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The Drosophila Boundary Element-Associated Factors BEAF-32A and BEAF-32B Affect Chromatin Structure

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ABSTRACT

Binding sites for the Drosophila *b*oundary *e*lement-*a*ssociated *f*actors BEAF-32A and -32B are required for the insulator activity of the scs′ insulator. BEAF binds to hundreds of sites on polytene chromosomes, indicating that BEAF-utilizing insulators are an important class in Drosophila. To gain insight into the role of BEAF in flies, we designed a transgene encoding a dominant-negative form of BEAF under GAL4 UAS control. This BID protein encompasses the *B*EAF self-*i*nteraction *d*omain. Evidence is provided that BID interacts with BEAF and interferes with scs′ insulator activity and that BEAF is the major target of BID *in vivo*. *BID* expression during embryogenesis is lethal, implying that BEAF is required during early development. Expression of *BID* in eye imaginal discs leads to a rough-eye phenotype, and this phenotype is rescued by a third copy of the *BEAF* gene. Expression of *BID* in salivary glands leads to a global disruption of polytene chromatin structure, and this disruption is largely rescued by an extra copy of *BEAF*. *BID* expression also enhances position-effect variegation (PEV) of the w^{m4h} allele and a *yellow* transgene inserted into the pericentric heterochromatin of chromosome *2R*, while a third copy of the *BEAF* gene suppresses PEV of both genes. These results support the hypothesis that BEAF-dependent insulators function by affecting chromatin structure or dynamics.

CHROMOSOMAL DNA in the nucleus of a eukaryotic cell is tens of thousands of times longer than the nuclear diameter. A high level of structural organization inside nuclei is required to allow chromosomes to function properly in processes such as transcription and mitosis. The first level of organization is the nucleosome, which is a 10-nm bead composed of 146 bp of DNA wrapped 1.6 times around an octamer of histone proteins. High-resolution crystal structures of nucleosomes have been solved (Luger *et al.* 1997; Muthurajan *et al.* 2004). Higher levels of chromatin structure are not well understood, progressing from 30-nm fibers to looped domains. Communication between enhancers and promoters involves long-range interactions and is also poorly understood (Bulger and Groudine 1999; Dorsett 1999). The physical organization of chromatin likely plays a functional role in this communication.

Chromatin domain insulators (also known as boundary elements) help establish patterns of gene expression by limiting possible interactions between regulatory elements and promoters (Labrador and Corces 2002; West *et al.* 2002; Kuhn and Geyer 2003). In enhancer-blocking assays, insulators interfere with enhancer–promoter communication only when positioned between the enhancer and the promoter. When located upstream or downstream, they have no effect. Transgenes bracketed by insulators are protected from chromosomal position effects. After integration into most chromosomal loci, similar levels of transgene expression are observed because expression is driven solely by regulatory elements in the transgenic construct (position-independent expression assays). It is likely that endogenous insulators divide chromosomes into functional domains such that regulatory elements and promoters can interact only if they are in the same domain. If this functional organization plays a role in the physical organization of chromosomes in nuclei, then insulators are candidate elements for linking chromatin organization and dynamics to gene regulation.

The *b*oundary *e*lement-*a*ssociated *f*actors BEAF-32A and BEAF-32B bind to the scs′ insulator as well as to hundreds of other sites on polytene chromosomes (Zhao *et al.* 1995; Hart *et al.* 1997). The BEAF-binding sites in scs′ are essential for its insulator activity, and other genomic BEAF-binding sites that have been tested also function as insulators (Cuvier *et al.* 1998). Thus BEAF-utilizing insulators are common in Drosophila. BEAF-32A and -32B are 32-kDa proteins derived from the same gene (Hart *et al.* 1997). They differ at their amino termini, which have different BED finger DNA-binding domains (Aravind 2000). The carboxy-terminal two-thirds of these proteins is identical. A BESS domain is found near the carboxy termini (Bhaskar and Courey 2002; Delattre *et al.* 2002) and is preceded by a potential leucine zipper domain. BEAF monomers

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interact with each other, presumably via interactions between BESS domains or leucine zippers or both. Evidence suggests that BEAF binds DNA as trimers, although larger complexes could also be involved (Hart *et al.* 1997). No other proteins copurify with BEAF, indicating that BEAF forms only stable complexes with itself.

To gain insight into the role of the BEAF proteins in Drosophila, we constructed a transgene encoding the BEAF *s*elf-*i*nteraction *d*omain (BID) but lacking a DNA-binding domain. This design is based on the Drosophila Emc and vertebrate Id proteins (Norton *et al.* 1998; Campuzano 2001). These proteins lack DNA-binding domains and so inhibit DNA binding by their partner transcription factors by forming dimers that lack one DNA-binding domain. The BID protein should similarly inhibit DNA binding by BEAF. The BID transgene is under GAL4 UAS control, allowing expression to be driven in different patterns by different GAL4 driver fly lines (Brand *et al.* 1994). We demonstrate that the BID protein inhibits BEAF activity and provide evidence that BEAF function influences chromatin structure or dynamics.

MATERIALS AND METHODS

DNA constructions: Four *P*-element plasmids were used to establish transgenic fly lines in this study: pUAS-BID, pC4-gBF, pC4-YG4, and pM2. To construct pUAS-BID, a 700-bp *Bam*HI fragment was isolated from a plasmid containing the *32A* cDNA (Zhao *et al.* 1995). This encodes the carboxy-terminal 141 amino acids of BEAF. This fragment was ligated into a modified pUAST-HN plasmid. pUAST-HN, kindly provided by J. A. Simon (University of Minnesota), has sequences encoding an HA epitope tag and an SV40 nuclear localization signal (NLS) located upstream of the *Eco*RI site of pUAST (Brand and Perrimon 1993). This was further modified by placing a *Bgl*II linker into the *Eco*RI site so that the *Bam*HI fragment would fuse *BEAF* sequences in the correct reading frame.

BEAF sequences were PCR amplified from genomic DNA and cut with *Bgl*II, resulting in a 5-kb fragment. The 5′-end is located in the first intron of the divergent *CG10155* gene, 2.5 kb upstream of the putative *32A* transcription initiation site. The 3′-end is located in the final exon of the convergent *knot* gene, 250 bp downstream of the putative *BEAF* polyadenylation site. This *Bgl*II fragment was ligated into the *Bgl*II site of pUC19, which had been modified by the insertion of a *Bgl*II linker into the *Sac*I site, resulting in pUC-gBF. For germline transformation of flies, the 5-kb *Bgl*II *gBF* fragment from pUC-gBF was ligated into the *Bam*HI site of pCaSpeR4 (Pirrotta 1988), upstream of the mini-*white* gene. In the resulting pC4-gBF plasmid, the *BEAF* gene is transcribed in the same direction as mini-*white*.

The transformation vector pC4-YG4 has *GAL4* coding sequences under the control of the *yellow* wing and body enhancers. This plasmid has a 2.9-kb PCR fragment from pCaSpeR4-yellow (kindly provided by V. Pirrotta, Rutgers University) that encompasses the *yellow* wing and body enhancers and promoter to +65, a 2.9-kb PCR fragment from pCL1 (CLONTECH, Palo Alto, CA) that encompasses the *GAL4* coding sequences, and a 490-bp *Hin*cII fragment from pCaSpeR4-yellow that encompasses the *yellow* polyadenylation region. The assembled *yellow-GAL4* gene construct was cloned into the *Not*I site of pCaSpeR4.

The mini-*white* position-independent expression vector pM2 is described in Cuvier *et al.* (1998).

