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Enhancer Blocking and Transvection at the Drosophila *apterous* Locus

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ABSTRACT

Intra- and interchromosomal interactions have been implicated in a number of genetic phenomena in diverse organisms, suggesting that the higher-order structural organization of chromosomes in the nucleus can have a profound impact on gene regulation. In Drosophila, homologous chromosomes remain paired in somatic tissues, allowing for *trans* interactions between genes and regulatory elements on the two homologs. One consequence of homolog pairing is the phenomenon of transvection, in which regulatory elements on one homolog can affect the expression of a gene in *trans*. We report a new instance of transvection at the Drosophila *apterous* (*ap*) locus. Two different insertions of boundary elements in the *ap* regulatory region were identified. The boundaries are inserted between the *ap* wing enhancer and the *ap* promoter and have highly penetrant wing defects typical of mutants in *ap*. When crossed to an *ap* promoter deletion, both boundary inserts exhibit the interallelic complementation characteristic of transvection. To confirm that transvection occurs at *ap*, we generated a deletion of the *ap* wing enhancer by FRT-mediated recombination. When the wing-enhancer deletion is crossed to the *ap* promoter deletion, strong transvection is observed. Interestingly, the two boundary elements, which are inserted ~10 kb apart, fail to block enhancer action when they are present in *trans* to one another. We demonstrate that this is unlikely to be due to insulator bypass. The transvection effects described here may provide insight into the role that boundary element pairing plays in enhancer blocking both in *cis* and in *trans*.

HIGHER-ORDER intrachromosomal and interchromosomal interactions play an important role in regulating gene expression. While such long-range regulatory interactions were first documented in Drosophila, recent studies indicate that they occur in many different organisms. For example, in mammalian cells, genes have been found to colocalize in a nonrandom fashion with one another and with RNA polymerase in transcription factories (Osborne *et al.* 2004). Intrachromosomal interactions have been found in the Igf2/H19 imprinting locus, as well as in the β-globin locus (Carter *et al.* 2002; Tolhuis *et al.* 2002; Murrell *et al.* 2004). Long-range interchromosomal interactions have also been detected between the Igf2/H19 imprinting locus and Wsb1/Nf1 (Ling *et al.* 2006), between the TH2 cytokine locus and the interferon-gamma gene (Spilianakis and Flavell 2004; Spilianakis *et al.* 2005), and between various olfactory receptor (OR) genes and the OR enhancer element (Lomvardas *et al.* 2006). In addition, chromosome pairing has been implicated in the regulation of many genetic phenomena in diverse organisms, such as paramutation in plants, X inactivation in mammals, and repeat-induced point mutation (RIP) in Neurospora (Wu and Morris 1999).

In Dipterans, such as Drosophila, somatic pairing between homologous chromosomes allows for cross talk between genes and regulatory elements on the two homologs (Stevens 1908; Metz 1916). To date, a number of *trans*-regulatory interactions that depend on chromosome pairing have been reported. For example, several types of pairing-dependent silencing have been observed, such as *trans* repression by the bw^D mutant and pairing-sensitive silencing mediated by polycomb response elements (PREs) (Henikoff and Dreesen 1989; Dreesen *et al.* 1991; Kassis 1994; Hagstrom *et al.* 1997; Müller *et al.* 1999; Sass and Henikoff 1999; Csink *et al.* 2002). Another pairing-dependent regulatory interaction is the phenomenon of transvection, in which regulatory elements such as enhancers or silencers on one homolog can affect the expression of a gene in *trans* (Duncan 2002). Transvection was first described by E. B. Lewis for the pairing-dependent complementation between bx^{34e} and Ubx^1, two alleles of the *Ultrabithorax* (*Ubx*) gene (Lewis 1954). Since then, transvection effects have been reported for over a dozen loci in Drosophila (reviewed in Wu and Morris 1999; Duncan 2002; Sipos and Gyurkovics 2005).

Detecting an instance of transvection genetically generally requires an allele that specifically affects the

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enhancer or regulatory element and a second allele that specifically affects the promoter or coding region. Transvection is most commonly observed as interallelic complementation between two such alleles. Since this special set of mutations is required to detect transvection, it is unknown exactly how prevalent transvection is in Drosophila. However, on the basis of known pairing frequencies of homologous loci (Golic and Golic 1996b; Vazquez *et al.* 2002; Lowenstein *et al.* 2004; Harmon and Sedat 2005) and the work of Chen *et al.* (2002), in which it was shown using a Cre and FLP-mediated transgene coplacement system that the Drosophila genome is generally permissive for transvection, it is likely that the dozen or so known instances of transvection represent only a small fraction of all *trans*-regulatory effects in Drosophila.

While mutations that inactivate regulatory elements can be used to uncover transvection effects, *trans*-regulatory interactions have also been detected when a boundary element is interposed between a regulatory element and its target promoter. Boundary elements, or insulators, are sequences that block the action of enhancers or silencers when interposed between the regulatory element and its cognate gene (Kellum and Schedl 1991, 1992; Mihaly *et al.* 1998; West *et al.* 2002). In fact, the bx^{34e} mutation that Lewis used to first demonstrate transvection in the bithorax complex (BX-C) is caused by a *gypsy* retrotransposon insertion that contains a boundary element (Peifer and Bender 1986).

The best-studied instance of transvection involving a boundary element is that observed at the *yellow* (*y*) locus. The y^2 allele is an insertion of the *gypsy* retrotransposon between the *y* gene and the *y* wing and body enhancers (Geyer *et al.* 1990; Morris *et al.* 1999a). The *gypsy* retrotransposon contains 12 degenerate binding sites for the Suppressor of Hairy-Wing (SuHw) protein, which are sufficient to function as a boundary element (Parkhurst *et al.* 1988; Spana *et al.* 1988; Geyer and Corces 1992). Both homozygous y^2 flies and y^2/Df flies have strong *yellow* phenotypes in their wings and bodies. However, when y^2 is crossed to a *y* promoter deletion ($y^{1\#8}$), the *trans*-heterozygotes have wild-type levels of *y* expression (Geyer *et al.* 1990). Studies of transvection at *y* have been particularly interesting not only because they have provided insight into the phenomenon of transvection, but also because they have been informative about the mechanism of insulator action (Morris *et al.* 1998; Golovnin *et al.* 2003; Parnell *et al.* 2003).

Here we describe a novel instance of transvection at the *apterous* (*ap*) locus. We show that two different boundary element insertions, one that contains the *suppressor of Hairy-wing* [*su(Hw)*] insulator and the other that contains the *Mcp* element, are able to block the activation of *ap* by the upstream wing enhancer. The *Mcp* element from the Drosophila BX-C (Karch *et al.* 1994; Müller *et al.* 1999) contains a separable boundary element and a PRE (Busturia *et al.* 2001; Gruzdeva *et al.* 2005), and, like the *su(Hw)* element, is able to mediate long-range pairing within or even between chromosomes (Sigrist and Pirrotta 1997; Müller *et al.* 1999; Vazquez *et al.* 2006). When crossed to an *ap* promoter deletion, both boundary inserts exhibit the interallelic complementation characteristic of transvection. We confirmed that transvection takes place at *ap* by testing for complementation between *ap* wing-enhancer and promoter deletions in the presence and absence of the *Mcp* and *su(Hw)* boundaries. We also present evidence that promoter tethering of the *ap* wing enhancer in *cis* occurs at *ap*, but to a lesser extent than that observed at the *y* locus. While both the *Mcp* and *su(Hw)* boundary elements can be bypassed by an enhancer in *trans*, the *trans* enhancer bypass does not occur when there is a second paired boundary on the other homolog. Interestingly, loss of boundary activity is observed when the two insulators are present in *trans* to one another. The loss of boundary activity is unlikely to be an instance of insulator bypass. Instead, we propose a transvection-based model to explain the unexpected complementation between the *Mcp* and *su(Hw)* inserts. These results are consistent with a model in which boundary element pairing functions to separate independent regulatory domains and in which pairing is integral to the mechanism of enhancer blocking. The transvection effects described here also provide insights into the conditions and chromosomal contexts that are permissive for insulator function and the role of chromosomal conformation/local chromosome topology in boundary function.

