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Integrity of the Mod(mdg4)-67.2 BTB Domain Is Critical to Insulator Function in *Drosophila melanogaster*▽†

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The Drosophila *gypsy* insulator contains binding sites for the Suppressor of Hairy-wing [Su(Hw)] protein. Enhancer and silencer blocking require Su(Hw) recruitment of Mod(mdg4)-67.2, a BTB/POZ domain protein that interacts with Su(Hw) through a carboxyl-terminal acidic domain. Here we conducted mutational analyses of the Mod(mdg4)-67.2 BTB domain. We demonstrate that this domain is essential for insulator function, in part through direction of protein dimerization. Our studies revealed the presence of a second domain (DD) that contributes to Mod(mdg4)-67.2 dimerization when the function of the BTB domain is compromised. Additionally, we demonstrate that mutations in amino acids of the charged pocket in the BTB domain that retain dimerization of the mutated protein cause a loss of insulator function. In these cases, the mutant proteins failed to localize to chromosomes, suggesting a role for the BTB domain in chromosome association. Interestingly, replacement of the Mod(mdg4)-67.2 BTB domain with the GAF BTB domain produced a nonfunctional protein. Taken together, these data suggest that the Mod(mdg4)-67.2 BTB domain confers novel activities to *gypsy* insulator function.

Enhancer-mediated promoter activation is a fundamental mechanism of gene regulation in eukaryotes (10, 44). Recently, sequences in different organisms have been identified that constrain enhancer action. These elements, known as insulators, block communication between an enhancer and promoter only when the insulator is positioned between these regulatory elements. Similarly, insulators prevent silencer interactions with promoters (6, 10, 30, 44, 45). The properties of insulators are exemplified by the *gypsy* insulator that originally was found in the *gypsy* transposable element (19, 24).

Genetic and molecular approaches have led to the identification and characterization of three proteins, Suppressor of Hairy wing [Su(Hw)], Mod(mdg4)-67.2, and CP190, that are required for the activity of the *gypsy* insulator (6, 36). Su(Hw) is a zinc finger protein that binds 12 directly repeated copies of a short sequence motif in the *gypsy* insulator (9, 42). In addition, Su(Hw) has two acidic domains located at the amino (N) and carboxyl (C) termini of the protein and a C-terminal enhancer-blocking region that is essential for insulation (23, 29). The *mod*(*mdg4*) gene, also known as *E*(*var*)*3-93D*, encodes a large set of protein isoforms with specific functions in regulating the chromatin structure of different genes (3). All isoforms encoded by *mod*(*mdg4*) contain a BTB/POZ domain and a glutamine-rich (Q) region in the N terminus (3, 7). The BTB (*b*road complex, *t*ramtrack, *b*ric-a-brac) or POZ (*po*xvirus and *z*inc finger) domain identifies a large family of proteins in organisms from yeast to humans (43, 47). This domain functions as a protein interaction domain that facilitates homodimer (2, 33, 34) and heterodimer formation as well as oligomerization (11, 28, 37). One of the *mod*(*mdg4*)-encoded protein isoforms, Mod(mdg4)-67.2, interacts with the enhancer-blocking domain of the Su(Hw) protein (12, 20) through a C-terminal acidic domain. This domain is affected in two viable mutations $mod(mdg4)^{u1}$ and $mod(mdg4)^{T6}$ (12, 15). The third component of the insulator complex, CP190, also contains a BTB domain (38). It was suggested that Mod(mdg4)-67.2 and CP190 interact through their BTB domains.

The $mod(mdg4)^{u1}$ and $mod(mdg4)^{T6}$ mutations have varying effects on insulator function, resulting in partial restoration of enhancer-promoter communication in some cases, while transforming the insulator into a silencer in others (4, 12, 13, 14, 15, 22). The domains of the Mod(mdg4)-67.2 protein required for the insulator and antirepression activity are not determined. Although the essential role of the BTB domain for Mod(mdg4)-67.2 activity was predicted in the previous studies, this postulate has not been proven (12, 20). Here we examined the role of the BTB domain in the functional activities of the Mod(mdg4)-67.2 protein.

The structure of the BTB domain has been examined for mammalian transcriptional repressors PLZF and Bcl-6 (33, 34). The high degree of sequence identity between the BTB domains of Mod(mdg4), Bcl-6, and PZLF (1) allowed us to predict key residues of the Mod(mdg4)-67.2 BTB domain to

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test for function. In these studies, we used a combination of in vitro and in vivo analyses to define the regions of Mod(mdg4)-67.2 required for insulator function. Using yeast two-hybrid analyses, we define a second homodimerization domain that substitutes for the BTB domain when its homodimerization capacity is reduced by mutation. We find that a complete absence of Mod(mdg4)-67.2 homodimerization results in the loss of insulator activity. Finally, our studies indicate that the BTB domain of Mod(mdg4)-67.2 makes specific contributions to the formation of a functional insulator complex, as a fusion protein containing the GAF BTB domain and the Mod(mdg4)-67.2 C terminus, while retaining the ability to dimerize, does not reconstitute enhancer blocking.

MATERIALS AND METHODS

***Drosophila melanogaster* strains, plasmid constructions, germ line transformation and genetic crosses.** All flies were maintained at 25°C on standard yeast medium. The plasmid constructions are described in the supplemental material. The transposon constructs, together with P25.7wc, a *P* element with defective inverted repeats used as a transposase source, were injected into *y ac* w^{1118} preblastoderm embryos (27). The generation of transgenic lines and introduction into the *mod*$(mdg4)^{u1}$ or *mod*$(mdg4)^{T6}$ background were done as described previously (14).

The effects of the various Mod(mdg4) proteins produced from homozygous expression vectors were scored independently by two authors. To express transgenes regulated by the *hsp70* promoter, flies homozygous for the construct were heat shocked for 1 h every day from the second larval to middle pupal stages of fly development. To determine the *yellow*, *cut*, and *sc* phenotypes, we examined 3- to 5-day-old males developing at 25°C. For *yellow* phenotypes, wild-type expression in abdominal cuticle, wings, and bristles was assigned an arbitrary score of 5, while the absence of *y* expression was ranked 1. Flies with the previously characterized *y* allele were used as a reference to determine *y* pigmentation levels. The representative abdomens and wings displayed in the figures were selected by arranging several abdomens or wings in order of the severity of their mutant phenotype and selecting the average mutant phenotype to photograph.

Mutation of Mod(mdg4)-67.2. Mod(mdg)-67.2 cDNA cloned in pGEX2T was kindly provided by D. Dorsett. To make mutations in BTB, Mod(mdg)-67.2 BTB cDNA was subcloned in pBluescript II SK+ digested with EcoRI and DraII. One half of the BTB domain (first 122 bp of BTB domain) was PCR amplified with a primer containing one substitution and M13 reverse sequencing primer. The second half of the BTB domain was amplified with a primer complementary to the BTB domain (in case of one amino acid substitution) or with a primer containing the second substitution (in case of double substitutions) and M13 forward sequencing primer. PCR products were digested with DraII and EcoRI and cloned in pBluescript II SK+ digested with EcoRI and DraII. The following primers were used to produce mutations: S25A, 5′-CATAGCGCCTCGTGG-3′; D33N, 5′-GGCCCTCGGCGGCCAGCGAGACGTTCACC-3′; H46D, 5′-AAA TAGTGAAGGCCGACCG-3′; R47Q, 5′-AAATAGTGAAGGCCCACCAATT G-3′. To prepare Mod(mdg4)$^{\Delta Q}$, Mod(mdg)-67.2 cDNA in pGEX2T was digested with BlpI, filled in with Klenow fragment, and self-ligated. The BTB domain of GAF was PCR amplified from GAF cDNA in pET3 with the primer 5′-AATACGACTCACTATAG-3′ and 5′-CCGCGGCGGTGCCAGTCCCTG AATG-3′ containing a SacII site. The PCR product was digested with SacII and ligated in vector pSK containing Mod(mdg4)-67.2 cDNA, which was first digested with EcoRI, blunted, and digested with SacII.

Construction of plasmids expressing Mod(mdg4)-67.2 and its mutant derivatives in flies. The *Su*(*Hw*) promoter (29) was kindly provided by D. Dorsett. To construct transposons, Mod(mdg4)-containing mutant BTB domains were cloned in pCaSpeR4 under control of the *hsp70* promoter in the case of the H46D, H46D/D33N, and D33N/S25A mutants or under control of the *Su*(*Hw*) promoter in the case of the R47Q, D33N, R47Q/D33N, and Mod(mdg4)^{Gaf} mutants. Vectors were digested with EcoRI and BamHI and ligated with the 1.8-kb EcoRI-BamHI fragment of Mod(mdg)-67.2 containing mutations described above.

Construction of plasmids for in vitro experiments. For protein expression, Mod(mdg4) was cloned in frame with a six-His tag in pET23a (Novagene). pGEX2TMod was digested with BamHI, and the end was filled in with Klenow fragment. After EcoRI digestion, the gene fragment was cloned in pET23a that was first digested with EagI and then filled in and digested with EcoRI, producing pET23mod. To make an expression vector with mutant Mod(mdg4) proteins, we replaced the EcoRI-Eco72I fragment of pET23mod with the same fragment of the pCaSpeR4 construct containing mutations.