Fly stocks and germline transformation: Flies were maintained at 25° or 18° on standard cornmeal, yeast, and sugar medium with Tegosept. The following *yellow* enhancer-blocking lines, described in Kuhn *et al.* (2004), were used: 2scs′ inserted at 19D; scs inserted at 60A; gypsy inserted at 25C. The *ey-GAL4/TM6b* line was kindly provided by T. E. Haerry (Florida Atlantic University). Lines from the Bloomington Drosophila Stock Center were *da-GAL4* (BSC-8641), *ey-GAL4/CyO* (5535), *salivary-gland-GAL4* (1824 and 1967), *CNS-GAL4/TM3* (3742), *UAS-GFP.S65T* (1521 and 1522), and w^{m4h} (isolated from 6234). The *y* variegating line KV20, located at 39-40H of chromosome arm *2R*, was kindly provided by G. H. Karpen (University of California at Berkeley). Transgenic flies were generated by co-injecting plasmids (0.4 μg/μl) with the pπ25.7wc helper plasmid (0.1 μg/μl) into preblastoderm y^1 w^{67c23} embryos (Spradling 1986). Names of fly lines generated in this study refer to the relevant transgene, followed by a designation of the chromosome onto which the transgene is inserted, and a letter for each independent line.

Immunoprecipitations: To prepare nuclear extracts, embryos were homogenized in nuclear isolation buffer [3.75 mm Tris (pH 7.4), 0.05 mm spermine, 0.125 mm spermidine, 0.5 mm EDTA (pH 7.4), 20 mm KCl, 0.5% thiodiglycol, 0.05% Empigen BB, 0.1 mm PMSF, 2 mg of aprotinin/ml] using a Dounce homogenizer and A and B pestles. Three hundred-microliter buffer was used per 100 mg embryos. Nuclei were filtered through Miracloth (Calbiochem, La Jolla, CA) and pelleted by centrifugation at 2000 × *g* for 10 min in a refrigerated microfuge. The supernatant was saved as cytoplasmic extract. The nuclei were washed twice in nuclear isolation buffer, then resuspended in 80 μl of nuclear extraction buffer [10 mm HEPES (pH 7.6), 360 mm KCl, 3 mm $MgCl_2$, 0.1 mm EDTA, 1 mm dithiothreitol, 10% glycerol, 4 mg of aprotinin/ml, 0.2 mm PMSF, 5 mg each of leupeptin, antipain, pepstatin A, and chymostatin/ml] per 100 mg of embryos, and incubated for 30 min at 4° with gentle agitation. Extracts were centrifuged at 16,000 × *g* for 30 min in a refrigerated microfuge. The supernatant was aliquoted, flash frozen, and stored at −80°.

Affinity-purified antibodies against BEAF were previously described (Zhao *et al.* 1995; Hart *et al.* 1997). Forty microliters of extract was incubated with 2 μl anti-32A or 2 μl anti-32B antibodies for 2 hr at 4°. Immunoprecipitates were recovered with protein A–agarose beads (Roche) and washed five times with 350 mm NaCl, 10 mm HEPES (pH 7.6), 0.1% Tween-20, and proteins were eluted with SDS sample buffer. After 10% SDS–PAGE and transfer to nitrocellulose, proteins were detected using anti-BEAF antibody (1:2000) followed by horseradish-peroxidase-conjugated goat anti-rabbit antibody (1:10,000) (Bio-Rad, Hercules, CA). Signals were developed using an ECL detection kit (Amersham, Buckinghamshire, UK).

Scanning electron microscopy: Flies were fixed in FAA (16% formaldehyde, 5% acetic acid, 45% ethanol) for at least 24 hr and then put through a dehydration series with ethanol (10 min each of 75, 87, 94, 97, and 4× 100%) followed by 2× 30 min in 100% hexamethyldisilazane. Flies were dried overnight in a hood and stored in a dessicator. Flies were sputter coated and observed in a Cambridge Stereoscan 260 SEM.

Immunostaining polytene chromosomes: Polytene chromosomes were prepared from salivary glands of healthy, wandering third instar larvae and immunostained as previously described (Zhao *et al.* 1995). Affinity-purified rabbit anti-BEAF antibody was used at a 1:50 dilution, and Texas Red-conjugated goat anti-rabbit antibody (Jackson, West Grove, PA) was used at a 1:500 dilution. Chromosomes were stained with 100 ng/ml DAPI. Slides were viewed with a Zeiss

Axioskop microscope equipped with a Spot RT Slider CCD camera.

Position-effect variegation assays: w^{m4h} females were crossed to *ey-GAL4/CyO*; *BID.3A/BID.3A* males and the eyes of w^{m4h}; *ey-GAL4/+*; *BID.3A/+* males were compared to those of their w^{m4h}; *CyO/+*; *BID.3A/+* male siblings. Because the ey-GAL4 and BID.3A transposons are marked with mini-*white*, crosses were conducted to determine the eye pigmentation of w^{m4h}; *ey-GAL4/+*; +/+ males, w^{-}; *ey-GAL4/+*; *BID.3A/+* males, w^{-}; +/+; *BID.3A/+* males, and w^{-}; *ey-GAL4/CyO*; +/+ males. To determine the effect of an extra copy of the BEAF gene, *gBF.3C/gBF.3C* males were crossed to w^{m4h} females or y^{1} w^{67c23} females, and the eyes of w^{m4h} males, w^{m4h}; *gBF.3C/+* males, and w^{-}; *gBF.3C/+* males were compared. Four- to 5-day-old males were etherized and photographed using darkfield illumination with a ×4 objective on a Ziess Axioskop microscope equipped with a Spot RT Slider CCD camera. To quantitate the pigment, heads of 20 males were homogenized in 200 μl 0.1% ammonium hydroxide and extracted once with chloroform, and the OD_{480} was determined (Ashburner 1989).

For *y* variegation, KV20 males were crossed to females of the genotypes indicated in Figure 7 and abdomens of 2- to 3-day-old males were photographed with a dissecting scope.

Mitotic analysis: Brains were dissected out of wandering third instar larvae, fixed in 3.7% formaldehyde in PBS for 30 min, transferred to 45% acetic acid for 3 min, and squashed in 60% acetic acid (Bonaccorsi *et al.* 2000). Chromosomes were stained with 100 ng/ml DAPI. In most cases, at least 50 fields/brain were scored for mitotic figures, where a field was defined as the region visible at ×100 magnification with a ×1 eyepiece on a Zeiss Axioskop microscope. For two brains, 25 fields were scored. The mitotic index was calculated as the total mitotic figures divided by the total number of fields scored, and the average field was estimated to have ~300 cells. Mitotic figures from *UAS-GFP/+*; *CNS-GAL4/BID.3B* larvae, *UAS-GFP/UAS-GFP*; *CNS-GAL4/TM3* larvae, and y^{1} w^{67c23} larvae were compared. There was no difference in mitotic figures or mitotic index between *UAS-GFP/UAS-GFP*; *CNS-GAL4/TM3* larvae and y^{1} w^{67c23} larvae.