MATERIALS AND METHODS

Fly methods and stocks: Flies were grown on standard cornmeal agar. All crosses reported were carried out at 22°. ap^{f00451} (also known as PBac{WH}f00451), PBac{RB}e01573, and ap^{f08090} (PBac{WH}f08090), were obtained from the Exelixis stock collection at Harvard Medical School. ap^{UGO35} was generously provided by Stephen Cohen. ap^{UGO35} was created by imprecise excision of the enhancer trap insert ap^{rk568} (Cohen *et al.* 1992). ap^{rk568} is inserted 42 bp 5′ of the longest *ap* cDNA (Cohen *et al.* 1992). This places ap^{rk568} 23 bp 5′ of the annotated *ap* transcription start site (FlyBase). Published information suggests that the distal ap^{UGO35} breakpoint coincides with the ap^{rk568} insertion site. Thus, ap^{UGO35} likely deletes the *ap* transcription start site and parts of the promoter. $su(Hw)^v$, $su(Hw)^f$, and $mod(mdg4)^{u1}$ mutants were generously provided by Victor Corces. ap^4 [Bloomington (BL)#223], ap^{56f} (BL#4189), ap^{rk568} (BL#5374), Df(2R)nap1 (BL#1006), Df(2R)nap2 (BL#6386), P{*hsFLP*}12, y^1 w^* (BL#1929), TM6B, P{Crew}DH2, Tb^1 (BL#1501), w^{1118}; CyO, P{Tub-PBac\T}2/$wg^{Sp\text{-}1}$ (BL#8285), and P{w[+mC]=ActGFP} JMR1 (BL#4533) were all obtained from the Bloomington Drosophila Stock Center.

Construction of the Flipper 2 element: The construction of Flipper 2 (see Figure 1A) was a multi-step cloning procedure. Details can be obtained upon request. In brief, the backbone of Flipper 2 consists of the intronless *yellow* gene (referred to as Dint in Geyer and Corces 1987) cloned into the *P*-element

vector Carnegie 4 (Rubin and Spradling 1983). In this plasmid (from here on referred to as C4yellow), the wing and body-color enhancers are located 5′ of the *yellow* cDNA. It was kindly provided by Pam Geyer. The *mini-white* gene (Pirrotta 1988) was introduced into the *Sal*I- and *Xba*I-restricted C4yellow as an *Xho*I–*Xba*I fragment. The resulting plasmid is called pC4YM. This *P*-element vector contains unique *Xho*I and *Not*I sites on the 5′ side of the *mini-white* gene. These two sites were used to introduce an *Xho*I–*Not*I fragment consisting of two parts, one of which is the 661-bp *Nde*I–*Pst*I *bxd* element (Sigrist and Pirrotta 1997) flanked by FRT sites (Golic and Lindquist 1989). The FRT-*bxd*-FRT cassette was excised from plasmid pBSscriptII+FPREF, which was kindly provided by Christian Sigrist. The other part is the 2.9-kb *Eco*RI *Mcp* element (Müller *et al.* 1999) flanked by LoxP sites (Siegal and Hartl 1996). The orientation of *Mcp* is such that the end normally adjacent to *iab-4* is closer to FRT-*bxd*-FRT.

***P*-element-mediated transformation:** Flipper 2-containing transgenic lines were generated according to standard procedures (Spradling and Rubin 1982). DNA was co-injected along with the P-turbo helper plasmid into Df(1) w^{67c23}, y^- embryos. Transformants were detected by rescue of the *white*$^-$ eye-color phenotype and/or the rescue of the *yellow*$^-$ body-color and wing phenotypes. A total of 30 independent Flipper 2 lines, which will be described in more detail elsewhere (M. Müller, I. Hogga and V. Pirrotta, unpublished results), were established. One of these lines (isolation no. 81.38.2) was found to be inserted in the *apterous* gene and from here on will be referred to as $ap^{MM\text{-}Mcp\text{-}bxd}$. The *mini-white* reporter is dominantly suppressed in $ap^{MM\text{-}Mcp\text{-}bxd}$ flies and its derivatives, but the insert could be identified thanks to strong *yellow*$^+$ expression in the wings and variegated expression in the adult abdominal cuticle. In the abdominal cuticle, *yellow*$^+$ expression is dosage dependent. There is no sign of pairing-dependent silencing.

Deletion of *Mcp* and *bxd* from the Flipper 2 element: *yw*; $ap^{MM\text{-}Mcp\text{-}bxd}$/SM6a females were crossed with *yw*;TM6B P[w^+, *cre*]/MKRS, *hsFLP* males (Siegal and Hartl 1996; stock obtained from Francois Karch). The progeny of this cross were heat-shocked twice for 1 hr during late embryogenesis and the first instar larval stage. Among the emerging adults, *yw*; $ap^{MM\text{-}Mcp\text{-}bxd}$/+;TM6B P[$w^+$, *cre*]/+ and *yw*; $ap^{MM\text{-}Mcp\text{-}bxd}$/+; MKRS, *hsFLP*/+ males were collected and crossed with *yw*;*l(2)*/SM6a virgins. The progeny of these two crosses were screened for loss of *Mcp* or loss of *bxd*, respectively. On the basis of experience with other Flipper 2 transgenes, a change in the expression of the *yellow* and/or the *mini-white* reporter gene was expected. However, compared to $ap^{MM\text{-}Mcp\text{-}bxd}$/+ control flies, apart from a moderate increase in *yellow* expression on the abdomen, no striking differences were apparent. Therefore, a number of single putative *yw*;$ap^{MM\text{-}Mcp}$/SM6a and *yw*;$ap^{MM\text{-}bxd}$/SM6a males were selected and independent stocks were established. The presence of a deletion chromosome was confirmed with the following PCR reactions: $ap^{MM\text{-}bxd}$, primer 1 (mini-white 5′, AAGGCGGACATTGACG) and primer 2 (5328, TGGAGTACGAAATGCG). On an agarose gel, the loss of *Mcp* is accompanied by the change of a 4.5-kb band to a 1.3-kb band: $ap^{MM\text{-}Mcp}$, primer 1 (miniwhite 5′, see above) and primer 2 (Mcp22/7, CTTCCCTTTCCGAGCG). On an agarose gel, the loss of *bxd* is accompanied by the change of a 1.26-kb band to a 0.42-kb band.

ap^{MM} was made by deleting *bxd* from $ap^{MM\text{-}bxd}$. Briefly, 0- to 24-hr embryos from P{*hsFLP*}12, y^1 *w** (from BL#1929); $ap^{MM\text{-}bxd}$/CyO flies were collected in bottles, allowed to age for 24 hr, and then heat-shocked for 1.5 hr/day in a 38° water bath until the majority of larvae had formed pupae. Heat-shocked P{*hsFLP*}12, y^1 *w**; $ap^{MM\text{-}bxd}$/CyO females were collected and crossed to *yw*; *bTf*/CyO males and multiple stocks were established and screened for the loss of *bxd* by PCR. The following primers were used to confirm the deletion of *bxd* in ap^{MM}: primer 1 (8-2, TGTTCAGATGCTCGGCAGATGG) and primer 2 (PEP5′in, GTGACTGTGCGTTAGGTCCTGTT).

Determining the insertion site of $ap^{MM\text{-}Mcp\text{-}bxd}$ by inverse PCR: Inverse PCR was performed as previously described Bellen *et al.* (2004), with several minor modifications. Briefly, DNA was isolated from $ap^{MM\text{-}Mcp\text{-}bxd}$ flies. A total of 50 flies were frozen in liquid nitrogen, homogenized in lysis buffer (0.1 M Tris–HCl, pH 8.0, 0.4 M NaCl, 25 mM EDTA, 1% SDS), mixed with an equal volume of Tris-buffered phenol, and centrifuged to remove debris. The supernatant was then phenol–chloroform extracted three times, washed once with chloroform, ethanol precipitated, and resuspended in 100 μl dH_2O. After treatment with 10 μg of RNaseA for 1 hr at 37°, 10 μl of $ap^{MM\text{-}Mcp\text{-}bxd}$ DNA was digested with *Hin*P1I (New England Biolabs, Beverly, MA). The *Hin*P1I enzyme was heat inactivated by incubation at 65° for 30 min, and the sample was diluted 40-fold and ligated with T4 DNA ligase (New England Biolabs). The ligation reaction was carried out overnight at 4° to favor intramolecular ligation. DNA was isolated from the ligation reaction by ethanol precipitation and amplified by nested PCR using the following primers: 5′-end, PCR 1 (plac1, CACC CAAGGCTCTGCTCCCACAAT, and pwht1, GTAACGCTAAT CACTCCGAACAGGTCACA); 5′-end, PCR 2 (sp1, ACACAA CCTTTCCTCTCAACAA, and pwht1, see above); 3′-end, PCR 1 (pry1, CCTTAGCATGTCCGTGGGGTTTGAAT, and pry4, CAATCATATCGCTGTCTCACTCA); and 3′-end, PCR 2 (pry2, CTTGCCGACGGGACCACCTTATGTTATT, and pry1, see above).