Construction of plasmids for yeast two-hybrid system. For yeast two-hybrid assays, the coding regions described above were cloned in vector pGBT9 and pGAD424 (Clontech) using EcoRI and BamHI sites. Su(Hw) was PCR amplified from pGEM3ZfSu(Hw) plasmid with primers 5′-AATGAGTGCCTCCAAGG AGGGC-3′ (upstream) and 5′-CCGTCGACTCAAGCTTTCTCTTGTTC-3′ (downstream) containing the SalI site. The PCR product was digested with SalI and cloned in vectors pGBT9 and pGAD424 digested with SmaI and SalI. Su(Hw) lacking the C-terminal end was PCR amplified as previously described, but the next downstream primer used was 5′-TTTGTCGACTTCGCCTGTGA C-3′ (also with the SalI site). The PCR product was digested with SalI and cloned in vector pGBT9 digested with SmaI and SalI, so we have pGBT9Su(Hw). To clone only the domain of SuHw interacting with Mod(mdg4), pGBT9Su(Hw) was digested with EcoRI and SalI and ligated with pGBT9 and pGAD424 vectors digested with the same enzymes. To make a plasmid carrying the Gal4-activating domain at the C-terminal end of the fusion protein, all pGAD plasmids with mutated or wild-type Mod(mdg4) were digested with EcoRI and then HindIII, filled in by Klenow, and self-ligated [the resulting plasmid was called pGAD(−)]. The activation domain was PCR amplified from pGAD424 with primers 5′-AG CGGATCCATGGATAAAGCGG-3′, containing a BamHI site, and 5′-GACA GATCTCTCTTTTTTTGGGTTTGGT-3′, containing a BglII site, digested with BamHI and BglII, and ligated with pGAD(−) digested with the same endonuclease. These operations produced plasmids containing fusions of all mutant forms and the wild type of Mod(mdg4) with the activation domain of Gal4 on the C-terminal end, designated pGDA. CP-1901-765 was PCR amplified with primers 5′-CATGGGTGAAGTCAAGTC-3′ and 5′-TTCAGATCTTTCCAGGTTG TCAATGG-3′, containing the BglII site. This PCR product was cloned in pGDA vector digested with EcoRI, filled in by Klenow and then BamHI. To prepare Mod(mdg4)1-273, PCR amplification from pGBT Mod 67.2 with the help of 5′-ATAGGATCCTTGCGGCACAAGTTG-3′, containing the BamHI site, and GAL DNA binding primers was done. The PCR product was digested with EcoRI and BamHI enzymes and cloned in either a pGBT or pGDA vector. PCR amplification with one primer 5′-TATGGATCCCTTCTTCTTGT TCTG-3′ containing a BamHI site and another primer containing EcoRI were used to prepare Mod(mdg4)$^{234\text{-}610}$ (5′-TATGAATTCATGTCCTCGAGCGCC-3′), Mod(mdg4)$^{317\text{-}610}$ (5′-ACCGAATTCATGTACTCTGAAGAC-3′), and Mod(mdg4)$^{390\text{-}610}$ (5′-ATAGAATTCATGGTCGACACCAGCGGG-3′) mutants. PCR products were cloned in either pGBT or pGDA vector as previously described. Deletion of DD was done by PCR amplification (primers 5′-ATAA GGCCTGGGCAATTCCATGGGGAG-3′ and 5′-ATAAGGCCTGTCGACA CCAGCGGG-3′) following StuI digestion and self-ligation of the resulting plasmid.

Two-hybrid and in vitro interactions. Two-hybrid assays were carried out using yeast strain pJ694A, plasmids, and protocols obtained from Clontech (Palo Alto, CA). For growth assays, plasmids were transformed into yeast strain pJ694A by the lithium acetate method as described by the manufacturer and plated on media lacking tryptophan and leucine. After 2 days of growth at 30°C, the cells were plated on selective media lacking tryptophan, leucine, histidine, and adenine, and growth was compared. Liquid culture assays were performed according to protocols described in the yeast protocols handbook (Clontech).

To express His-tagged proteins in vitro, the vector pET23mod was transformed in *Escherichia coli* strain BL21(DE3), grown in LB with ampicillin at 37°C to an optical density at 600 nm (OD_{600}) of 0.5, and then induced with 1 mmol of isopropyl-β-D-thiogalactopyranoside (IPTG), followed by growth at 18°C for 6 h. Protein purification was done with Talon superflow resin (Clontech) under native conditions according to the manufacturer's instructions. To express N-terminally glutathione *S*-transferase (GST)-tagged Mod(mdg4) protein, the pGEX2Tmod plasmid was transferred to *E. coli* strain BL21(DE3) and induced as described before. Purification was performed on glutathione-Sepharose 4B (Amersham) according to the manufacturer's instructions.

To study homodimerization in vitro, we performed polyacrylamide gel electrophoresis (PAGE) of six-His-tagged protein under native conditions. Gel electrophoresis in 7% gels was done in Tris-glycine buffer for 3 h at 20 V/cm. The proteins were blotted on a polyvinylidene difluoride membrane, incubated with primary antibody specific for the Mod(mdg4) 67.2 isoform, and developed with the ECL-plus kit (Amersham).

To investigate the possibility of heteromultimerization, we performed GST pull-down experiments. GST-Mod(mdg4) protein was incubated with glutathione-Sepharose 4B beads in binding buffer (20 mM HEPES-KOH, pH 7.6, 200 mM KCl, 2.5 mM MgCl, 10% glycerol, and 0.05% NP-40) for 2 h. Beads were blocked

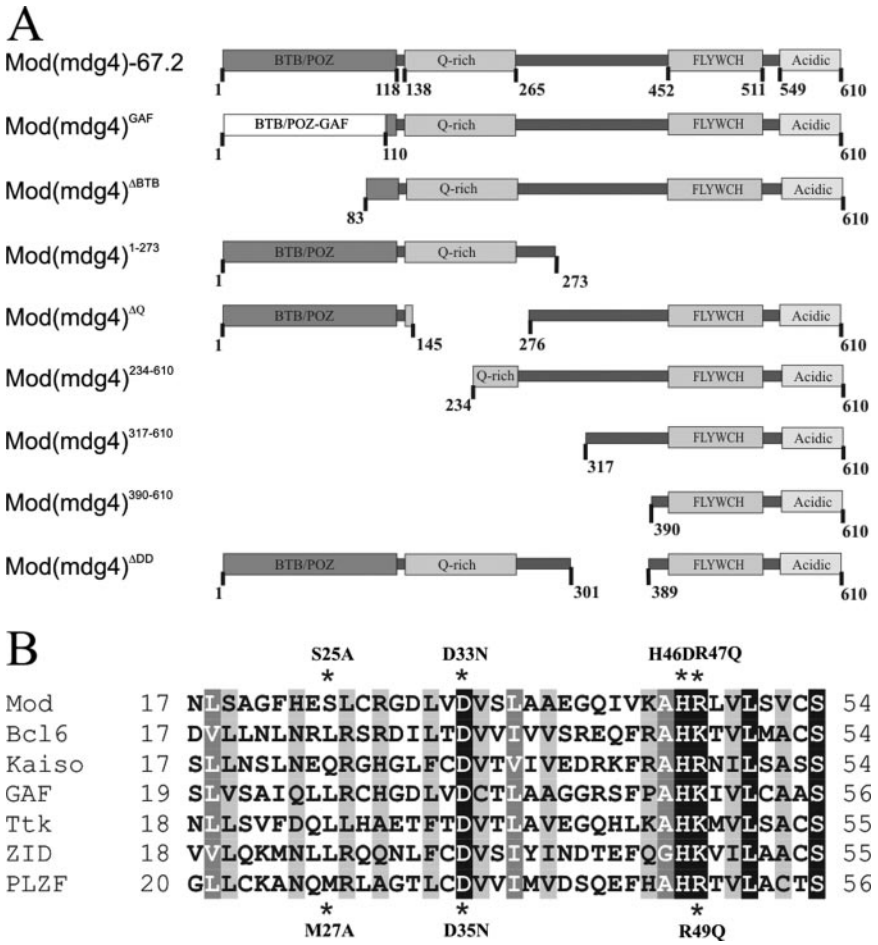

FIG. 1. Mod(mdg4)-67.2 protein domains and sequence comparisons. (A) Schematic representation of the Mod(mdg4)-67.2 protein. Mod(mdg4)-67.2 contains discrete functional domains, including the N-terminal BTB domain, glutamine-rich domain (Q-rich), conserved Cys2His2 motif, named FLYWCH motif (8), and the C-terminal acidic domain that interacts with the Su(Hw) protein. The structure of the Mod(mdg4)-67.2, Mod(mdg4)^{Gaf}, Mod(mdg4) $^{\Delta BTB}$, Mod(mdg4) $^{\Delta Q}$, Mod(mdg4) $^{\Delta DD}$, Mod(mdg4)$^{1-273}$, Mod(mdg4)$^{234-610}$, Mod(mdg4)$^{317-610}$, and Mod(mdg4)$^{390-610}$ proteins are shown. (B) Sequence alignment of the N terminus of the Mod(mdg4)-67.2 BTB domain with other BTB domains. This region of the BTB corresponds mainly to the charged pocket. Darker shading indicates more highly conserved residues. Conserved residues selected for mutational analysis are marked with an asterisk. The boxes indicate the charged residues in the pocket.

in 5% bovine serum albumin for 1 h and incubated with six-His-tagged protein for 3 h. After incubation, the beads were washed three times in wash buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.2% NP-40, 400 mM NaCl). Washed beads were boiled in Laemmli buffer and separated on an 8% sodium dodecyl sulfate-polyacrylamide gel. The proteins were blotted on a polyvinylidene difluoride membrane, which was then incubated with anti-six-His antibody (Amersham).

