanojevic *et al.,* 1991). The lower case letters below the *iab-2* sequence indicate a mutation that abolishes giant regulation of the *iab-2* enhancer (Shimell *et al.,* 2000).

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two mismatches (Fig. 7). Full giant activity may also be mediated on the native CD element through the additional high-affinity CD2 site.

Other factors besides binding site affinity can affect giant's activity, and possibly its CtBP-dependence. We have previously demonstrated that small alterations in the location of giant binding sites is sufficient to strongly affect the ability of giant to repress in transgenic embryo assays (Hewitt *et al.,* 1999). Thus, we need to consider location and affinity of giant sites in studying CtBP-dependent repression. We do not believe that differences in the nature of the activators explain CtBP-dependence or -independence, because both AD and CD enhancers of *Kr* are activated by bicoid protein (Jacob *et al.,* 1991; Hoch *et al.,* 1991), as is the *eve* stripe 2 enhancer. Detailed studies illuminating how the general properties of short-range transcriptional repressors are integrated into the design of promoter elements will promote our understanding of the control of complex developmentally regulated genes.

*Note added in proof.* Material cited as Nibu and Levine, personal communication, has now appeared as Nibu, Y. and Levine, M. S. (2001) CtBP-dependent activities of the short-range giant repressor in the *Drosophila* embryo. *Proc. Natl. Acad. Sci. USA* **98,** 6204–6208.

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