The PCR products were excised from a gel and isolated using the QIAquick gel extraction kit (QIAGEN, Valencia, CA). The PCR products were then sequenced using the sp1 (5′) and pry2 (3′) primers. The insertion site of $ap^{MM\text{-}Mcp\text{-}bxd}$ was determined to be 403 bp upstream of the *apterous* transcriptional start site (*D. melanogaster* Genome Release 5.1 coordinates 1614738). The insertion site and orientation were confirmed by PCR and sequencing between sp1 and apPromR and between plac1 and apPromR:
apPromR, TGGTCTGCAGCTGATCTA.

Scoring the *apterous* wing phenotypes: In general, crosses were set up between five to six virgin females and three to four males. Two to three vials were set up in duplicate (for a total of four to six vials). These replicates were compared to calculate a standard deviation. Flies were allowed to lay eggs for 4 days and then the crosses were brooded into new vials. Wing phenotypes were scored each day, until all of the flies in each vial had eclosed. Individual wings were given a score from 1 to 5 on the basis of the severity of the wing defect (for representative wings, see Figure 1C). Wings that were wild type or that had only very minor bristle or wing-vein defects were scored as class 1. Wings with mild-to-severe notching were scored as class 2. Wings that were of approximately normal size, but were blistered or crumpled, were scored as class 3. Wings that were significantly reduced in size or were strap-like in appearance were scored as class 4. Finally, when little or no wing tissue was present, this was scored as class 5. All graphs depict the mean percentage of wings in each of the five classes. Error bars in each graph represent one standard deviation from the mean. Wing specimens shown in the various figures were dissected in 95% ethanol and mounted in Hoyer's medium. Pictures were taken using a Nikon DXM200F digital camera on a Nikon Microphot-SA light microscope.

***In situ* hybridizations:** *In situ* hybridizations were done as previously described (Tautz and Pfeifle 1989). Briefly, probes for *white* or *yellow* were prepared by *in vitro* transcription in the presence of digoxigenin (DIG)-labeled dNTPs (Roche). The probe for *white* was made using T7 polymerase from a

white-containing plasmid obtained from Jumin Zhou. A portion of the *yellow* coding region was amplified by PCR using the following primers: *yellow* for (GGATTCCGGCCACTCTGACC TAT) and *yellow* rev (CTGGTCTGAGGTTTCTGTGGCAA).

The *yellow* PCR product was cloned into the pCRII-TOPO vector (Invitrogen, San Diego), and the *yellow* probe was made using SP6 polymerase. $ap^{MM\text{-}Mcp}$ was balanced over CyO, P{w[+mC]=ActGFP}JMR1 (BL#4533) to select homozygous $ap^{MM\text{-}Mcp}$ larvae. Homozygous $ap^{MM\text{-}Mcp}$ or ap^{MM} larvae were selected and the imaginal discs and central nervous system (CNS) were dissected in PBS. Tissues were fixed in 4% formaldehyde in PBS for 20 min at room temperature, while rocking. The tissues were then washed thoroughly with phosphate-buffered saline + 0.1% Triton X-100 (PBST) and allowed to prehybridize in hybridization buffer (50% formamide, 5× SSC, 50 μg/ml heparin, 0.1% Tween 20, 100 μg/ml sonicated salmon sperm DNA) for 2 hr at 55°. The DIG-labeled probes were then diluted 1:100, heated to 80°, added to the tissue, and incubated at 55° overnight to hybridize. The probe was then removed and the sample was washed extensively with hybridization buffer, followed by PBST. The sample was then probed with 1:2000 HRP-conjugated anti-DIG antibody (Roche) for 1.5 hr. Upon removal of the antibody, the sample was washed extensively with PBST and then washed twice with developing solution (0.1 M NaCl, 0.1 M Tris–HCl, pH 9.0, 0.05 M $MgCl_2$, 0.1% Tween 20). The tissues in developing solution were transferred to a glass dish and 20 μl of solution [18.75 mg/ml Nitro blue tetrazolium chloride, 9.4 mg/ml 5-bromo-4-chloro-3′-indolyl phosphate, toluidine salt, in 67% dimethyl sulfoxide (w/v) (Roche)] was added. *In situ*s were developed for between 30 and 60 min. The reaction was stopped by washing twice with PBST. Imaginal discs and brains were then mounted on slides in 70% glycerol and pictures were taken using a Nikon DXM200F digital camera on a Nikon Microphot-SA light microscope.

Reverting the ap^{f00451} insertion by mobilizing the *piggyBac* element: ap^{f00451} virgins were crossed with w^{1118}; CyO, P{Tub-PBac\T}2/$wg^{Sp\text{-}1}$ (BL#8285) males. w; ap^{f00451}/CyO, P{Tub-PBac\T}2 males or females were selected from this cross and mated with *w*; *Sp Pin*/CyO virgins or males, respectively. A total of 19 independent crosses were set up. Among the progeny of these 19 crosses, ap^{f00451}revertant males with white eyes due to the loss of the PBac{WH} transposon were isolated and individually crossed with *w*; *Sp Pin*/CyO virgins. In this way, nine independent ap^{f00451} revertant stocks were established. All nine stocks were homozygous viable and had normal wings.

Deleting the *apterous* wing enhancer: The region containing the *apterous* wing enhancer was deleted by FLP-mediated recombination between the FRT site present in $ap^{MM\text{-}Mcp}$ and the FRT site present in PBac{RB}e01573 (Golic and Golic 1996a; Parks *et al.* 2004; Thibault *et al.* 2004). Briefly, P{*hsFLP*}12, y^1 *w** (from BL#1929); $ap^{MM\text{-}Mcp}$/CyO virgin females were crossed to PBac{RB}e01573 males. Embryos (0–24 hr) were collected in bottles, allowed to age for 24 hr, and then heat-shocked for 1.5 hr/day in a 38° water bath until the majority of larvae had formed pupae. P{*hsFLP*}12, y^1 *w**; $ap^{MM\text{-}Mcp}$/PBac{RB}e01573 males were selected and crossed to *yw*; *bTf*/CyO virgin females. In the next generation, progeny were scored for the absence of the yellow marker from $ap^{MM\text{-}Mcp}$ and the white marker from PBac{RB}e01573. Two $y^- w^-$ flies (indicative of a deletion of the intervening DNA, $ap^{DG\text{-}Mcp}$) were recovered, as well as one $y^+ w^+$ fly (indicative of a duplication of the *ap* wing-enhancer region, ap^{2xE}). ap^{2xE} homozygotes carrying a duplication of the *ap* wing enhancer had no obvious phenotype. Presence of a recombinant *P* element in $ap^{DG\text{-}Mcp}$ and ap^{2xE} was confirmed by PCR and sequencing. The following primers were used: $ap^{MM\text{-}Mcp}$ 5′, Mcpout (CCACAGAACTT CTTCCCTTTCCGA); $ap^{MM\text{-}Mcp}$ 3′, 8-2 (see above); PBac{RB}e01573 5′, w2Down (GACCTGTTCGGAGTGATTAGCGTT); and PBac{RB}e01573 3′, RB2 (GCCCAATTCGCCCTTGAAGATCTA).

PCR was also done on DNA isolated from wild type and $ap^{DG\text{-}Mcp}$ and ap^{2xE} homozygotes to show that primers to the deleted region failed to form a product in $ap^{DG\text{-}Mcp}$ flies. The following primers were used: apE1 (CCCCGGTTAAGTCGG AACTGATT), apE2 (AGGTTCCTGCCCCCTTCTTTTACA), apE5 (GAGCCCGGCTCTATTCACACTTT), apE6 (CTCGCCCTT CCAGGACTATGTTT), apPromF2 (TACCGACTTTGGTCTG CAGCTGAT), and apPromR2 (GCTACCGCTGCCTTATTCA CGTT).