Immunofluorescence analyses. Antibodies against residues 403 to 610 of the Mod(mdg4)-67.2 were generated in chickens. Cy3-conjugated anti-chicken antibody (1:500; Chemicon) was used as a secondary antibody. Fixation and squashing of salivary glands and antibody staining were performed as originally described by Platero (40). Antibodies to lamin were generated in rabbits or mice and detected by secondary Cy5-conjugated antibody. Antibodies to Su(Hw) protein were generated in rabbits and detected by secondary fluorescein isothiocyanate-conjugated antibody. Imaginal disk staining was performed as described previously (31) with the same antibody.

RESULTS

Design of mutations in the BTB domain of the Mod(mdg4)-67.2 protein. To examine the role of the BTB domain in Mod(mdg4)-67.2 activity, we made several derivatives and tested the function of the resulting protein. First, we deleted 83 amino acids from the BTB domain [Mod(mdg4)$^{\Delta BTB}$]. These amino acids represent the most conserved and functionally important region of the BTB domain (33). Second, we made point mutations in critical amino acids within the charge pocket (Fig. 1). Melnick et al. (33, 34) identified two conserved charged residues in the BTB domain, an aspartate at position 35 and arginine at position 49 (Fig. 1B). Each monomer of the BTB dimer contributes a wall of the pocket containing the D35 (negatively charged) and R49 (positively charged) residues, leading to the formation of a coordinately charged pocket containing two positive and two negative charges. Since Mod(mdg4)-67.2 has both conserved residues, we made the same single, ModD33N (aspartate to asparagine at position 33) and ModR47Q (arginine to glutamine at position 47), and double, ModD33N/R47Q, substitutions. Third, we changed histidine 46, which is the most conserved residue among BTB domains (42), to an acidic aspartate in the ModH46D mutant. Histidine 46 is not involved in formation of the charge pocket. We also made the double mutant ModD33N/H46D in which the alter-

TABLE 1. Summary of interactions between Mod(mdg4) mutants and Mod(mdg4)-67.2[a]

Interacting proteins (GAL4BD, GALAD)	Strength of interaction with AD fused:	
	Before protein	Behind protein
Mod(mdg4), Mod(mdg4)	+++	+++
Mod(mdg4), ModD33N/H46D	−	+++
ModD33N/H46D, Mod(mdg4)	−	+++
ModD33N/H46D, ModD33N/H46D	−	+
Mod(mdg4), ModH46D	−	++
ModH46D, Mod(mdg4)	−	+++
ModH46D, ModH46D	−	+
Mod(mdg4), ModD33N	−	++
ModD33N, Mod(mdg4)	−	++
ModD33N, ModD33N	−	−
Mod(mdg4), ModR47Q	+++	+++
ModR47Q, Mod(mdg4)	++	+++
ModR47Q, ModR47Q	++	+++
Mod(mdg4), ModD33N/R47Q	+	+++
ModD33N/R47Q, Mod(mdg4)	+	+++
ModD33N/R47Q, ModD33N/R47Q	−	++
Mod(mdg4), Mod(mdg4)^{GAF}	−	+++
Mod(mdg4)^{GAF}, Mod(mdg4)	−	+++
Mod(mdg4)^{GAF}, Mod(mdg4)^{GAF}	−	+++
Mod(mdg4), Mod(mdg4)$^{\Delta BTB}$	−	++
Mod(mdg4)$^{\Delta BTB}$, Mod(mdg4)	−	++
Mod(mdg4)$^{\Delta BTB}$, Mod(mdg4)$^{\Delta BTB}$	−	++

[a] No growth occurred after transformation with single plasmids, indicating that interactions between the proteins are required for expression of the reporter genes (data not shown). The GAL4AD is fused in front of or behind the tested protein. The + symbol refers to the relative strength of the two-hybrid interaction. The − symbol indicates the absence of interaction. Equivalent expression of the chimeric proteins in yeast was confirmed by immunoblotting with GAL4BD or AD monoclonal antibodies (Fig. 2A and data not shown).

ation in charge by the H46D substitution is compensated by the neutralization of a negative charge in the D33N substitution. Finally, we constructed a BTB swap derivative. Read et al. (41) demonstrated that the BTB domain of the Mod(mdg4) protein substituted for that of GAF in transcription stimulation. To determine whether the GAF BTB domain is functionally equivalent to the Mod(mdg4) BTB domain, we replaced the first 108 residues of Mod(mdg4) with the first 122 residues of the GAF protein, retaining the position of the BTB domain with respect to the GAF protein, to form Mod(mdg4)^{Gaf} (Fig. 1A).

Study of dimerization of the Mod(mdg4) mutants. Each of the Mod(mdg4) mutants was tested in the yeast two-hybrid system for its ability to interact with Su(Hw). Recently we found that the acidic domains and DNA binding region of Su(Hw) partially repress transcription in yeast (32), complicating the interpretation of results obtained using this system. For this reason, we used a truncated version of the Su(Hw) protein that contains only the Mod(mdg4)-interacting domain [Su(Hw)^{MID}], Su(Hw)^{MID} and Mod(mdg4)-67.2 were fused in frame with either the yeast GAL4 DNA binding domain (GAL4BD) or activation domain (GAL4AD). As expected, Su(Hw)^{MID} interacts strongly with Mod(mdg4)-67.2 in both reciprocal two-hybrid tests (see Table S1 in the supplemental material).

As a first step in our studies, we tested interactions between the Mod(mdg4) BTB mutants and Su(Hw)^{MID} (Table 1; also see Table S1 in the supplemental material). Unexpectedly, we found that most of the mutant forms of Mod(mdg4) displayed a polar effect in the two-hybrid system, as the interaction was strong only when the Mod(mdg4) derivative was fused to GAL4BD. Fusion of the Mod(mdg4) derivatives to GAL4AD produced only weak colony growth on selective plates. To overcome this problem, we fused the GAL4AD domain to the C-terminal part of the Mod(mdg4) mutants. In this case, all Mod(mdg4) mutants showed robust interactions with Su(Hw)^{MID} in the reciprocal two-hybrid tests, suggesting that the Mod(mdg4) mutants accumulate stably in yeast. These data were supported by immunoblot analysis of Mod(mdg4) proteins demonstrating a comparable level of accumulation in yeast cells (Fig. 2A).

We examined whether the mutated forms of the Mod(mdg4) protein were able to self-associate (Table 2). Note that the

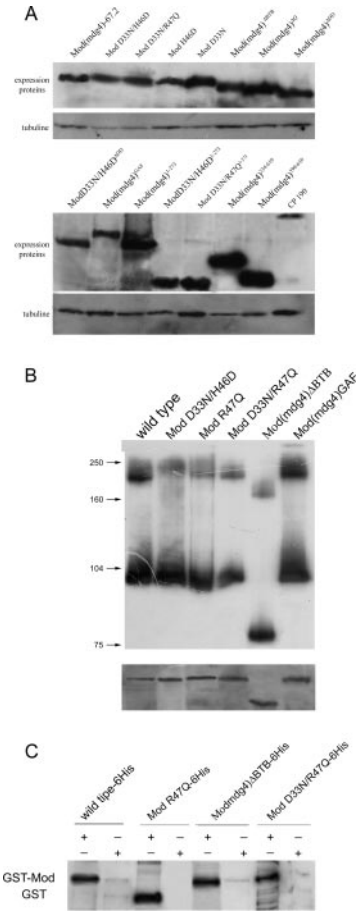

FIG. 2. Analysis of the mutant Mod(mdg4) proteins in vitro. (A) Western analyses of yeast extracts carrying different Mod(mdg4) mutants. The panel shows the expression Mod(mdg4) proteins fused to GAL4 binding domain detected with GAL4 antibodies. In the lower panel, the same filter was stripped and reprobed with anti-tubuline antibodies. (B) Western blot analyses of *E. coli*-expressed and purified mutant Mod(mdg4) proteins. The native PAGE and sodium dodecyl sulfate-PAGE of the same proteins are shown in the upper and lower panels, respectively. Experimental details are described in Materials and Methods. (C) Interaction of the Mod(mdg4) mutants with Mod(mdg4)-67.2 by GST pull-down assay. The interactions of mutant Mod(mdg4) proteins with wild-type Mod(mdg4)-67.2 were visualized by Western blot analysis using the anti-His tag monoclonal antibodies. All results were reproduced in three independent experiments.