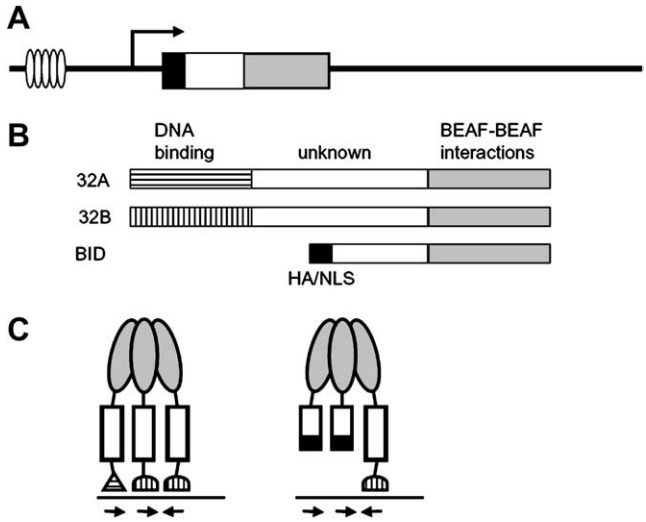

Figure 1.—Design of the BID protein. (A) The carboxy-terminal half of the BEAF-coding sequences were joined in frame to sequences encoding an HA epitope tag and SV40 NLS (solid box). The *BID* sequence is in pUAST (Brand and Perrimon 1993) and so is under GAL4 UAS control (open ovals) and has a downstream SV40 polyadenylation site (not shown). (B) BEAF-32A and -32B have unique amino-terminal DNA-binding domains of 80 amino acids (hatched boxes). The rest of the proteins are identical, being derived from the same exon. The identical portion includes a 120-amino-acid central region of unknown function (open box) and an 80-amino-acid carboxy-terminal domain that mediates interactions among BEAF proteins (shaded box) (Hart *et al.* 1997). The BID protein has an amino-terminal HA epitope tag and SV40 NLS joined to the carboxy-terminal half of BEAF. (C) Evidence suggests that BEAF forms trimers and that trimer formation occurs independently of DNA binding (Hart *et al.* 1997). The BID protein should form complexes with 32A and 32B, inhibiting DNA binding by BEAF complexes.

RESULTS

Rationale for the design of the *BID* transgene: The *BEAF* gene encodes two 32-kDa proteins, BEAF-32A and -32B. There are no mutations available in this gene. To circumvent the lack of mutations, we designed an inducible transgene that should inhibit DNA binding by the BEAF proteins (*BID*). Expression of the *BID* transgene is under GAL4 *UAS* control (Figure 1A). Many driver lines that express *GAL4* transgenes in different, known patterns are available. When crossed to these driver lines, *BID* expression will be driven in the same pattern as that of *GAL4*. The BID protein has part of the common portion of the BEAF proteins. This part of the BEAF proteins is involved in interactions with other BEAF molecules, but lacks a DNA-binding domain (Figure 1B; Hart *et al.* 1997). No other proteins copurify with BEAF, and evidence from immunoprecipitations and gel filtration columns indicates that BEAF subunits stably interact in solution; DNA binding is not necessary. Data indicate that BEAF forms trimers (Figure 1C), although cooperative binding to two sites separated by 200 bp suggests that larger BEAF complexes also form, at least transiently (Hart *et al.* 1997). The design of *BID* is based on the Drosophila Emc protein (Campuzano 2001) and the vertebrate family of Id proteins (Norton *et al.* 1998). Emc and Id lack DNA-binding domains and form heterodimers with certain DNA-binding transcription factors. The lack of one DNA-binding domain prevents stable binding to DNA. Thus these proteins are dominant-negative antagonists of their transcription factor partners. This plays an important role in developmental processes such as sensory organ development, myogenesis, and differentiation of blood cells. The BID protein should similarly act as a dominant negative by eliminating DNA-binding domains from BEAF complexes, thereby drastically reducing their affinity and specificity for DNA (Figure 1C).

Developmental effects of *BID* expression: Six transgenic fly lines containing single inserts of the *BID* transgene were generated by *P*-element-mediated germline transformation of microinjected embryos. As an initial test of the effects of *BID* expression, these flies were crossed to a *da-GAL4* line to drive ubiquitous expression during embryogenesis. Both transgenes were

TABLE 1

Phenotypes of flies expressing the *BID* transgene

BID fly line	Crossed to *da-GAL4*	Crossed to *ey-GAL4*
BID.2A	Male lethal (pupae); rough eyes	Rough eyes
BID.3A	Lethal (embryo/larvae)	Rough eyes
BID.3B	Lethal (embryo/larvae)	Rough eyes
BID.3C	Viable	Mild rough eyes
BID.4A	Viable; rough eyes	Rough eyes
BID.4B	Viable	Mild rough eyes

The *BID* transgene is under GAL4 UAS control. Six independent fly lines were generated and tested by crossing to GAL4-producing driver lines. Line names indicate the chromosome that the transgene is on followed by a letter for each independent insertion found; each line has a single insert. *da-GAL4*: GAL4 protein produced under control of the *daughterless* promoter, ubiquitous expression. *ey-GAL4*: GAL4 protein produced under control of the *eyeless* promoter, eye imaginal disc expression. The phenotypes correlate with the level of BID expression, as determined by semiquantitative Western blots.

heterozygous in the resulting embryos. This resulted in embryonic lethality with two *BID* fly lines, with a few embryos giving rise to first instar larvae (Table 1). For a third *BID* line, adult females eclosed but had a rough-eye phenotype. Males died as pharate adults. This could indicate an effect on dosage compensation, which involves chromatin modifications that double the activity of expressed genes on the single *X* chromosome in males. For the other three *BID* lines, viable adults were obtained. One of these three lines exhibited a rough-eye phenotype. The different phenotypes observed could be due to chromosomal position effects that affect the level of GAL4-mediated activation of the *BID* transgene in the different lines. In support of this, the levels of BID protein detected on Western blots of embryo protein extracts correlated with the severity of the phenotypes (data not shown).

It has previously been reported that overexpression of a *BEAF-32A* transgene in eye imaginal discs results in a rough-eye phenotype (Yamaguchi *et al.* 2001). In those experiments, it would be expected that 32A-utilizing insulators would be functional but insulators whose function relies on the 32B DNA-binding activity would be impaired. In our experiments, the function of all BEAF-utilizing insulators should be impaired. When our *BID* lines were crossed to *ey-GAL4* lines to drive expression in eye imaginal discs, all lines exhibited a rough-eye phenotype (Table 1 and Figure 2). As expected, the severity of the rough-eye phenotype increased when the *BID* chromosome was homozygous in the presence of an *ey-GAL4* driver. This was done for the three lines that were lethal in the presence of the *da-GAL4* driver (*BID.2A*, *BID.3A*, and *BID.3B*). Both *ey-GAL4* lines that we used in these experiments were recessive lethal; one had *ey-GAL4* balanced over *CyO* and the other had it balanced over *TM6B*. The resulting flies were sickly, and only the *ey-GAL4/CyO*; *BID.3A/BID.3A* line could be maintained as a stock.