The two primer pairs for the *ap* wing-enhancer region (apE1/apE2 and apE5/apE6) did not form a product in $ap^{DG\text{-}Mcp}$ flies, while the amplification of the primer pair in the vicinity of the *ap* promoter (apPromF2/apPromR2) was normal (data not shown).

ap^{DG} was generated by excising *Mcp* from $ap^{DG\text{-}Mcp}$ using Cre recombinase (TM6B, P{Crew}DH2, Tb^1: BL#1501). PCR and sequencing was done to confirm the presence (primer pair RB2/Mcpout; see above for sequence) or absence (primer pair RB2/PEP5′in; see above) of *Mcp*. Both $ap^{DG\text{-}Mcp}$ and ap^{DG} delete the ~26.8-kb region between $ap^{MM\text{-}Mcp}$ and PBac{RB}e01573 (*D. melanogaster* Genome Release 5.1 coordinates 1614738–1641533).

RESULTS

***apterous* phenotype caused by an *Mcp*-containing *P*-element insertion:** The *ap* gene encodes a LIM-homeodomain transcription factor that is necessary for specifying dorsal cell fate and defining the dorsal/ventral compartment boundary in the developing wing (Bourgouin *et al.* 1992; Cohen *et al.* 1992). Perturbing *ap* expression in the wing disc can lead to defects in the adult wing blade. Weak *ap* mutants cause a held-out wing phenotype (Wilson 1981), while stronger hypomorphic alleles lead to reductions in wing size, as well as blistering or crumpling of the wing blade. Null mutants in *ap*, such as ap^{UGO35}, cause a complete loss of wings and halteres in adult flies (Cohen *et al.* 1992). In addition to its role in wing patterning, *ap* is also expressed in the haltere, leg, and eye-antennal imaginal discs in the developing CNS, the peripheral nervous system, brain, and in a subset of embryonic muscle precursors (Bourgouin *et al.* 1992; Cohen *et al.* 1992). The enhancers that drive the expression of *ap* in the wing and CNS are located ~6–12 kb upstream of the *ap* promoter (Lundgren *et al.* 1995), while the embryonic muscle enhancer is located in downstream of the *ap* transcriptional start site in intronic regions (Capovilla *et al.* 2001).

An insertion of the Flipper 2 transposon carrying the *Mcp* element that had a strong *ap* phenotype was isolated (see materials and methods) (Figure 1A). This insertion, called $ap^{MM\text{-}Mcp}$, failed to complement *Df(2R)nap1* and *Df(2R)nap2*, two deficiencies that delete the *ap* gene. $ap^{MM\text{-}Mcp}$/*Df* as well as homozygous $ap^{MM\text{-}Mcp}$ flies have wing defects that range from a complete lack of wings to wings that are severely blistered or crumpled (Figure 1, B and D) and also frequently lack halteres. In addition, like other strong *ap* alleles, $ap^{MM\text{-}Mcp}$ flies are short lived and cannot be maintained as a homozygous

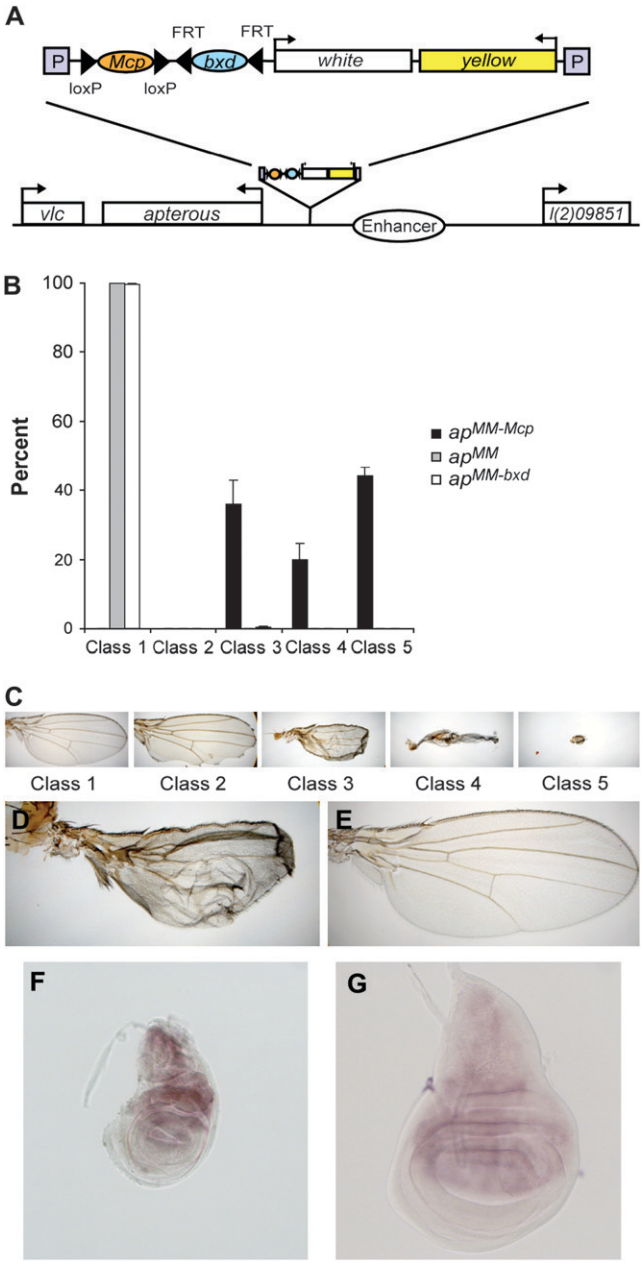

Figure 1.—Insertion of an *Mcp*-containing *P* element in the *apterous* regulatory region. (A) A schematic of the Flipper 2 element (top). $ap^{MM\text{-}Mcp\text{-}bxd}$ is an insertion of the Flipper 2 element 403 bp upstream of the *ap* transcriptional start site (*D. melanogaster* Genome Release 5.1 coordinates 1614738). (B) Wing phenotypes of the $ap^{MM\text{-}Mcp}$, ap^{MM}, and $ap^{MM\text{-}bxd}$ inserts. $ap^{MM\text{-}Mcp}$ homozygotes have wing defects ($N \geq 684$). (C) The following method was used for scoring the severity and penetrance of the *ap* phenotype: class 1, wild-type wings; class 2, mild-to-severe notching; class 3, approximately normal amount of wing tissue present, but wing blistered or crumpled; class 4, strap-like wings; and class 5, very little or no wing tissue. (D) A representative $ap^{MM\text{-}Mcp}$ homozygous (class 3) wing. (E) When the *Mcp* element is deleted, the wings of ap^{MM} flies are completely wild type. (F) Expression of *white* in $ap^{MM\text{-}Mcp}$ wing discs. Because *ap* expression is disrupted by the *Mcp* insert, the wing disc is reduced in size and malformed; however, *white* expression is evident. (G) In $ap^{MM\text{-}Mcp}/+$ wing discs, *white* is clearly expressed in the *ap* pattern.

stock. The insertion site of $ap^{MM\text{-}Mcp}$ was mapped by inverse PCR (Bellen *et al.* 2004). $ap^{MM\text{-}Mcp}$ was determined to be inserted 403 bp upstream of the *ap* transcriptional start site between the wing enhancer and the *ap* promoter (Figure 1A). Using the Cre recombinase, a derivative of $ap^{MM\text{-}Mcp}$ lacking *Mcp* was created (ap^{MM}). The wings of ap^{MM} flies are completely normal (Figure 1, B and E), implying that the *Mcp* element is responsible for the wing defect seen in $ap^{MM\text{-}Mcp}$ flies. In addition, ap^{MM} flies are homozygous viable and a homozygous stock has been maintained for many generations.

Since the *Mcp* element present in $ap^{MM\text{-}Mcp}$ contains both a boundary element and a PRE (Busturia *et al.* 1997; Müller *et al.* 1999; Gruzdeva *et al.* 2005), two possible models could account for the *ap* phenotype in $ap^{MM\text{-}Mcp}$ flies. It is possible that the *Mcp* PRE silences *ap*; alternately, the *Mcp* boundary may block the wing enhancer, which is located 6–12 kb upstream of the *ap* gene (Lundgren *et al.* 1995).