TABLE 2. Identification of the second domain required for dimerization of Mod(mdg4)-67.2[a]

Interacting proteins	Strength of interaction
Mod(mdg4)$^{\Delta BTB}$, Mod(mdg4)$^{\Delta Q}$	+++
Mod(mdg4)$^{\Delta BTB}$, Mod(mdg4)$^{1-273}$	−
Mod(mdg4)$^{1-273}$, Mod(mdg4)$^{1-273}$	+++
ModD33N, Mod(mdg4)$^{1-273}$	−
ModH46D, Mod(mdg4)$^{1-273}$	−
ModD33N/H46D, Mod(mdg4)$^{1-273}$	+
ModD33N/R47Q, Mod(mdg4)$^{1-273}$	+
Mod(mdg4)^{GAF}, Mod(mdg4)$^{1-273}$	++
ModR47Q, Mod(mdg4)$^{1-273}$	+++
ModR47Q$^{1-273}$, ModR47Q$^{1-273}$	+++
ModR47Q$^{1-273}$, Mod(mdg4)$^{1-273}$	+++
ModD33N/H46D$^{1-273}$, ModD33N/H46D$^{1-273}$	−
ModD33N/H46D$^{1-273}$, Mod(mdg4)$^{1-273}$	+
ModD33N/R47Q$^{1-273}$, ModD33N/R47Q$^{1-273}$	−
ModD33N/R47Q$^{1-273}$, Mod(mdg4)$^{1-273}$	+
Mod(mdg4)$^{234-610}$, Mod(mdg4)$^{234-610}$	++
Mod(mdg4)$^{317-610}$, Mod(mdg4)$^{317-610}$	++
Mod(mdg4)$^{390-610}$, Mod(mdg4)$^{390-610}$	−
Mod(mdg4)$^{\Delta DD}$, Su(Hw)^{MID}	+++
ModD33N/H46D$^{\Delta DD}$, Su(Hw)^{MID}	+++
Mod(mdg4)$^{\Delta DD}$, Mod(mdg4)$^{\Delta DD}$	+++
ModD33N/H46D$^{\Delta DD}$, Mod(mdg4)$^{\Delta DD}$	+
ModD33N/H46D$^{\Delta DD}$, Mod(mdg4)	+
ModD33N/H46D$^{\Delta DD}$, ModD33N/H46D$^{\Delta DD}$	−

[a] The relative strength of the two-hybrid interaction was similar in both directions. The GAL4AD was on the C terminus of the fused proteins. No growth occurred after transformation with single plasmids, indicating that interactions between the proteins are required for expression of the reporter genes (data not shown). Equivalent expression of the chimeric proteins in yeast was confirmed by immunoblotting with GAL4 BD or AD monoclonal antibodies (Fig. 2A and data not shown). Designations are as defined for Table 1.

yeast two-hybrid assay does not discriminate between homodimerization and multimerization. According to previous observations (33, 34), we expected that alterations in the BTB domain would compromise self-association. We tested this in two ways. First, we tested for interactions with full-length Mod(mdg4)-67.2. Second, we tested for homologous interactions between BTB domain mutants. Surprisingly, in these tests, we found that deletion or alteration of the BTB domain did not eliminate homodimerization, as previously reported (20). This difference may result from our use of the C-terminal fusion proteins, as, for example, we also failed to observe interactions when GAL4AD was fused to the N-terminal part of the Mod(mdg4)$^{\Delta BTB}$ protein. As in the case of Mod(mdg4)$^{\Delta BTB}$, most Mod(mdg4) mutants supported yeast growth on selective plates when the GAL4AD domain was on the C terminus of the fusion protein. For ModH46D, ModD33N/H46D, and ModD33N/R47Q, growth on the selective plates was weaker, suggesting that these proteins were able to self-associate with lower efficiency. Only ModD33N lost the ability to self-associate in the two hybrid assay. Thus, the properties of ModD33N contrast with those of Mod(mdg4)$^{\Delta BTB}$. These results might be explained by unfolding of the mutant BTB domain that reduces the ability of the ModD33N protein to self-associate. Indeed, a similar D33N mutation in PLZF and BCL-6 BTB domains led to unfolding of the proteins (33, 34).

The ability of several Mod(mdg4) proteins to self-associate was confirmed in vitro by native PAGE (Fig. 2B). Mod(mdg4)-67.2 was found both as monomer and dimer. The Mod(mdg4)$^{\Delta BTB}$, ModD33N/H46D, ModR47Q, ModD33N/R47Q, and Mod(mdg4)^{Gaf} proteins were detected in the same two forms. Thus, all tested Mod(mdg4) mutants preserved the ability to self-associate in vitro, confirming the results obtained by the yeast two-hybrid method.

Finally, we confirmed, by the GST pull-down assay, interactions of Mod(mdg4)-67.2 with Mod(mdg4)$^{\Delta BTB}$, ModD33N/R47Q, and ModR47Q mutants (Fig. 2C). Equal amounts of purified GST or GST fused to full-length Mod(mdg4)-67.2 were mixed with purified recombinant mutant Mod(mdg4) proteins fused to a histidine tag. All mutant forms of Mod(mdg4) were retained on the glutathione beads when incubated with the GST-Mod(mdg4)-67.2. The lack of binding to GST alone confirmed the specificity of the interaction with Mod(mdg4)-67.2.

Mod(mdg4)-67.2 contains a second homodimerization domain. As Mod(mdg4)$^{\Delta BTB}$ is able to self-associate, we tried next to identify a second interaction domain in the Mod(mdg4)-67.2 protein. Mod(mdg4)-67.2 contains a glutamine-rich (Q-rich) region (between 138 amino acids [aa] and 265 aa) in common with other Mod(mdg4) isoforms (3, 7). Glutamine-rich (Q) domains are frequently involved in homodimerization (46). To examine an involvement of the Q domain in self-association of the Mod(mdg4) protein, we made Mod(mdg4)$^{\Delta Q}$ that contains a deletion extending from 145 aa to 277 aa and Mod(mdg4)$^{1-273}$ that includes both the BTB and Q domains (Fig. 1A). In the yeast two-hybrid system, Mod(mdg4)$^{\Delta BTB}$ interacted with Mod(mdg4)$^{\Delta Q}$ but not with Mod(mdg4)$^{1-273}$ (Table 2). At the same time, Mod(mdg4)$^{1-273}$ was able to self-interact. These results suggest that the Q domain is not involved in self-association of the Mod(mdg4)-67.2 protein.

To define the region of the putative dimerization domain in Mod(mdg4)-67.2, we tested interactions between Mod(mdg4)$^{1-273}$ and various mutant Mod(mdg4) proteins (Table 2). Mod(mdg4)$^{1-273}$ interacted strongly with ModR47Q and Mod(mdg4)^{Gaf}, interacted weakly with ModD33N/H46D and ModD33N/R47Q, and did not interact with ModD33N and ModH/46D. Next, we determined whether BTB mutants that were truncated would retain the ability to self-associate. We constructed derivatives of ModD33N/H46D, ModD33N/R47Q, and ModR47Q that contain 273 aa (Table 2). As expected, ModR47Q$^{1-273}$ showed self-association, as this mutation retained a functional BTB. In contrast, ModD33N/H46D$^{1-273}$ and ModD33N/R47Q$^{1-273}$ lost the ability to self-associate while retaining the ability to weakly interact with Mod(mdg4)$^{1-273}$. These results suggest that the C-terminal part of Mod(mdg4)-67.2 is essential for the interaction with Mod(mdg4) mutants.

To refine the C-terminal domain required for self-association, we made three N-terminally truncated versions of the Mod(mdg4)-67.2 protein: Mod(mdg4)$^{234-610}$, Mod(mdg4)$^{317-610}$, and Mod(mdg4)$^{390-610}$ (Fig. 1). In the yeast two-hybrid system, only Mod(mdg4)$^{234-610}$ and Mod(mdg4)$^{317-610}$ proteins show growth on the selective media, indicating that these proteins are able to self-associate (Table 3). Mod(mdg4)$^{390-610}$ was unable to self-associate, implying that at least a part of a putative dimerization domain, named DD, might be located between positions 317 and 390.