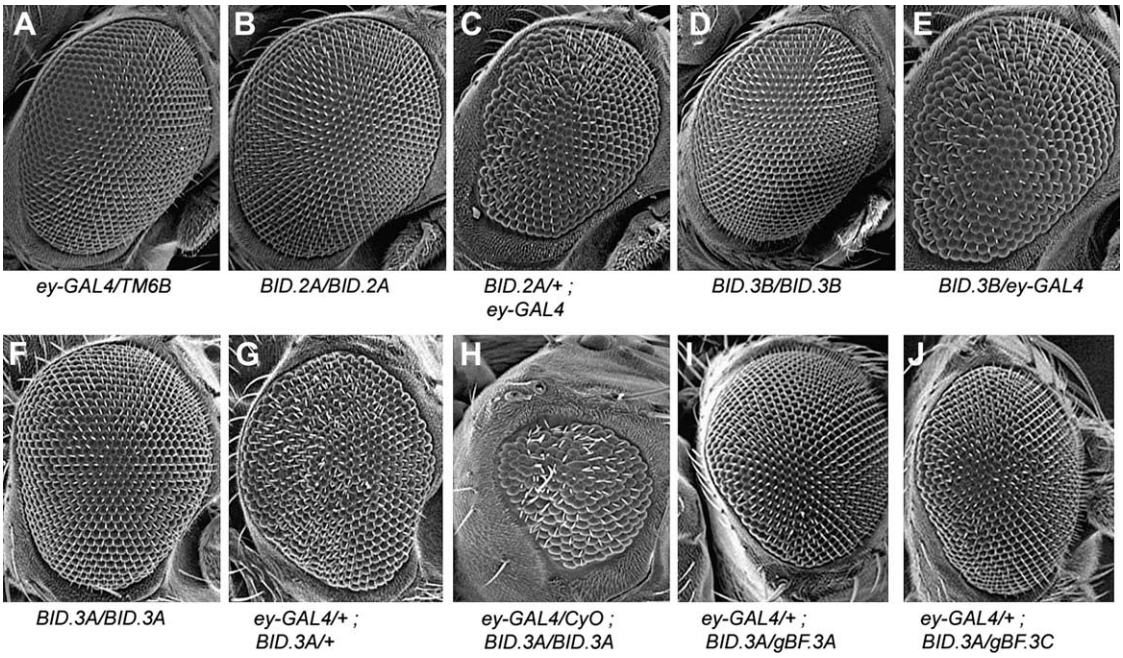

Figure 2.—*BID*-dependent rough-eye phenotype and rescue by *gBF*. Scanning electron micrographs show that the *ey-GAL4/TM6B* and *BID* fly lines have wild-type eye morphology (A, B, D, and F). The same is true for the *ey-GAL4/CyO* line (not shown). Flies heterozygous for both *ey-GAL4* and *BID* exhibit a rough-eye phenotype (C, E, and G). This phenotype is more extreme when *BID* is homozygous (H) and is rescued to near wild type when a third copy of the *BEAF* gene is introduced by a transgene (I and J). Two fly lines with the *gBF* rescue transgene inserted at different locations rescued the rough-eye phenotype.

To address the specificity of the BID protein for BEAF, transgenic lines that contained a 5-kb *Bgl* II fragment of genomic DNA that spans the *BEAF* gene were generated. This DNA, which we refer to as *gBF*, includes portions of genes located upstream (*CG10155*) and downstream (*kn*) of *BEAF*. Therefore it is likely that this region contains all control elements necessary for proper expression of the *BEAF* gene. When these flies were crossed to the *ey-GAL4/CyO*; *BID.3A/BID.3A* line, the *gBF* transgene rescued the rough-eye phenotype in the resulting heterozygous *GAL4/+*; *BID.3A/gBF.3A* or *gBF.3C* progeny (Figure 2). The ability of an extra copy of the *BEAF* gene to overcome the effects of *BID* expression indicates that BID specifically interferes with BEAF function to result in the rough-eye phenotype.

BID co-immunoprecipitates with BEAF: To further explore *in vivo* interactions between BID and BEAF proteins, we performed a co-immunoprecipitation assay. *BID.3B* males were crossed to *da-GAL4* females, resulting in ubiquitous expression of the *BID* transgene in embryos. Because this is a lethal combination, most of the embryos do not hatch. Embryos were collected on grapejuice agar plates for 16 hr or less. Nuclear extracts were prepared from these embryos as well as from y^1 w^{67c23} embryos that did not have the *BID* transgene. In addition, we tested the cytoplasmic fractions and found that some cytoplasmic extracts from *da-GAL4/+*; *BID.3B/+* embryos contained BID but not BEAF protein. BID was immunoprecipitated only if BEAF was present in the extract (Figure 3). Therefore BID forms complexes with BEAF *in vivo*.

***BID* expression interferes with scs′ insulator function:** The *BID*-dependent rough-eye phenotype is rescued by an extra copy of the *BEAF* gene, and BID physically interacts with BEAF *in vivo*. We next wanted to determine if *BID* expression would interfere with scs′ function in a transgene assay. We used two assays, a position-independent expression assay and an enhancer-blocking assay. For the position-independent expression assay, we generated transgenic fly lines in which the mini-*white* gene was bracketed by the M2 derivative of scs′ (on the 5′ side) and scs (on the 3′ side) (Cuvier *et al.* 1998). The M2 insulator has two copies of the high-affinity BEAF-binding site present in scs′. The bracketed mini-*white* gene is insulated from most chromosomal position effects; ~90% of fly lines have yellow or light-orange eyes. In the absence of the 5′ insulator, <50% of fly lines have such light eye pigmentation (Cuvier *et al.* 1998). Therefore if BID interferes with BEAF function, about half of the *M2* lines will have darker eye pigmentation in the presence of *BID* expression. To test whether BID interferes with insulator function, flies homozygous for *M2* insertions were crossed to the *ey-GAL4/CyO*; *BID.3A/BID.3A* line. Eye pigmentation in 3-day-old female flies heterozygous for *GAL4*, *BID.3A*, and *M2* was compared to their siblings that were heterozygous for *CyO*, *BID.3A*, and *M2*. Two of four *M2* lines tested had significantly darker eye pigmentation when *BID* expression was driven by the *ey-GAL4* driver, while very little effect was observed in the other two lines (Figure 4A).

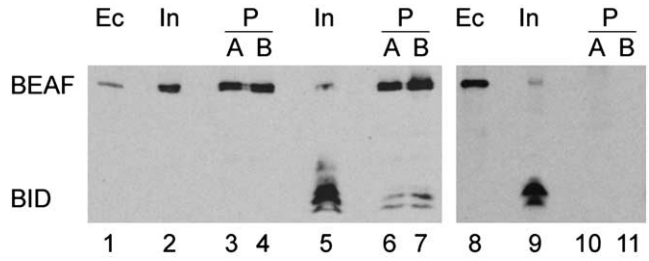

Figure 3.—BID interacts with BEAF *in vivo*. Nuclear extracts were prepared from y^1 w^{67c23} embryos (lanes 2–4) or embryos heterozygous for *da-GAL4* and *BID.3B* (lanes 5–7). A cytosolic extract prepared from embryos heterozygous for *da-GAL4* and *BID.3B* was found to have BID protein but essentially no BEAF and was used as a negative control (lanes 9–11). Immunoprecipitations were performed with antibodies specific for the unique amino termini of 32A (lanes 3, 6, and 10) or 32B (lanes 4, 7, and 11). Proteins on the Western blots were detected with an antibody that recognizes both BEAF and BID proteins. BID co-immunoprecipitated with BEAF-32A (lane 6) and -32B (lane7). BEAF proteins immunoprecipitated in the absence of BID (lanes 3 and 4), but BID did not immunoprecipitate in the absence of BEAF (lanes 10 and 11). Ec, 32B protein produced in *Escherichia coli* used as a Western control; In, input extract; P, immunoprecipitated proteins.

It is unlikely that the mini-*white* transgenes in the *BID* and *ey-GAL4* transposons account for the darker eye pigmentation described above. Flies heterozygous for the *ey-GAL4* and the *BID.3A* transposons together have light-orange eyes, indicating that only a low level of pigment is produced. The only difference between the flies compared in the assay was the presence or absence of the *ey-GAL4* transposon, which by itself results in a pale-yellow eye color. Two of the *M2* lines tested apparently are not subject to chromosomal position effects, and the additive effect of the *ey-GAL4* transposon on eye pigmentation was slight. We conclude that the other two *M2* lines tested are susceptible to chromosomal position effects and that the BID protein interferes with M2 insulator function to result in darker eye pigmentation.