If the wing phenotype observed in $ap^{MM\text{-}Mcp}$ flies is due to the silencing of the *ap* gene by the *Mcp* PRE, one would predict that the *y* and *w* transformation markers in the transposon would also be silenced in the wing. On the other hand, if the *Mcp* boundary prevents the wing enhancer from activating the *ap* gene, then the $ap^{MM\text{-}Mcp}$ transposon transformation markers would likely be expressed in an *ap* pattern. Our results are consistent with the boundary model. First, in contrast to *ap*, *y* is strongly expressed in the wings of $ap^{MM\text{-}Mcp}$ flies (Figure 1, D and E) and variegated *y* expression is also seen in the abdomen (data not shown). The *y* expression observed in the adult wing of $ap^{MM\text{-}Mcp}$ flies is likely driven by a combination of the *y* wing enhancer in the Flipper 2 transgene and the upstream *ap* wing enhancer. *y* expression is also seen in the *ap* pattern in the developing wing disc of $ap^{MM\text{-}Mcp}$ flies by *in situ* hybridization (data not shown). Second, while the *w* gene in the transposon is silenced in the eye and $ap^{MM\text{-}Mcp}$ flies have white eyes, *w* is not silenced in the wing disc. Instead, *w* is expressed in the developing wing disc of $ap^{MM\text{-}Mcp}$ flies in the *ap* pattern (Figure 1, F and G).Thus neither of the reporter genes present in the Flipper 2 *P* element are silenced in the wing. Instead, both *w* and *y* appear to be expressed under the control of the *ap* wing enhancer.

Other lines of evidence argue that the effects of *Mcp* on *ap* in the wing are due to its boundary activity and not due to silencing by the PRE. Since silencing by PREs is often pairing sensitive, if the wing phenotype of $ap^{MM\text{-}Mcp}$ were due to silencing of *ap* by the *Mcp* PRE, the silencing might be expected to be stronger when the *P* element is homozygous, as opposed to hemizygous. As seen in Figure 2A, flies that are homozygous or hemizygous for $ap^{MM\text{-}Mcp}$ have identical wing phenotypes.

Finally, we tested whether substituting the well-characterized *bxd* PRE for *Mcp* could recapitulate the *ap* phenotype observed with $ap^{MM\text{-}Mcp}$. $ap^{MM\text{-}bxd}$ was made by

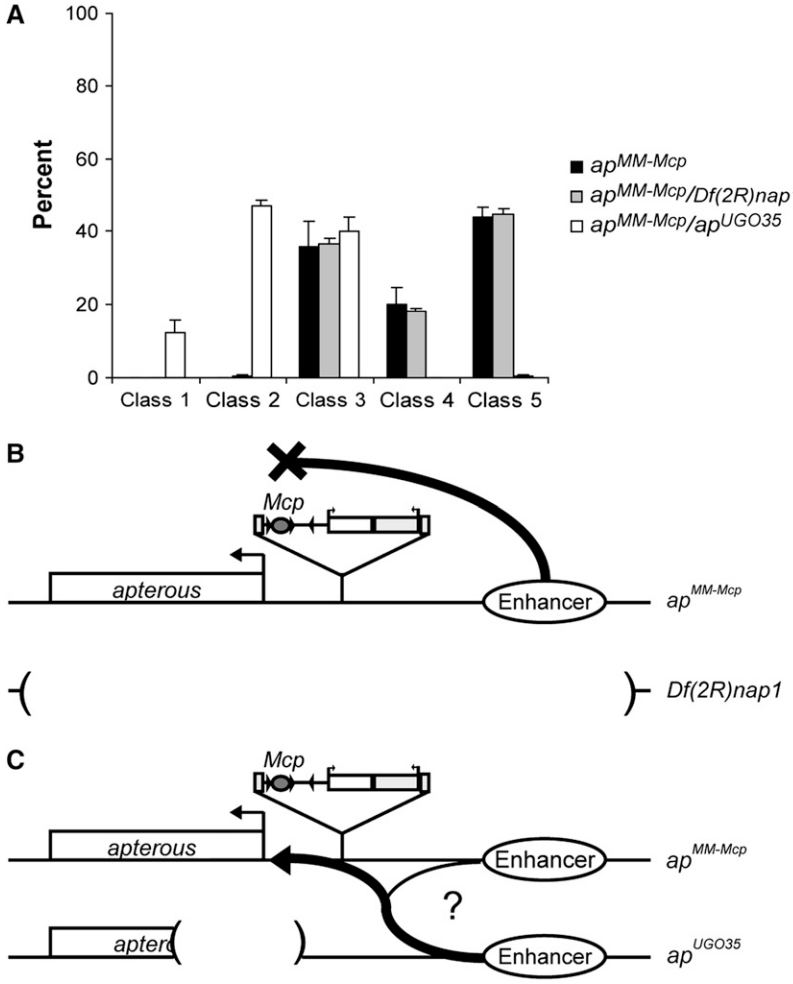

FIGURE 2.—Transvection at the *apterous* locus uncovered by $ap^{MM\text{-}Mcp}$. (A) Similar wing defects are observed in $ap^{MM\text{-}Mcp}$ and $ap^{MM\text{-}Mcp}/Df(2R)nap1$ flies, while the *apterous* phenotype of $ap^{MM\text{-}Mcp}$ is strongly suppressed when crossed to ap^{UGO35}, a null mutant that deletes the *ap* promoter and first exon ($N \geq 536$). (B) A schematic depicting the chromosomes of $ap^{MM\text{-}Mcp}/Df(2R)nap1$. In this genotype, the wing enhancer (open oval) is blocked by *Mcp* in *cis*. (C) A schematic depicting the chromosomes of $ap^{MM\text{-}Mcp}/ap^{UGO35}$. In this genotype, the *trans* and/or *cis* wing enhancers are able to partially bypass the *Mcp* boundary.

using the Cre recombinase to excise *Mcp* from the original Flipper 2 insert (Figure 1A, $ap^{MM\text{-}Mcp\text{-}bxd}$). The wings of $ap^{MM\text{-}bxd}$ flies were completely normal (Figure 1B), suggesting that enhancer blocking by *Mcp* and not silencing by the *Mcp* PRE is responsible for the wing defect seen in $ap^{MM\text{-}Mcp}$ flies. Like ap^{MM} flies, $ap^{MM\text{-}bxd}$ flies are homozygous viable and a homozygous stock has been maintained for many generations. Taken together, these observations suggest that the wing defects observed in $ap^{MM\text{-}Mcp}$ flies are due to the enhancer blocking activity of the *Mcp* boundary element and not due to silencing by the *Mcp* PRE.

Transvection at the *apterous* locus: The fact that the *ap* phenotype of $ap^{MM\text{-}Mcp}$ flies is due to *Mcp* enhancer blocking is further supported by the fact that interallelic complementation characteristic of transvection is observed when $ap^{MM\text{-}Mcp}$ is crossed to other *ap* alleles. When $ap^{MM\text{-}Mcp}$ is crossed to *Df(2R)nap1*, a deficiency that deletes the *ap* gene, the wing defects due to the *Mcp* insertion are unchanged (Figure 2, A and B). In contrast, when $ap^{MM\text{-}Mcp}$ is crossed to ap^{UGO35}, a null mutation that deletes the *ap* transcriptional start site as well as the first exon (COHEN *et al.* 1992), the wing defects are strongly suppressed (Figure 2, A and C). The simplest explanation of this interallelic complementation is that the wing enhancer on the ap^{UGO35} chromosome is able to act in *trans* on the $ap^{MM\text{-}Mcp}$ chromosome, a phenomenon known as transvection (Figure 2C). It is also possible that the enhancer in *cis* to the *Mcp* boundary is able to bypass the boundary due to structural disruption of the *ap* locus when $ap^{MM\text{-}Mcp}$ is crossed to ap^{UGO35} (Figure 2C; MORRIS *et al.* 1998). And, while the wing defects of $ap^{MM\text{-}Mcp}/ap^{UGO35}$ are significantly less severe than those of $ap^{MM\text{-}Mcp}$ homozygotes, the wings are not completely wild type. This would suggest that, in the presence of the *Mcp* boundary, the activation of *ap* in *trans* by the wing enhancer is less efficient than *cis* activation.