To test the role of the DD domain in self-association of Mod(mdg4)-67.2, we made the Mod(mdg4)$^{\Delta DD}$ (Fig. 1A) and D33N/H46D$^{\Delta DD}$ mutants carrying a deletion between posi-

TABLE 3. Summary of results obtained with the Mod(mdg4) mutants[a]

Protein	Dimerization	Enhancer blocking	Transcription stimulation	Binding to chromosomes	Speckle pattern	Interaction with CP190
Mod(mdg4)-67.2	+	+	+	+	+	+
Mod(mdg4)$^{\Delta BTB}$	+/−	−	−	−	−	−
Mod(mdg4)$^{\Delta DD}$	+	+	+	+	+	+
Mod(mdg4)^{GAF}	+	−	−	−	+/−	+
ModD33N	−	−	−	−	−	−
ModH46D	+/−	−	−	−	−	−
ModR47Q	+	+	+	+	+	+
ModD33N/R47Q	+/−	+/−	+/−	+	+/−	+/−
ModD33N/H46D	+/−	+	+	+	+	+/−
ModD33N/H46D$^{\Delta DD}$	−	−	−	−	−	+/−

[a] +, wild-type level of functionality [like Mod(mdg4)-67.2]; +/−, weak functionality; −, almost no functionality. The enhancer blocking mediated by the Mod(mdg4) mutants was examined with the aid of the y^2 sc^{D1}, sc^{ms}, ct^6, and ct^k alleles (Fig. 4; see also Table S1 in the supplemental material). Transcription stimulation was examined with aid of the y^2 (*yellow* expression in bristles) (Fig. 4) and sc^{v2} (see Table S1 in the supplemental material) alleles.

tions 301 and 389. In the yeast two-hybrid system, both proteins efficiently interacted with Su(Hw)^{MID} (Table 2). Mod(mdg4)$^{\Delta DD}$ was able to self-associate, most likely because the BTB domain is intact (Table 2). In contrast, the D33N/H46D$^{\Delta DD}$ protein does not show interaction. Like ModD33N/H46D$^{1-273}$, D33N/H46D$^{\Delta DD}$ showed poor growth on selective plates, which indicated a weak interaction with Mod(mdg4) and Mod(mdg4)$^{\Delta DD}$. These results support the proposal that the DD plays a critical role in self-association of D33N/H46D.

Genetic systems used to study the Mod(mdg4) activities. To determine the in vivo effects of mutations in the BTB domain, we used three gypsy-induced alleles in the *yellow*, *scute*, and *cut* loci. Two activities were demonstrated for the Mod(mdg4)-67.2 protein interacting with Su(Hw): participation in enhancer blocking and transcription stimulation. The features of these model systems are detailed below.

The first system we used involved the *yellow* gene. In the y^2 mutation (Fig. 3A), a *gypsy* element is inserted between the *yellow* promoter and the enhancers controlling *yellow* expression in the wing and body (17). As a result, males hemizygous for the y^2 allele show brown abdominal pigmentation in the fifth and sixth abdominal segments instead of the black pigmentation observed in wild-type males (Fig. 4). These effects are caused by the *gypsy* insulator that blocks the wing and body enhancers but not the bristle enhancer that is located in the *yellow* intron (17, 18). The *mod*(*mdg4*)^{u1} and *mod*(*mdg4*)^{T6} mutations enhance the mutant y^2 phenotype by repressing *yellow* expression in bristles and other derivative cuticle structures (14, 15). At the same time, *mod*(*mdg4*)^{u1} causes a partial loss of the enhancer-blocking activity of the *gypsy* insulator. In the *mod*(*mdg4*)^{u1} background, y^2 males display a variegated cuticular phenotype resulting from different expression levels of the *yellow* gene in adjacent groups of cells in the abdominal segments (16). In some cuticle cells, the effect of the *gypsy* insulator is reversed, resulting in normal expression of the *yellow* gene; in other cells, the effect of the *gypsy* insulator is enhanced due to direct repression of *yellow*. Thus, in the y^2 allele, binding of the Mod(mdg4)-67.2 protein strengthens the enhancer-blocking activity of the *gypsy* insulator and prevents repression of the *yellow* promoter.

The second model system (Fig. 3B) utilized in our studies involved genes in the Achaete-Scute complex (AS-C), located adjacent to the *yellow* gene (5). The expression of the *ac* and *sc* genes is confined to the proneural clusters that determine the precise positions of macrochaetae (36). A complex pattern of *ac* and *sc* expression is mediated by the action of site-specific enhancer-like elements distributed over about 90 kb of the AS-C cluster (5, 36). Several AS-C alleles were tested. The sc^{D1} mutation is caused by an insertion of *gypsy* 20 kb downstream of the *sc* gene (5) that blocks the communication between many bristle-specific enhancers and the *sc* promoter. The *mod*(*mdg4*)^{u1} mutation only partially suppresses the *sc* mutant phenotype, suggesting that Mod(mdg4)-67.2 is not critical for the block of the *sc* enhancers by the *gypsy* insulator. Recently, the first endogenous insulator 1A-2, containing two Su(Hw) binding sites, was found to separate the *yellow* gene from the AS-C (21, 39). In the sc^{ms1} and sc^{ms2} mutants (Fig. 3B), the 1A-2 insulator was duplicated between the *sc* gene and its enhancers (21). In contrast to its effects on the sc^{D1} allele, *mod*(*mdg4*)^{u1} almost completely suppresses the mutant phenotype of the sc^{ms} alleles. Finally, we used the *In*(*1*)sc^{v2} mutation (Fig. 3B), which carries an inversion with one breakpoint located very close to the 3′ end of the *ac* coding region and a second in centric heterochromatin (5). Despite the close proximity to centric heterochromatin, *In*(*1*)sc^{v2} causes only a weak mutant phenotype (5). However, in the *mod*(*mdg4*)^{u1} background, this inversion strongly enhances *ac* and *sc* phenotypes, suggesting that the Mod(mdg4)-67.2 protein blocks heterochromatin-mediated repression. Thus, the AS-C mutations allow us to test the ability of mutant Mod(mdg4) proteins to function at the *gypsy* insulator as well as an endogenous Su(Hw) insulator.

The third genetic model system is the *cut* locus, where ct^6 and ct^K are two *gypsy*-induced alleles. In the ct^6 allele, a *gypsy* element is inserted close to and completely blocks a wing margin enhancer located nearly 85 kb upstream of the *cut* promoter (12, 26), producing a cut wing phenotype (Fig. 4). The *mod*(*mdg4*)^{u1} and *mod*(*mdg4*)^{T6} mutations almost completely suppress the ct^6 mutant phenotype, suggesting that Mod(mdg4)-67.2 is essential for blocking the wing enhancer (Fig. 4). In the ct^K allele, the wing margin enhancer of *cut* is only partially blocked, resulting in an intermediate cut wing phenotype (Fig. 4), presumably because the inserted *gypsy* contains fewer Su(Hw)-binding sites than most *gypsy* elements (25). The ct^K mutant phenotype is more sensitive to the levels of Su(Hw) and Mod(mdg4)-67.2 activity than most *gypsy* inser-

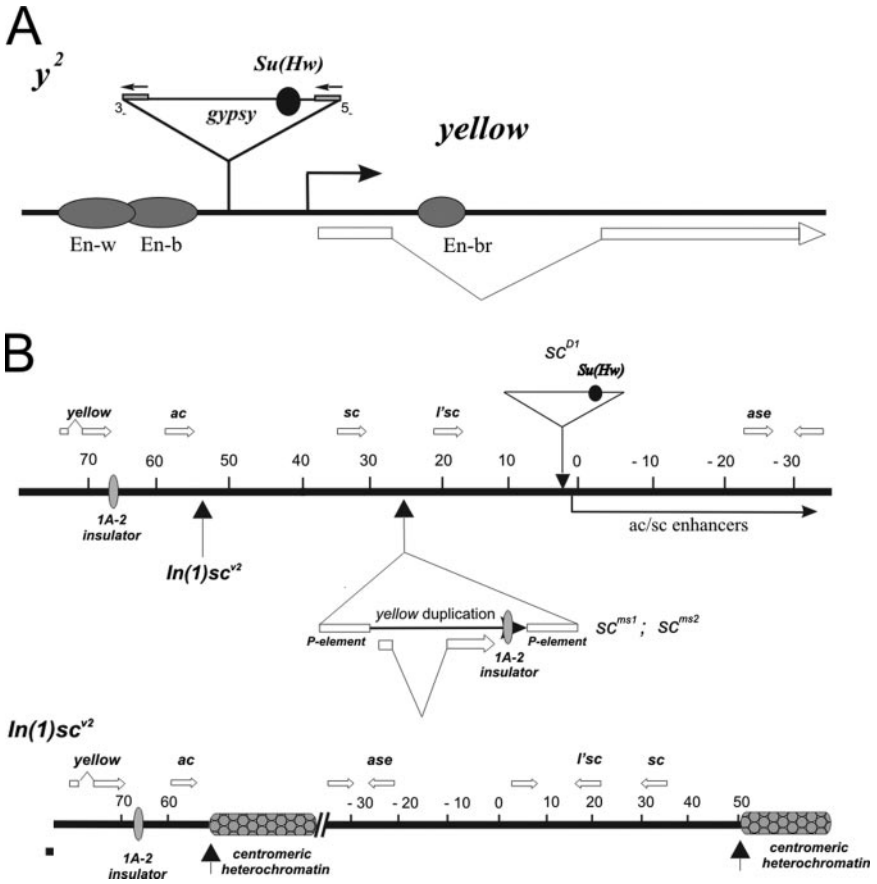

FIG. 3. (A) Structure of the *yellow* locus in wild-type and y^2 alleles. Exons of the *yellow* gene are represented by white rectangles, and filled ovals represent various tissue-specific transcriptional enhancers that control *yellow* gene expression in the respective tissues. The *gypsy* insertion responsible for the y^2 allele is represented by a triangle. Closed boxes flanking *gypsy* represent the long terminal repeats. The Su(Hw) insulator is represented by a closed circle located in the 5′ transcribed untranslated region of *gypsy*. (B) Schematic representation of the *yellow*/*ac*/*sc* region in the sc^{D1}, sc^{ms}, and $In(I)sc^{V2}$ mutations (5, 21). The coordinates of the AS-C region are as defined in Campuzano et al. (5). Vertical arrows indicate the position of chromosomal breakpoints associated with the $In(I)sc^{v2}$ mutation. Arrows with a triangle show insertions of *gypsy* (sc^{D1}) and of *P* elements (sc^{ms}) associated with duplication of the *yellow* sequences. Thick horizontal white arrows show the positions and direction of *yellow* and AS-C genes transcripts. The filled oval indicates the endogenous 1A-2 insulator and the Su(Hw) binding sites in *gypsy*.

tions and is almost completely suppressed by heterozygous *su*(*Hw*) mutations or *mod*(*mdg4*)^{u1} (12). Thus, the ct^K allele provides a sensitive assay to examine the ability of the Mod(mdg4) mutant proteins to support the blocking of the wing enhancer.