The enhancer-blocking assay that we employed utilized a *yellow* transgene rather than mini-*white*. An scs′ dimer (2scs′), scs, or gypsy insulator was located between the *yellow* wing and body enhancers and the *yellow* gene. This allows insulators that do not have BEAF-binding sites to be tested. "Sibling" lines in which the insulator was removed by the *Cre* recombinase were also used as controls for the presence and absence of an insulator at the same chromosomal locus. Using these fly lines, it was previously found that insulators do not block the propagation of heat-shock puffs in polytene chromosomes (Kuhn *et al.* 2004). To use this assay, an appropriate *GAL4* driver line was required. For this

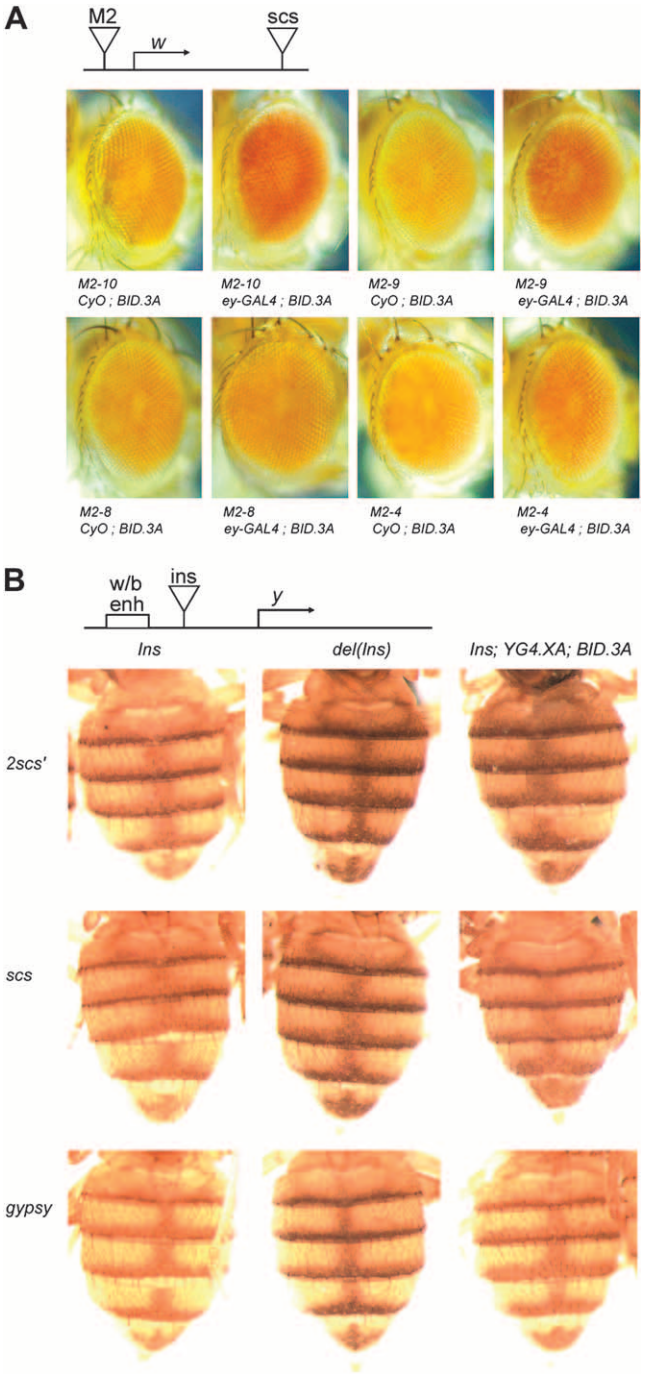

Figure 4.—BID interferes with scs′ insulator activity, but not with scs or *gypsy* insulator activities. (A) *BID* expression inactivates the BEAF-dependent M2 insulator, an scs′ derivative, in a position-independent expression assay. Eyes of 3- to 4-day-old females heterozygous for all indicated transposons are shown. See text for details. (B) *BID* expression inactivates an scs′ dimer, but has minimal effects on the scs or *gypsy* insulators in an enhancer-blocking assay in transgenic flies. Abdomens of 3- to 4-day-old females heterozygous for the indicated transposons, with (Ins) or without [del(Ins)] the indicated insulator between the enhancer and promoter, are shown. See text for details.

purpose, we made a construct that has the *yellow* wing and body enhancers and promoter upstream of the *GAL4*-coding sequences and the *yellow* poly(A) region downstream (hereafter referred to as *YG4*). Transgenic flies with the *YG4* construct were crossed to *UAS–GFP* flies to confirm that GAL4 protein was produced. A fly line homozygous for *YG4* (on the *X* chromosome) and *BID.3A* (on the third chromosome) was constructed and crossed to fly lines homozygous for the enhancer-blocking constructs. As controls for body pigmentation, the enhancer-blocking lines and their "siblings" lacking the insulators were crossed to $y^1\ w^{67c23}$ flies. The resulting progeny were heterozygous for all transposons that were present. The level of pigmentation in the dorsal abdomen of 3- to 4-day-old females was recorded. As shown in Figure 4B, control flies with the insulators had less pigment than their "sibling" lines without the insulators. *BID* expression inactivated the scs′ dimer, resulting in pigmentation similar to that in flies lacking the insulator. There was no effect of *BID* expression on the function of the gypsy insulator. The effect of *BID* expression on the function of the scs insulator was less clear. The level of pigmentation appeared to be intermediate between the insulated and uninsulated controls, suggesting some effect on scs function. It has been shown that BEAF and Zw5, the scs′- and scs-binding proteins, can interact with each other (Blanton *et al.* 2003). Perhaps this interaction accounts for the effect observed. The main conclusion is that *BID* expression strongly interferes with scs′ function.

***BID* expression interferes with polytene chromosome structure:** Some models of insulator function hypothesize that insulators affect chromatin structure or dynamics. To determine whether *BID* expression affects chromatin structure, polytene chromosomes were prepared from salivary glands of third instar larvae after crossing *BID* lines to lines that produced GAL4 in salivary glands (*SG-GAL4* driver). A BID-dependent global disruption of polytene chromosome organization was observed (Figure 5). In the presence of a *SG-GAL4* driver, animals heterozygous for *BID* had smaller salivary glands than did wild-type animals while those homozygous for *BID* often had tiny salivary glands with chromatin that easily fragmented. These *SG-GAL4*; *BID* homozygous flies were crossed with flies containing the *gBF* transgene, resulting in progeny that were heterozygous for the *SG-GAL4* driver, *BID* and *gBF*. By introducing a third copy of the *BEAF* gene in this way, the defect in polytene chromosome organization was largely rescued (Figure 5). This provides further evidence that the BEAF proteins are the main target of BID.