A *piggyBac* insertion containing the *su(Hw)* boundary element inserted in the *apterous* regulatory region: A second boundary-element-containing insertion in the *ap* regulatory region was obtained from the Harvard–Exelixis stock collection (PARKS *et al.* 2004; THIBAULT *et al.* 2004). This *piggyBac* WH element, ap^{f00451}, contains the *su(Hw)* boundary element and is inserted ~10.1 kb upstream of the *ap* transcriptional start site. ap^{f00451} has a weak, but highly penetrant, *ap* phenotype (Figure 3, A and C). The wing defect of ap^{f00451} is attributable to the presence of the *piggyBac* insertion, as nine of nine

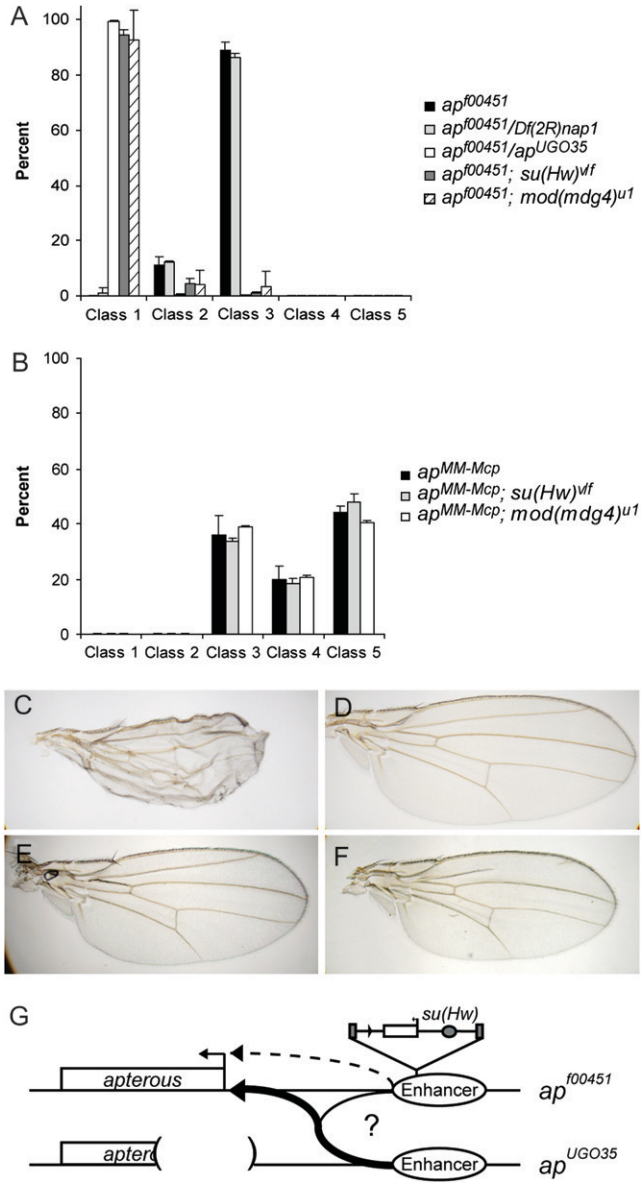

FIGURE 3.—A *su(Hw)*-insulator-containing *P* element also blocks the *apterous* wing enhancer and supports transvection. (A) As with $ap^{MM\text{-}Mcp}$, the wing defects of homozygous ap^{f00451} flies and $ap^{f00451}/Df(2R)nap1$ flies are similar, but are strongly suppressed when ap^{f00451} is crossed to ap^{UGO35}. The *ap* phenotype of ap^{f00451} is also strongly suppressed by the $su(Hw)^v/su(Hw)^f$ and $mod(mdg4)^{u1}$ mutants, which interfere with *su(Hw)* element enhancer-blocking activity ($N \geq 142$). (B) $ap^{MM\text{-}Mcp}$ is not affected by either $su(Hw)^v/su(Hw)^f$ or $mod(mdg4)^{u1}$ mutants. This suggests that *su(Hw)* and *mod(mdg4)* are not involved in *Mcp* boundary activity; neither do they affect the normal regulation of the *ap* gene, so the effects on ap^{f00451} seen in A, E, and F are specific to the *su(Hw)* boundary ($N \geq 72$). (C) A representative ap^{f00451} (class 3) wing. (D) A representative ap^{f00451}/ap^{UGO35} (class 1) wing. (E) A representative ap^{f00451}; $su(Hw)^v/su(Hw)^f$ (class 1) wing. (F) A representative ap^{f00451}; $mod(mdg4)^{u1}$ (class 1) wing. (G) A schematic depicting the chromosomes of ap^{f00451}/ap^{UGO35}. In this genotype, the *trans* and/or the *cis* wing enhancers are able to bypass the *su(Hw)* boundary. The *ap* gene is also likely activated by the unblocked, *ap* proximal portion of the wing enhancer (dashed arrow).

revertants obtained by mobilizing the *piggyBac* transposon are homozygous viable and have wild-type wings (data not shown). The insertion site of ap^{f00451} is near the middle of an ~6-kb fragment that is capable of driving reporter gene expression in the *ap* pattern in the wing disc and CNS (LUNDGREN *et al.* 1995). The fact that ap^{f00451} has a weak *ap* phenotype suggests that this insert is able to partially block the wing enhancer (perhaps blocking elements of the enhancer that are distal to the insertion site, but not affecting the gene proximal portions of the wing enhancer).

This suggestion is supported by analysis of the effects of mutations in two of the *trans*-acting factors that are required for enhancer blocking by the *su(Hw)* element. Both the Su(Hw) and Modifier of *mdg4* [Mod(mdg4)] proteins are necessary for *su(Hw)* element enhancer blocking. Su(Hw) is a DNA-binding protein containing 12 zinc-finger domains, which binds to the YRTTGCA TACCY repeats present in the *su(Hw)* element from the *gypsy* reterotransposon (PARKHURST *et al.* 1988; GEYER and CORCES 1992; PARNELL *et al.* 2006; RAMOS *et al.* 2006). Mod(mdg4) is a BTB/POZ domain-containing protein that can interact with Su(Hw), other components of the *su(Hw)* insulator, and itself to form insulator bodies (PARKHURST *et al.* 1988; GEYER and CORCES 1992; GERASIMOVA and CORCES 1998; GERASIMOVA *et al.* 2000; GHOSH *et al.* 2001). To test whether the *ap* wing phenotypes observed in ap^{f00451} flies are due to the presence of the *su(Hw)* boundary element, ap^{f00451} flies were crossed to mutants in *su(Hw)* and *mod(mdg4)*. When ap^{f00451} was crossed to the hypomorphic combination $su(Hw)^v/su(Hw)^f$, the wing defects were nearly completely suppressed (Figure 3, A and E). Similarly, when ap^{f00451} was crossed into a homozygous $mod(mdg4)^{u1}$ mutant background, the wing defects were also strongly suppressed (Figure 3, A and F). The wings of ap^{f00451}; $mod(mdg4)^{u1}$ flies are nearly wild type, with the exception of a disrupted L2 wing vein; however, this is likely due to the *mod(mdg4)* mutation rather than ap^{f00451}, as the wing-vein defect is also present in $+/+$; $mod(mdg4)^{u1}$ flies. The fact that both $su(Hw)^v/su(Hw)^f$ and $mod(mdg4)^{u1}$ strongly suppress the wing defect of ap^{f00451} suggests that, like $ap^{MM\text{-}Mcp}$, the phenotype of ap^{f00451} is due to disruption of the ability of the wing enhancer to activate *ap* by the *su(Hw)* boundary element.