Role of the BTB and DD domains in insulation and transcription stimulation mediated by the Mod(mdg4)-67.2 protein. We constructed transgenic flies bearing the mutant Mod(mdg4) transgenes under the control of two promoters, the inducible *hsp70* promoter [Mod(mdg4)-67.2; ModH46D and ModD33N/H46D] or the ubiquitously expressed *su*(*Hw*) promoter [Mod(mdg4)-67.2; Mod(mdg4)$^{\Delta BTB}$, Mod(mdg4)$^{\Delta DD}$ ModD33N/H46D, ModD33N/H46D$^{\Delta DD}$, ModR47Q, ModD33N, ModD33N/R47Q, and Mod(mdg4)^{Gaf}]. The activities of these mutant Mod(mdg4) proteins were assessed in *mod*(*mdg4*)^{u1} and *mod*(*mdg4*)^{T6} mutant backgrounds. At least eight independent transgenic lines were examined for rescue in both backgrounds by each Mod(mdg4) mutant to exclude position effects. If expression of the transgene did not rescue the mutant *mod*(*mdg4*)^{u1} phenotype, then we established lines that contained either two or three copies of the homozygous transgenes to reveal weak phenotypic effects. These studies revealed similar results with both mutant backgrounds. For this reason, we refer only to the *mod*(*mdg4*)^{u1} mutation in the subsequent text.

In 14 lines, expression of the Mod(mdg4)67-2 transgene under control of either the *hsp70* or *Su*(*Hw*) promoter completely reverted the phenotypes associated with the *mod*(*mdg4*)^{u1} mutation (Fig. 4 and Table 3; see also Fig. S1 in the supplemental material). Thus, either promoter produces sufficient levels of functional protein to complement the loss of the Mod(mdg4)-67.2 protein. Interestingly, expression of Mod(mdg4) carrying a deletion of the BTB domain failed to rescue the mutant phenotypes, indicating a complete inactivation of the protein. These data confirm the essential role of Mod(mdg4)67.2 BTB domain.

We examined the function of proteins bearing point mutations that affect the integrity of the charge pocket of the BTB domain (Fig. 4 and Table 3; see also Fig. S1 in the supplemental material). The ModH46D protein has a substitution in the most conserved residue of the BTB domain that changes the uncharged histidine (47) to the negatively charged aspartic acid (H46D). This mutation almost completely inactivated all

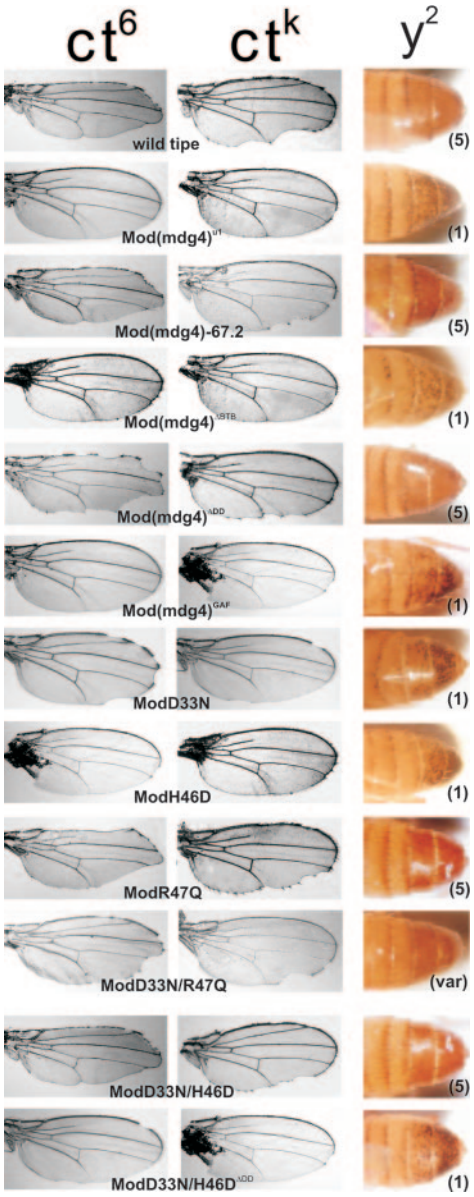

FIG. 4. Phenotypic effects of the mutant Mod(mdg4) proteins. Effects of the Mod(mdg4) mutants on the cut wing phenotype in the ct^K and ct^6 alleles and on abdomens of 3-day-old males. Mod (mdg4)-67.2 and the mutant Mod(mdg4) proteins were expressed in ct^6 or ct^K or y^2; $mod(mdg4)^{u1}/mod(mdg4)^{u1}$ flies. Figures in parentheses indicate the levels of *yellow* expression in bristles, as follows: 1, loss of pigmentation in all bristles; var, parts of bristles are pigmented; 5, pigmentation of all bristles like in wild-type flies.

insulator functions. In contrast, the ModR47Q mutant manifested the same levels of activity as the wild-type Mod(mdg4)-67.2 protein. Neutralization of a negative charge in the BTB pocket of ModD33N resulted in almost complete loss of the insulating and antirepression activities. ModD33N only slightly rescued the enhancer-blocking activity of the *gypsy* insulator in the ct^6 allele but was unable to restore enhancer blocking in sc^{ms}, sc^{D1}, and $In(1)sc^{V2}$ mutants and ct^K mutations. However, the loss of negative charge at one position in the pocket can be compensated by creation of a negative charge elsewhere in the pocket. For example, the ModD33N/H46D mutant had near wild-type function. These results suggest that inversion of the two most conserved residues preserves the function of BTB.

Additionally, the double mutant ModD33N/R47Q showed moderate levels of insulator and antirepression activities. ModD33N/R47Q restored the enhancer blocking activity of the *gypsy* insulator in the ct^6 allele but did not significantly affect the mutant phenotype of the ct^K, sc^{D1}, and sc^{ms} alleles. Interestingly, sc^{D1} and sc^{ms} depend upon an endogenous insulator whose enhancer blocking effects are only partially dependent on the Su(Hw) and Mod(mdg4) proteins. ModD33N/R47Q reversed the variegation of abdominal pigmentation in the y^2 flies but only partially suppressed inhibition of *yellow* expression in bristles. Finally, ModD33N/R47Q only slightly suppressed the heterochromatin-mediated repression in $In(1)sc^{V2}$ flies.

Taken together, our studies suggest that the charge pocket is important in Mod(mdg4) function. However, the absolute charge may not be the critical factor determining activity. The most severe effects on insulator function were observed when a conserved negative amino acid (D33N) was changed, whereas neutralizing a positive charge (R47Q) had little effect. Combining the D33N mutation with changes in positively charged residues restored function to a degree dependent upon the identity of the amino acid. Based on these observations, we propose that residues in the charge pocket may make specific contributions to the function of the BTB domain.

We were interested in determining the role of the newly discovered DD in *gypsy* insulator activities mediated by Mod(mdg4)-67.2 (Fig. 4 and Table 3; see also Fig. S1 in the supplemental material). Expression of $Mod(mdg4)^{\Delta DD}$ completely reverted the phenotypes associated with the $mod(mdg4)^{u1}$ mutation, suggesting that DD is not essential for the Mod(mdg4)-67.2 function. In contrast, if the DD was deleted in mutants that affect the BTB domain, such as $ModD33N/H46D^{\Delta DD}$, then there was a complete loss of function. These results suggest that the DD domain is essential when the mutant BTB domain partially loses the ability to dimerize.

Models of insulator function propose that the Mod(mdg4) BTB domain plays an important role in directing protein interactions with other BTB proteins to form looped chromatin domains (6). Based on this postulate, we predicted that the BTB domain of Mod(mdg4) could be replaced with another BTB domain and retain insulator function. To test this prediction, we chose the related BTB domain of GAF (41). We found that the $Mod(mdg4)^{Gaf}$ protein was completely inactive in all assays, suggesting that the GAF BTB domain does not substitute for the Mod(mdg4) BTB (Fig. 4 and Table 3; see also Fig. S1 in the supplemental material). We propose that these effects are not due to the inability of the BTB swap protein to fold properly, as $Mod(mdg4)^{Gaf}$ formed homodimers and interacted with the Su(Hw) protein in the yeast two-hybrid system. These data imply that the Mod(mdg4) BTB domain mediates specific interactions with unidentified protein complexes required for the functional potency of Mod(mdg4)-67.2.