Immunostaining indicated that animals heterozygous for *SG-GAL4* and *BID* had reduced levels of BEAF on their polytene chromosomes, and the BEAF banding pattern observed on normal polytene chromosomes was absent. Chromosomes prepared from larvae homozygous for *SG-GAL4* and *BID* had virtually no BEAF

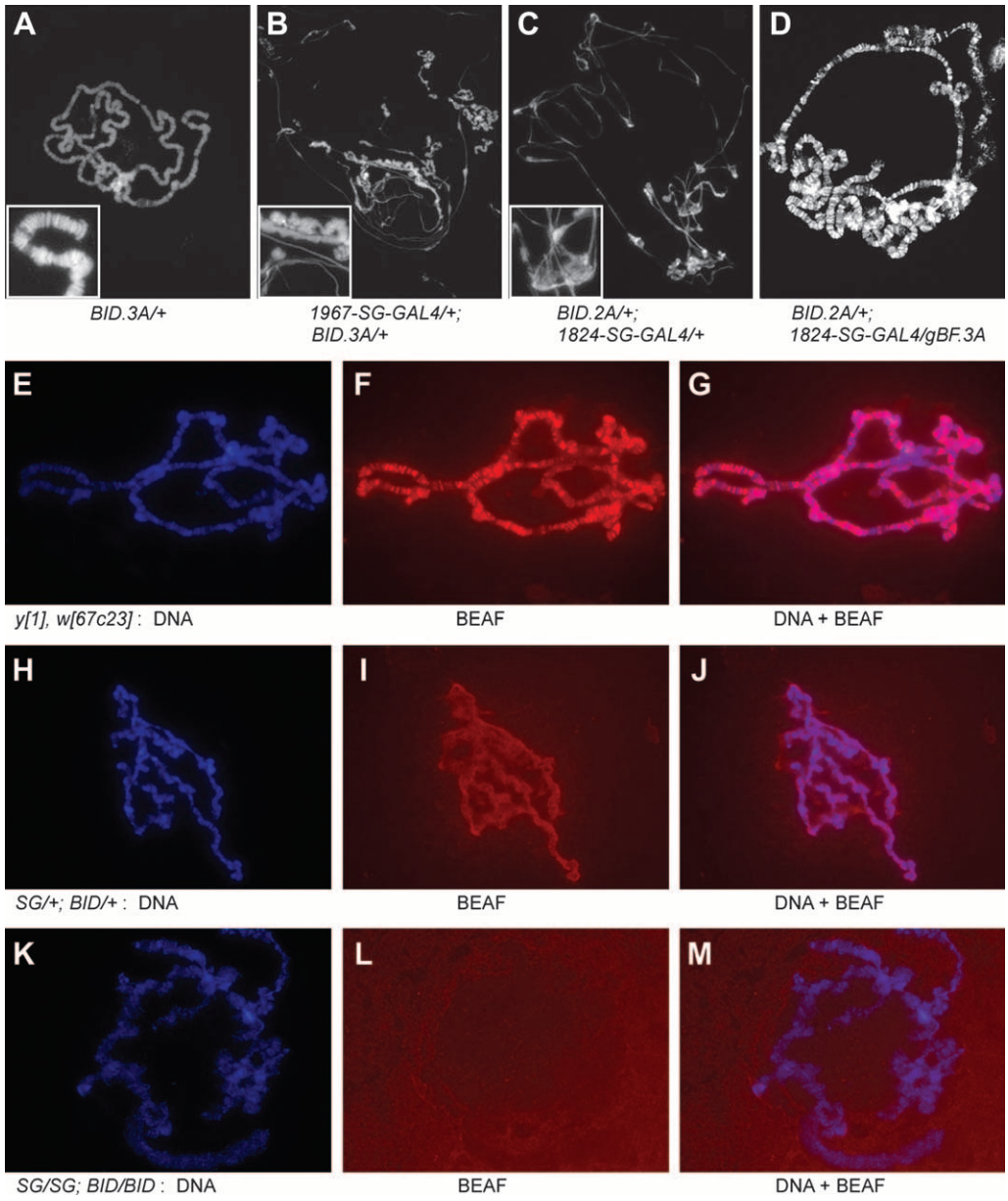

FIGURE 5.—Expression of *BID* in salivary glands leads to a global disruption of polytene chromosome structure and to a loss of the BEAF immunostaining pattern. Polytene chromosomes from salivary glands of a wild-type third instar larva have a well-defined banding pattern (A). Polytene chromosomes from salivary glands of third instar larva with two different salivary gland GAL4 (*SG-GAL4*) drivers and different *BID* transgenes lack this defined pattern and are easily overstretched (B and C). Adding a third copy of the *BEAF* gene via a *gBF* transgene largely rescues the BID-associated defect in polytene chromosome organization (D). Immunostaining of wild-type polytene chromosomes for BEAF gives a characteristic banding pattern. BEAF binds to several hundred interbands and band/interband junctions (E, DAPI; F, BEAF; G, overlay). Immunostaining of polytene chromosomes from larvae heterozygous for *1967-SG-GAL4* and *BID.3A* shows a reduced level of BEAF on the chromosomes and a lack of any defined banding pattern of BEAF (H, DAPI; I, BEAF; J, overlay). Immunostaining of polytene chromosomes from larvae homozygous for *1967-SG-GAL4* and *BID.3A* shows an apparent lack of BEAF staining (K, DAPI; L, BEAF; M, overlay).

staining and had a more extreme morphology (Figure 5). In addition, there appeared to be a higher background level of staining. This could indicate a higher level of BID protein remaining on the slide after fixation, even though it is a soluble protein (*i.e.*, it is not chromatin bound). These results indicate that BID interferes with the ability of BEAF to associate with chromatin *in vivo* and that the chromatin structure of the resulting polytene chromosomes is globally affected.

***BID* expression does not affect mitotic chromosomes or mitosis:** Many proteins participate in chromosome condensation during mitosis. The condensin complex clearly plays a key role, although disruption of condensin only partially interferes with mitotic chromosome condensation. Therefore, condensin cannot determine all levels of compaction. BEAF remains on mitotic chromosomes (HART *et al.* 1999). If interphase organization is utilized in a modified form to produce highly condensed metaphase chromosomes, BEAF might also play a role in mitotic chromosome organization. If that were the case, *BID* expression should affect chromosome condensation. This hypothesis was addressed by observing mitotic cells in brain squashes from third instar larvae.

The *CNS-GAL4* driver line used in these experiments has a third chromosome insertion that is sickly when homozygous. We generated a line with the *CNS-GAL4* driver chromosome balanced over *TM3* with a homozygous *UAS-GFP* responder second chromosome. This allowed identification of larvae that were producing GAL4 protein, and therefore also of both GFP and BID, after crossing these flies to *BID* flies. Both *UAS-GFP/UAS-GFP*; *CNS-GAL4/TM3* and y^1 w^{67c23} larvae were used

TABLE 2

***BID* expression does not affect the mitotic index in larval brains**

	Wild type	*BID.3B*
Mitotic index	6.45 ± 0.79	6.60 ± 1.35

The mitotic index ± standard deviation is shown for two genotypes: wild type, *UAS-GFP/UAS-GFP*; *CNS-GAL4/TM3* and *BID.3B, UAS-GFP/+*; *BID.3B/CNS-GAL4*. Data for wild type are from five brains (50 fields/brain); data for *BID.3B* are from six brains (50 fields/brain for four brains, 25 fields/brain for two brains).

as controls with similar results. *BID* expression did not affect the health of animals and did not affect the size of the larval brain or the mitotic index of neuroblasts obtained from DAPI-stained brain squashes (Table 2). In addition, we did not observe any defects in chromosome condensation, premature sister-chromatid separation, aneuploidy, or anaphase problems. We conclude that BEAF does not play a role in mitosis. It is possible that BEAF remains on mitotic chromosomes to provide a molecular memory of the location of insulators.

***BID* expression affects position-effect variegation:** To further explore the apparent link between BEAF function and chromatin structure, we tested the effect of the BID protein on position-effect variegation (PEV) of the w^{m4h} gene. Due to a chromosomal inversion on the *X* chromosome, this gene is near pericentric heterochromatin. This rearrangement results in variegated expression in eyes, which is detected as varying numbers of pigmented ommatidia (Tartof *et al.* 1989). The level of variegation is very sensitive to mutations that directly or indirectly affect chromatin organization.