We also tested whether *su(Hw)* or *mod(mdg4)* mutations have any effect on the boundary activity of the *Mcp* element in $ap^{MM\text{-}Mcp}$. Neither $su(Hw)^v/su(Hw)^f$ nor $mod(mdg4)^{u1}$ had an effect on the wing defects observed with $ap^{MM\text{-}Mcp}$, indicating that *su(Hw)* and *mod(mdg4)* do not affect the *Mcp* boundary, nor do they affect the regulation of *ap* in the absence of the ap^{f00451} insert (Figure 3B). In addition, $mod(mdg4)^{u1}$ was crossed to the $Beadex^1$ (Bx^1) mutation. The *Bx* gene is a direct transcriptional target of *ap*, and the Bx^1 mutation has been used to screen for other genes involved in the regulation of *ap* (MILAN *et al.* 2004). $mod(mdg4)^{u1}$ had no effect on

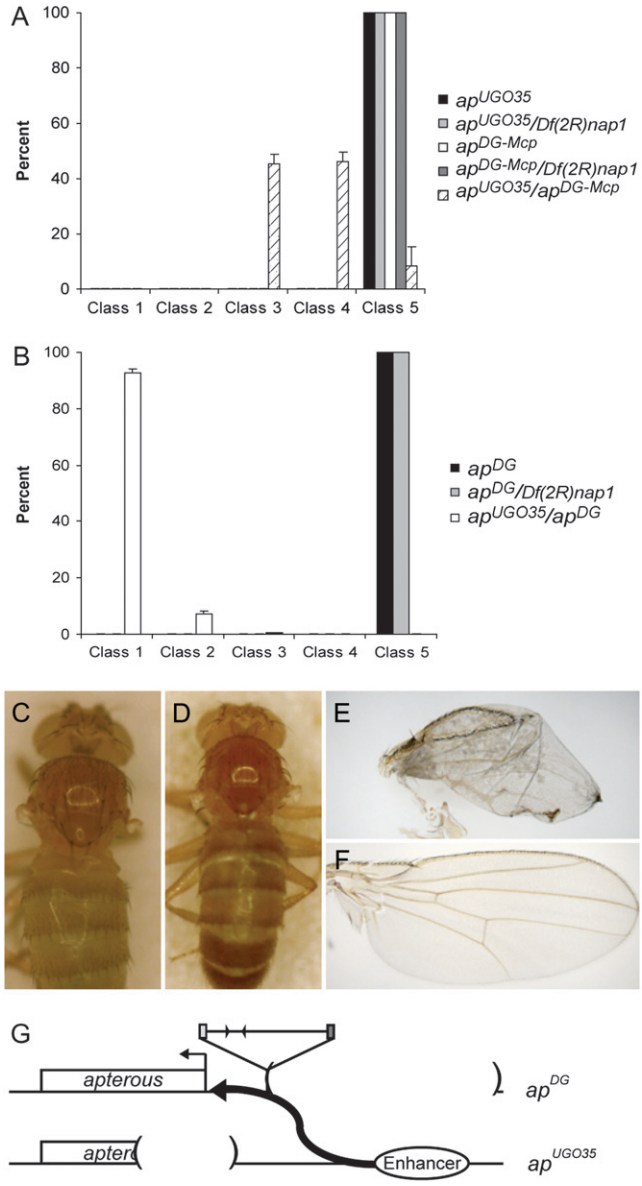

FIGURE 4.—Deletion of the *apterous* wing enhancer. (A) Both the *Mcp*-containing enhancer deletion $ap^{DG\text{-}Mcp}$ and ap^{UGO35} are completely defective in forming wings either as homozygotes or over *Df(2R)nap1*. However, the wing defects of these mutants are significantly suppressed in $ap^{DG\text{-}Mcp}/ap^{UGO35}$ *trans*-heterozygotes ($N \geq 196$). (B) As with $ap^{DG\text{-}Mcp}$, both homozygous ap^{DG} and $ap^{DG}/Df(2R)nap1$ flies fail to form any wing material. When the enhancer deletion ap^{DG} is crossed to ap^{UGO35}, >90% of the wings are completely wild type ($N \geq 158$). (C) A homozygous $ap^{DG\text{-}Mcp}$ fly. (D) A homozygous ap^{DG} fly. (E) A representative $ap^{DG\text{-}Mcp}/ap^{UGO35}$ (class 3) wing. (F) A representative ap^{DG}/ap^{UGO35} (class 1) wing. (G) A model for the transvection observed in ap^{DG}/ap^{UGO35} flies.

the wing defects observed with Bx^1, indicating that *mod(mdg4)* is not normally involved in the *ap* pathway (data not shown).

As in the *Mcp*-containing insert, $ap^{MM\text{-}Mcp}$, interallelic complementation characteristic of transvection was observed for ap^{f00451}. The phenotype of ap^{f00451}/Df(2R)nap1 is as severe as that of ap^{f00451} homozygotes (Figure 3A). As with the *Mcp* insert, the wing defect of ap^{f00451} was strongly suppressed when ap^{f00451} was crossed to the promoter deletion, ap^{UGO35}, suggesting that this allele is also able to support transvection (Figure 3, A, D, and G).

Deletion of the *apterous* wing enhancer—testing the transvection hypothesis: To provide further evidence that transvection occurs at the *ap* locus, the region containing the *ap* wing enhancer was deleted by FLP-mediated recombination between FRT sites in $ap^{MM\text{-}Mcp}$ and the insert PBac{RB}e01573 (GOLIC and GOLIC 1996a; PARKS *et al.* 2004; THIBAULT *et al.* 2004). The resulting deletion, $ap^{DG\text{-}Mcp}$, deletes an ~26.8-kb region spanning the *ap* wing enhancer. $ap^{DG\text{-}Mcp}$ is homozygous viable, indicating that it does not disrupt the function of the neighboring gene, *l(2)09851*, which is ~500 bp from the deletion breakpoint. As expected for an *ap* wing-enhancer deletion, $ap^{DG\text{-}Mcp}$ homozygotes completely lack wings (Figure 4, A and C). Likewise, $ap^{DG\text{-}Mcp}/Df(2R)nap1$ flies fail to form wings (Figure 4A). However, robust interallelic complementation is seen between $ap^{DG\text{-}Mcp}$ and ap^{UGO35} (Figure 4, A and E). While neither $ap^{DG\text{-}Mcp}$ nor ap^{UGO35} homozygotes have any observable wing tissue, the majority of $ap^{DG\text{-}Mcp}/ap^{UGO35}$ flies have either class 3 (crumpled or blistered) or class 4 (strap) wings. To test whether the *Mcp* element in $ap^{DG\text{-}Mcp}$ attenuates enhancer action in *trans*, we generated an *ap* wing-enhancer deletion derivative that lacks the *Mcp* element, ap^{DG}, using Cre recombinase. The transvection effect is much more striking when the *Mcp* element is excised from the enhancer deletion. ap^{DG}/ap^{UGO35} flies have almost completely wild-type wings (Figure 4, B and F). The fact that transvection is stronger in ap^{DG}/ap^{UGO35} flies compared with $ap^{DG\text{-}Mcp}/ap^{UGO35}$ flies indicates that *Mcp* is able to block the enhancer on the ap^{UGO35} chromosome in *trans*. It is interesting to note that enhancer action in *trans* in the ap^{DG}/ap^{UGO35} combination is sufficient for nearly wild-type levels of expression (Figure 4G).

The role of the promoter in *ap* transvection: We also tested whether transvection was observed for several additional *ap* mutations that, unlike ap^{UGO35}, are likely to have an intact *ap* promoter. Two spontaneous *ap* mutants, ap^4 and ap^{56f}, the *P*-element insertion ap^{rk568}, and the *piggyBac* WH insertion ap^{f08090} were tested for transvection in combination with the *Mcp* boundary insertion ($ap^{MM\text{-}Mcp}$), the enhancer deletion (ap^{DG}), and the enhancer deletion linked to the *Mcp* boundary ($ap^{DG\text{-}Mcp}$). While no molecular information is available for ap^4 and ap^{56f}, it is likely that these mutants disrupt the *ap*-coding region, and not the regulatory elements, as they fail to complement ap^{UGO35} (data not shown). ap^{f08090} is an insertion in the second large intron of *ap*, just upstream of the *ap-RB* transcriptional start site (*D. melanogaster* Genome Release 5.1 coordinates 1597428). On the basis of complementation data and the fact that ap^{f08090} is not suppressed by mutations in *su(Hw)* or *mod(mdg4)* (data not shown), the *ap* mutant phenotype observed with this