Localization of the Mod(mdg4) mutants on polytene chromosomes and in diploid cells. To further investigate the mechanism associated with the loss of insulator function among the BTB domain mutants, we examined the chromosome associa-

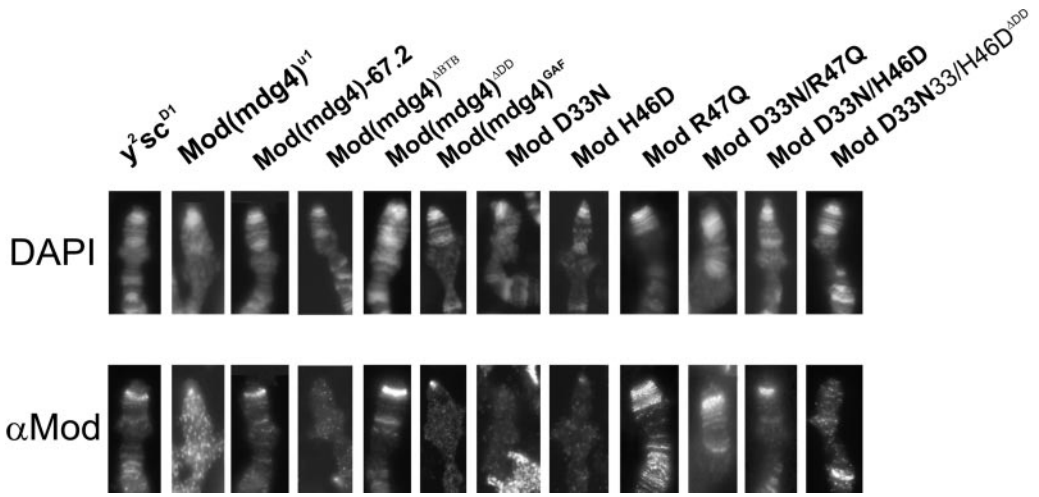

FIG. 5. Localization of mutant Mod(mdg4) proteins on polytene chromosomes of the y^2sc^{D1}; $mod(mdg4)^{u1}/mod(mdg4)^{u1}$ line. Salivary glands were dissected from third-instar larvae. The upper panel shows 4′,6′-diamidino-2-phenylindole (DAPI)-stained chromosomes. The lower panel represents the localization of Mod(mdg4) on polytene chromosomes; the white arrows indicate the Mod(mdg4) bands. The mutant Mod(mdg4) proteins were detected using a polyclonal antibody against the Mod(mdg4)-67.2 isoform and Cy3-conjugated secondary antibodies.

tion of these proteins. First, we determined whether these mutant proteins were bound to the *gypsy* transposon present in the y^2 and sc^{D1} genes using a polytene chromosome assay. We used antibodies raised against the unique C-terminal domain of Mod(mdg4)-67.2 to specifically detect the insulator protein. In flies wild type for Mod(mdg4)-67.2, the sites corresponding to *gypsy* insertions in the y^2 and sc^{D1} mutations bind this protein, as demonstrated by strong bands of protein localization at the tip of the X chromosome (Fig. 5). In $mod(mdg4)^{u1}$ flies expressing Mod(mdg4)-67.2, ModR47Q, ModD33N/R47Q, ModD33N/H46D, and Mod(mdg4)$^{\Delta DD}$, immunolocalization was similar to $mod(mdg4)^{+}$ flies (Fig. 5), confirming that these proteins interact with Su(Hw). Proteins lacking insulator function did not associate with chromosomes, including Mod(mdg4)$^{\Delta BTB}$, ModH46D, ModD33N, Mod(mdg4)^{Gaf}, and ModD33N/H46D$^{\Delta DD}$.

In a second assay, we determined whether mutant Mod(mdg4) proteins reconstituted nuclear speckles that represent a coalescence of the Su(Hw)-Mod(mdg4) protein complexes in diploid nuclei (Fig. 6). Previous studies suggest that formation of these speckles correlates with insulator function (16, 38). In the transgenic lines, Mod(mdg4)-67.2, ModR47Q, ModD33N/H46D, and Mod(mdg4)^{DDD} proteins displayed a wild-type punctate pattern (Fig. 6A). We also found that the Mod(mdg4)-67.2, ModD33N/H46D, and Mod(mdg4)$^{\Delta DD}$ proteins colocalize with the Su(Hw) protein (Fig. 6B), confirming that the interaction between these proteins is nuclear. The Mod(mdg4)^{Gaf} protein produced a more diffuse pattern with many fuzzy speckles. The Su(Hw) and Mod(mdg4)^{Gaf} proteins displayed only partial colocalization on the nuclear periphery. Other mutant proteins, Mod(mdg4)$^{\Delta BTB}$, ModD33N/R47Q, ModH46D, and ModD33N, showed diffuse nuclear localization predominantly at the nuclear periphery (Fig. 6A). Interestingly, ModD33N/H46D$^{\Delta DD}$ displayed unusual patterns: it formed spackles at the nuclear periphery and cytoplasm. The Su(Hw) protein only occasionally colocalizes with ModD33N/R47Q or ModD33N/H46D$^{\Delta DD}$ aggregates, suggesting that these Mod(mdg4) mutants and Su(Hw) failed to interact in nuclei.

Thus, functional inactivation of the BTB domain or deletion of the DD domain is directly correlated with the inability of the mutant protein to interact with Su(Hw) on polytene chromosomes and to form nuclear speckles.

The functionality of the mutant Mod(mdg4) proteins does not correlate with their ability to interact with CP190. A second insulator protein that interacts with Mod(mdg4)-67.2 is CP190 (38). If this interaction is critical for the Mod(mdg4)-67.2 activity, then the function of the mutant Mod(mdg4) proteins might be directly correlated with an ability to interact with CP190. To test this assumption, we determined whether the mutant Mod(mdg4) proteins interacted with CP190 in the yeast two-hybrid system.

Pai et al. (38) reported that CP190 fails to interact with itself but associates with Mod(mdg4)-67.2 in the yeast two-hybrid assay. This unexpected finding might be explained if CP190 showed the same orientation effects described for Mod(mdg4)67.2. For this reason, we tested interactions using C-terminal fusions of the GALAD. When GAL4AD was fused to the C terminus of CP190 (CP190-GAL4AD), a weak interaction was observed (data not shown). Further tests indicate that the C-terminal part of CP190 negatively influenced the activity of the fused GAL4AD, complicating the interpretation of these results. Tests of a C-terminally truncated CP190$^{1-765}$ coexpressed with the full-length GAL4BD-CP190 supported strong interaction (Table 4). As deletion of the C-terminal acidic domain of CP190 does not significantly influence function (38), we used the CP190$^{1-765}$-GALAD protein in further experiments.

Each of the Mod(mdg4) mutants was tested in the yeast two-hybrid assay for its ability to interact with CP190 (Table 4). CP190 was unable to interact with Mod(mdg4)$^{\Delta BTB}$, ModH46D, and ModD33N and displayed weak interactions with ModD33N/R47Q, ModD33N/H46D, and ModD33N/H46D$^{\Delta DD}$ and strong interactions with Mod(mdg4), ModR47Q, Mod(mdg4)$^{\Delta DD}$, and