The w^{m4h} assay is complicated by the presence of mini-*white* genes on the *ey-GAL4* and *BID.3A* chromosomes. The level of pigmentation in the presence of these heterozygous chromosomes is relatively low and is not variegated (Figure 6). Males heterozygous for the *BID.3A* chromosome have more eye pigment than males heterozygous for the *ey-GAL4* chromosome. Males heterozygous for both chromosomes have still more eye pigment, despite having smaller, rough eyes. In a similar series of male flies hemizygous for w^{m4h}, males of the genotype w^{m4h}; *ey-GAL4/+*; *BID.3A/+* had the fewest red ommatidia and the lowest levels of eye pigment (Figure 6). The smaller, rough eyes cannot account for the lower

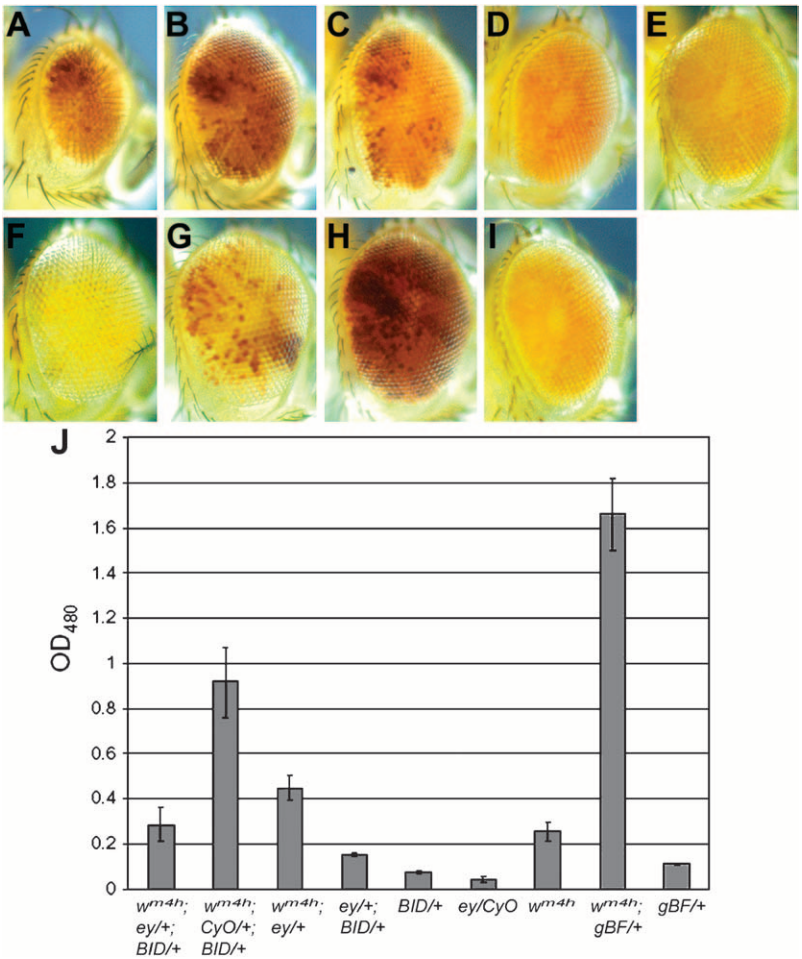

Figure 6.—Expression of *BID* in eye imaginal discs enhances w^{m4h} variegation, while expression of *gBF* suppresses w^{m4h} variegation. Eyes of 4- to 5-day-old males of the following genotypes are shown: (A) w^{m4h}; *ey-GAL4/+*; *BID/+*; (B) w^{m4h}; *CyO/+*; *BID /+*; (C) w^{m4h}; *ey-GAL4/+*; *+/+*; (D) w^{-}; *ey-GAL4/+*; *BID /+*; (E) w^{-}; *+/+*; *BID /+*; (F) w^{-}; *ey-GAL4/CyO*; *+/+*; (G) w^{m4h}; *+/+*; *+/+*; (H) w^{m4h}; *+/+*; *gBF/+*; (I) w^{-}; *+/+*; *gBF /+*. Note that all transgenes are marked with mini-*white* and result in yellow or light-orange eyes when heterozygous alone. (J) Pigment was extracted by homogenizing heads in 10 μl/head of 0.1% ammonium hydroxide and extracting 1× with chloroform. The OD_{480} values for the extracted pigment are shown in the same order as the eye pictures.

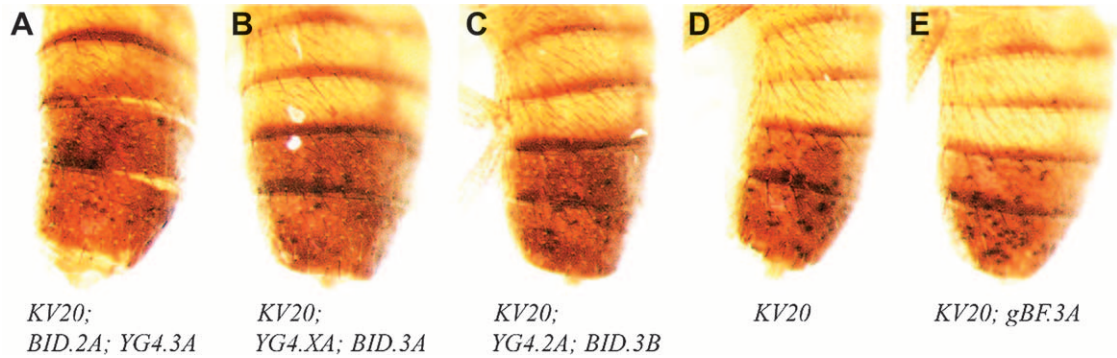

FIGURE 7.—Variegation of a *y* transgene located in the pericentromeric heterochromatin of chromosome arm *2R* is enhanced by *BID* expression and suppressed by a third copy of *BEAF*. The *y* transgene is in the *KV20* transposon. Abdomens of 2- to 3-day-old males heterozygous for the indicated transposons are shown. See text for details.

pigment levels, since *ey-GAL4/+*; *BID.3A/+* flies had more pigment than *ey-GAL4/+* or *BID.3A/+* flies. This indicates that *BID* expression enhances PEV, leading to a suppression of w^{m4h} expression.

If BID enhances PEV via effects on BEAF, then overproduction of BEAF should suppress PEV. This was tested with the *gBF* rescue transgene. Once again, the *gBF* transposon is marked with a mini-*white* gene that results in a low level of eye pigmentation. Males of the genotype w^{m4h}; *gBF/+* had more red ommatidia and more eye pigment than w^{m4h} males (Figure 6). While the increase in eye pigment is due in part to the mini-*white* gene associated with *gBF*, this is unlikely to account for the entire increase. As predicted on the basis of *BID* expression results, we conclude that BEAF is a triplo-suppressor of PEV. The involvement of BID and BEAF in pericentric PEV suggests that BEAF protects the w^{m4h} gene from being incorporated into heterochromatin, perhaps by forming a barrier that limits heterochromatin spreading.

We further tested the effect of BID on PEV associated with a different reporter gene and chromosome. For this purpose, we used the KV20 line, which has a *yellow* transgene inserted into the pericentric heterochromatin of chromosome arm *2R* (YAN *et al.* 2002). This circumvents the use of variegated *w* expression in a background that introduces transgenic mini-*white* genes. Male flies heterozygous for the *KV20* transposon and different *YG4* drivers and *BID* responders exhibited enhanced variegation relative to males heterozygous for only *KV20*. They had fewer dark spots on their posterior abdominal segments (Figure 7). Adding a third copy of *BEAF* via a *gBF* transgene suppressed PEV of the *y* transgene, resulting in a larger number of dark spots. Thus, two PEV assays, using different reporter genes on different chromosomes, indicate that BEAF interferes with the formation of pericentric heterochromatin.