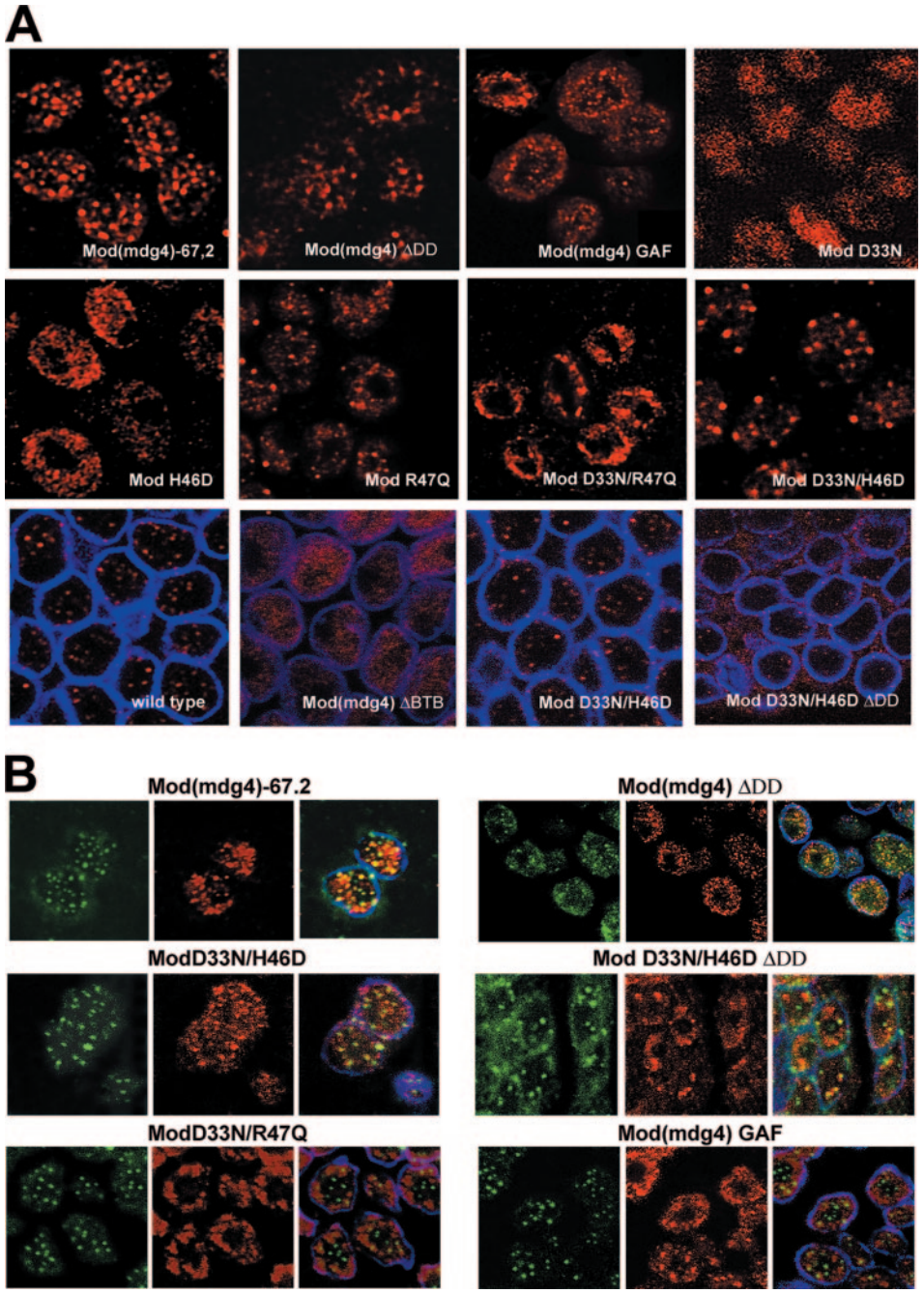

FIG. 6. Immunolocalization of insulator proteins in diploid cells of larval imaginal discs. (A) Localization of Mod(mdg4)-67.2 and the mutant Mod(mdg4) proteins. (B) Colocalization of Su(Hw) and Mod(mdg4) proteins. Interphase diploid cells were obtained from imaginal discs of y^2sc^{D1}; $mod(mdg4)^{u1}/mod(mdg4)^{u1}$ lines and y^2sc^{D1}; $mod(mdg4)^{u1}/mod(mdg4)^{u1}$ lines expressing Mod(mdg4)-67.2 or one of the mutant Mod(mdg4) proteins. The distribution of proteins was detected with polyclonal antibody against the Mod(mdg4)-67.2 isoform (red), anti-lamin (blue), and anti-Su(Hw) (green) antibodies.

$Mod(mdg4)^{Gaf}$. Interestingly, $Mod(mdg4)^{Gaf}$ and Mod(mdg4)-67.2 interact with CP190 with similar efficiency (Table 4). Pai et al. (38) showed that GAF did not interact with CP190 in the yeast two-hybrid assay, a finding supported in our studies (Table 4). Thus, these data suggest that Mod(mdg4)-67.2 interacts with CP190 in a BTB-independent manner. Further, the observation that $Mod(mdg4)^{Gaf}$ lacks insulator properties suggests that the functionality of Mod(mdg4) mutants is not directly correlated with their ability to interact with CP190.

DISCUSSION

The BTB domain is a versatile protein-protein interaction motif that participates in a wide range of cellular functions,

TABLE 4. Summary of interactions with CP190[a]

Interacting proteins	Strength of interaction
CP190, CP190	+++
CP190, Mod(mdg4)	+++
CP190, ModR47Q	+++
CP190, Mod(mdg4)$^{\Delta DD}$	+++
CP190, Mod(mdg4)$^{\Delta BTB}$	−
CP190, ModH46D	−
CP190, ModD33N	−
CP190, ModD33N/R47Q	+
CP190, ModD33N/H46D	+
CP190, ModD33N/H46D$^{\Delta DD}$	+
CP190, Mod(mdg4)^{GAF}	+++
CP190, GAF	−

[a] The GAL4AD was on the C terminus of the fused proteins. The GAL4BD domain was fused to full-length CP190, while GAL4AD was fused to the C terminus of the truncated CP190$^{1-765}$ protein. The relative strength of the two-hybrid interaction was similar in both directions. No growth occurred after transformation with single plasmids, indicating that interactions between the proteins are required for expression of the reporter genes (data not shown). Equivalent expression of the chimeric proteins in yeast was confirmed by immunoblotting with GAL4 BD or AD monoclonal antibodies (Fig. 2A and data not shown). Designations are as defined for Table 1.

including transcriptional regulation, cytoskeleton dynamics, ion channel assembly and targeting proteins for ubiquitination (43). Here we demonstrated that the integrity of the BTB domain is critical for the Mod(mdg4)-67.2 activity in insulation.

Melnick et al. (33, 34) showed that the alignment of charged residues within the BTB pocket of the PLZF and Bcl-6 proteins was required for autonomous transcriptional repression by the homodimers. When two conserved charged residues in the pocket, D35 and R49, were switched to polar amino acids, the full-length PLZF or Bcl-6 proteins harboring the double mutant BTB domain were severely impaired for transcriptional repression yet could still oligomerize and localize to characteristic nuclear speckles. Here we demonstrate that the Mod(mdg4)-67.2 protein carrying mutations in analogous residues in the BTB domain loses much of its function as a part of the *gypsy* and 1A2 insulators. Elimination of a negative charge in the pocket by the single alteration D33N almost completely compromised the integrity of the BTB domain of Mod(mdg4)-67.2 and inactivated its function, suggesting that the net charge may be important for proper folding of the BTB domain. The opposite charge alteration, replacing a positive charge with a neutral amino acid (R47Q) had a minor effect, suggesting that the overall charge may be less important than the identity of the residue changed. The substitution of the most conserved H46 with a negatively charged aspartic acid (D) completely inactivated Mod(mdg4)-67.2. While H46 is not involved in formation of the pocket structure, the additional D33N substitution that reestablishes the overall negative charge in the pocket of the ModD33N/H46D double mutant restores Mod(mdg4)-67.2 insulator function, even though the D33N/H46D BTB domain partially loses the ability to dimerize. Taken together, we predict that the net charge is essential for proper folding of the BTB domain.

Here we found that Mod(mdg4)-67.2 contains a second domain involved in homodimerization of the protein. When the BTB domain partially lost the ability to dimerize, as in the mutant ModD33N/H46D protein, the second domain directed proper nuclear localization and formation of the insulator complex on the DNA. Thus, the DD domain contributes to dimerization of Mod(mdg4)-67.2 that is essential for insulator function.

Interestingly, we found that interaction between the Mod(mdg4)-67.2 and GAF proteins requires both the BTB domain and a second protein interaction domain (35). We show that the specificity of the interaction between Mod(mdg4)-67.2 and CP190 is determined by an unidentified domain beside BTB. Based on these findings, we suggest that interactions between BTB-containing proteins may be supported by additional protein-protein interaction domains.

Mod(mdg4)-67.2 facilitates binding of Su(Hw) to insulator sequences in vivo (16). Here we found that inactivation of the BTB domain by point mutations like H46D, D33N, or BTB deletion render the mutant Mod(mdg4) protein unable to associate with polytene chromosomes. These proteins also only partially colocalize with the Su(Hw) protein. As these mutant proteins still interact with the Su(Hw) protein in the yeast two-hybrid assay, we postulate that specific interactions mediated by the BTB domain of Mod(mdg4) with unidentified protein(s) are required for efficient recruitment of the Su(Hw)/Mod(mdg4) complex to the insulator sequences and nuclear bodies formed by the insulator proteins. This model is supported by the inability of the chimeric Mod(mdg4)^{Gaf} protein, containing the GAF BTB domain, to interact with Su(Hw) binding sites in vivo and extensively form the nuclear speckles with Su(Hw), while in two-hybrid assays Mod(mdg4)^{Gaf} interacts with Su(Hw) with the same efficiency as Mod(mdg4)-67.2. These data imply that the interaction with Su(Hw) is not sufficient to recruit Mod(mdg4) to Su(Hw) binding sites. Further, the interaction of Mod(mdg4)-67.2 with CP190 does not appear to be critical for the recruitment of Mod(mdg4)-67.2 to insulators. Based on these results, we speculate that an unidentified protein is required for recruiting the Su(Hw)-Mod(mdg4)-67.2 complex to the Su(Hw) insulator and propose that the BTB domain of the Mod(mdg4)-67.2 facilitates or directly interacts with this hypothetical protein.

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