DISCUSSION

To gain insight into BEAF function, we designed a gene encoding the BEAF self-interaction domain. The encoded protein should act as a dominant-negative form of the BEAF proteins by interfering with DNA binding. We have shown by co-immunoprecipitation that the BID protein physically interacts with BEAF *in vivo* and by immunostaining that it removes BEAF from polytene chromosomes. Adding a third copy of the *BEAF* gene rescues the BID-associated rough-eye phenotype and disruption of polytene chromosome structure. Furthermore, BID interferes with scs′ insulator function in both a position-independent expression and an enhancer-blocking assay. We conclude that BID interferes with BEAF function by reducing the level of chromatin-associated BEAF.

Could interactions between BID and proteins other than BEAF account for the effects of BID? No proteins copurify with BEAF, indicating that BEAF does not form stable complexes with other proteins. However, interactions between BEAF and other proteins have been reported. D1 is an abundant chromosomal protein that resembles mammalian HMGA (formerly HMG-I) proteins, except it is larger (ASHLEY *et al.* 1989). Whereas mammalian HMGA proteins have 3 AT-hook domains, D1 has 10 (at least 6 of which should be functional). Although D1 predominantly binds to AT-rich satellite DNA sequences (LEVINGER and VARSHAVSKY 1982; AULNER *et al.* 2002), it can cooperatively bind to certain DNA sequences with BEAF (CUVIER *et al.* 2002). The potential role of this in the effect of BEAF on PEV of the w^{m4h} and KV20 *y* alleles is discussed below. Another protein that interacts with BEAF is Zw5 (BLANTON *et al.* 2003), a protein that binds to the scs insulator (GASZNER *et al.* 1999). This interaction could account for the apparent weak effect of BID on scs insulator activity in the enhancer-blocking assay. A protein interaction map derived from a high-throughput yeast two-hybrid screen identified five proteins that can interact with BEAF (GIOT *et al.* 2003) (http://portal.curagen.com/cgi-bin/interaction/flyhome.pl). Four of these proteins are encoded by conceptual genes, and no functional information is available. The fifth protein is katanin-60, the catalytic component of a microtubule-severing complex. The two-hybrid screen did not identify D1 or Zw5,

and it is unknown if BEAF interacts with any of these five proteins *in vivo*. We cannot formally rule out the possibility that interactions with these or other proteins contribute to the effects of the BID protein. But the effect of BID on the activity of the scs′ insulator, the lack of effect on the gypsy insulator, the minimal effect on the scs insulator, and the rescue of the rough-eye and polytene chromosome phenotypes by a third copy of the *BEAF* gene suggest that BEAF is the major target of BID.

Ubiquitous expression of *BID* during embryogenesis is lethal, indicating that the BEAF proteins are essential during development. It was previously shown that expression of a *BEAF-32A* transgene in eye imaginal discs led to a rough-eye phenotype associated with increased apoptosis (Yamaguchi *et al.* 2001). Overproduction of 32A should affect the function of insulators that require 32B DNA-binding activity, but not those that require only 32A. The BID protein should affect all BEAF-dependent insulators. On the basis of the proposed role of BEAF in insulator function, we hypothesize that many genes are misregulated when BEAF insulator function is perturbed. This misregulation could be due in part to the transcription factor DREF (Hirose *et al.* 1996). Originally proposed to regulate DNA-replication-related genes, it has more recently been proposed that DREF functions as part of a core promoter selectivity factor for TRF2-utilizing promoters (Hochheimer *et al.* 2002; Ohler *et al.* 2002). There is evidence that BEAF and DREF compete for binding to certain DNA sequences (Hart *et al.* 1999); removing BEAF would facilitate binding by DREF to these sites. We propose that a breakdown in gene regulation disrupts the developmental program in the developing eye, resulting in a rough-eye phenotype. In the developing embryo, this breakdown is lethal.

BEAF and the D1 protein can cooperatively bind to DNA (Cuvier *et al.* 2002). However, their patterns of immunolocalization on polytene chromosomes are largely distinct. D1 binds an AT-rich sequence and largely immunolocalizes to heterochromatin, especially the AT-rich 1.672 and 1.688 g/cm^3 satellites (Rodriguez Alfageme *et al.* 1980; Aulner *et al.* 2002). These satellites are found in the pericentromeric heterochromatin of the *X* and *Y* chromosomes and of chromosome 4 (Lohe *et al.* 1993). BEAF binds to several hundred sites in euchromatin (Zhao *et al.* 1995). Despite their largely distinct chromosomal distributions, BEAF and D1 likely interact at the bases of the *X*, *2L*, and *2R* chromosome arms, where several hundred dispersed copies of a sequence (BE28) that has both BEAF- and D1-binding sites are found (Cuvier *et al.* 1998, 2002). The w^{m4h} gene is located near the base of the *X* chromosome, and the 1.688 g/cm^3 satellite is a component of the pericentromeric heterochromatin in this region. Interfering with D1 function suppresses w^{m4h} variegation (Monod *et al.* 2002). The BE28 repeats could be locations where BEAF and D1 normally interact to create a transition zone that is checkered with heterochromatin and euchromatin islands. Perhaps BID enhances the PEV of w^{m4h} by allowing D1-associated heterochromatin to spread farther, while extra BEAF blocks the spread. This could also occur for the KV20 *y* transgene, although *2R* does not have high concentrations of the 1.672 or 1.688 g/cm^3 satellites. Alternative possibilities include direct suppression of variegation by BEAF by some other currently unknown mechanism, or indirect suppression by affecting the activity or gene expression of other chromatin proteins that directly affect variegation.

The mechanism leading to disruption of polytene chromosome structure by BID is not known. It is possible that the D1 protein is involved, although as pointed out above, D1 is mainly associated with satellite heterochromatin and BEAF is mainly found on euchromatin. Furthermore, the chromosomes look puffy, not condensed like heterochromatin. It is possible that underreplication of the chromosomes could be involved, but that cannot account for the loss of banding patterns. Also, no effect on replication was apparent in our examination of mitotic figures in larval brain squashes. It has been shown in vertebrates and yeast that covalent histone modifications can differ on either side of insulators or barrier elements (Litt *et al.* 2001a,b; Noma *et al.* 2001). Perhaps impairing BEAF function allows these modifications to spread farther in a stochastic manner. Then individual chromosomes in the polytene bundle could have different patterns of histone modifications over the same sequences, causing a loss of banding and coherence between chromosomes. Similar phenotypes are observed in the presence of mutations known to affect proteins that act on chromatin. Examples include the JIL-1 histone H3 Ser10 kinase (Wang *et al.* 2001), the chromatin-remodeling factor ISWI (Deuring *et al.* 2000), SU(VAR)2-10 (Hari *et al.* 2001), and the Z4 interband-specific protein (Eggert *et al.* 2004). In all cases, the cause of the loss of polytene chromosome morphology remains unknown.

Some models propose that insulators limit communication between regulatory elements and promoters located in different domains by affecting chromatin structure or dynamics (Labrador and Corces 2002; West *et al.* 2002; Kuhn and Geyer 2003). Inhibiting the ability of BEAF to associate with chromatin leads to a global disruption of polytene chromosome structure and enhances PEV of the w^{m4h} and KV20 *y* alleles. These results provide strong support for a role of chromatin structure or dynamics in BEAF-dependent insulator function.

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