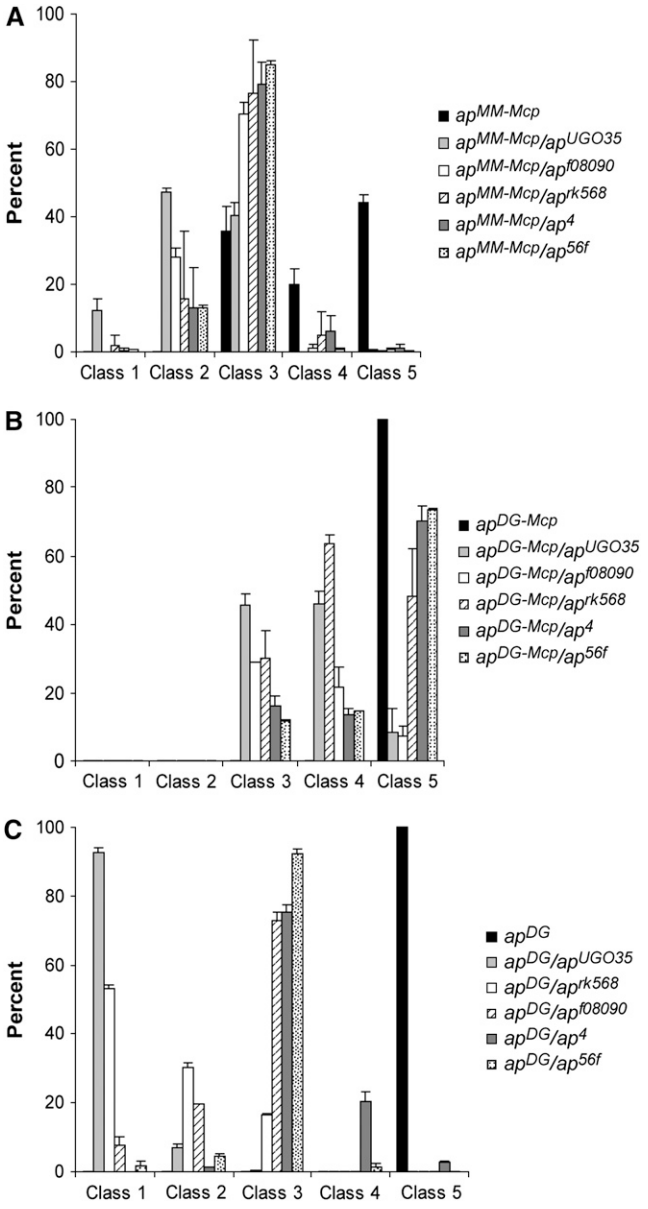

FIGURE 5.—Transvection observed with other *ap* alleles. Several other *ap* alleles were tested for transvection effects: (A) As with ap^{UGO35}, ap^{56f}, ap^{rk568}, ap^{f08090}, and ap^{4}, all exhibited transvection when crossed to the boundary insertion $ap^{MM\text{-}Mcp}$. These alleles fail to complement one another and *Df(2R)nap1* or *Df(2R)nap2* (data not shown). This suggests that, as with ap^{UGO35}, all these additional alleles affect the *ap*-coding region/promoter, and not the *ap* regulatory elements. Also, the transvection effect with ap^{UGO35} is the strongest of all the alleles. This is likely because ap^{UGO35} deletes the *ap* promoter, which releases the wing enhancer to act only in *trans* ($N \geq 406$). (B) ap^{56f}, ap^{rk568}, ap^{f08090}, and ap^{4} also exhibit transvection when crossed to the *Mcp*-containing enhancer deletion $ap^{DG\text{-}Mcp}$. The transvection seen with these four alleles is weaker than that observed in $ap^{UGO35}/ap^{DG\text{-}Mcp}$ flies ($N \geq 268$). (C) ap^{56f}, ap^{rk568}, ap^{f08090}, and ap^{4} also exhibit transvection when crossed to the enhancer deletion ap^{DG}. The transvection seen with these four alleles is weaker than that observed in ap^{UGO35}/ap^{DG} flies ($N \geq 312$).

allele is likely to be due to a disruption of the *ap* open reading frame (ORF), rather than enhancer blocking by the *su(Hw)* insulator present in the WH *piggyBac* transposon.

ap^{4}, ap^{56f}, ap^{rk568}, and ap^{f08090} all suppress the $ap^{MM\text{-}Mcp}$ wing phenotype (Figure 5A). However, the suppression observed with these other four *ap* alleles is weaker than that seen when $ap^{MM\text{-}Mcp}$ is crossed to ap^{UGO35}, suggesting that the *ap* wing enhancer can be tethered by an intact promoter in *cis*. On the other hand, since some transvection is still observed in these four mutants that likely do not disrupt the *ap* promoter, the *cis* tethering of enhancers at *ap* must be weaker than that observed at the endogenous *y* locus, where an intact promoter in *cis* largely suppresses transvection (MORRIS *et al.* 1999b; LEE and WU 2006). ap^{rk568} is an insertion of a *P* element 23 bp 5′ of the annotated *ap* transcription start site, suggesting that this insert may compromise, but not completely abolish, promoter function. Consistent with this observation, ap^{rk568} supports transvection at a level intermediate to ap^{UGO35} and the other *ap* alleles tested. Like ap^{4} and ap^{56f}, ap^{rk568} fails to complement ap^{UGO35} (data not shown).

We also tested combinations between ap^{DG} and the putative *ap*-coding region mutations that are expected to retain the promoter. As shown in Figure 5C, transvection is also observed when ap^{DG} is combined with these putative point mutations; however, the wing phenotype is not as completely suppressed as it is in the ap^{DG}/ap^{UGO35} combination. Although the suppression seen when ap^{DG} is combined with these putative ORF mutations is not as strong as when it is combined with the promoter deletion ap^{UGO35}, the transvection effects with these alleles are considerably stronger than those observed when these alleles are combined with the enhancer deletion that retains the *Mcp* element, $ap^{DG\text{-}Mcp}$ (Figure 5B). This again indicates that the *Mcp* element can partially interfere with *trans*-regulatory interactions.

Effect of *zeste* mutants on transvection at *apterous*: Previous studies have implicated the Zeste protein in transvection at some, but not all loci. Transvection effects at *white*, *yellow*, *Ubx*, *dpp*, and *eya* are all sensitive to *zeste* (*z*) mutants (LEWIS 1954; GELBART and WU 1982; GEYER *et al.* 1990; LEISERSON *et al.* 1994; DUNCAN 2002). However, other instances of transvection that are insensitive to mutations in *z*, such as those observed at *Scr*, *Abd-B*, and *vg* (HOPMANN *et al.* 1995; SOUTHWORTH and KENNISON 2002; COULTHARD *et al.* 2005), have been identified. These observations, coupled with the fact that *z* null mutants are viable and do not have notably disrupted chromosome pairing (GOLDBERG *et al.* 1989; PIRROTTA 1999), suggest that parallel or redundant mechanisms must exist for maintaining somatic chromosome pairing.

Two alleles of *zeste*, z^{1} and z^{a}, were tested to see if they influenced transvection at the *ap* locus. The z^{1} allele is a

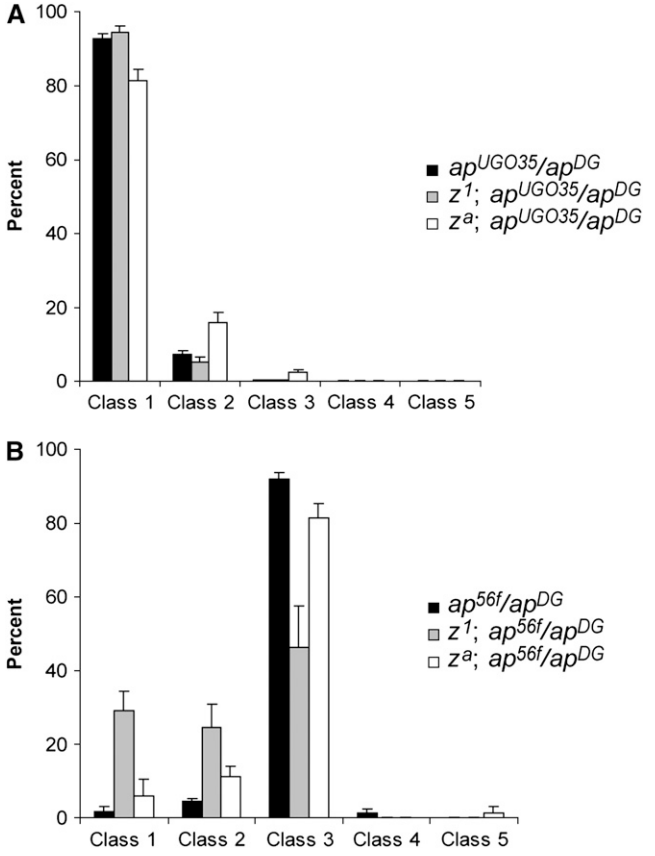

FIGURE 6.—Effects of *zeste* mutants on *apterous* transvection. (A) The gain-of function, hyperaggregating z^1 allele has little or no effect on ap^{UGO35}/ap^{DG} transvection, while the hypomorphic mutation, z^a, causes only a very modest decrease in the strength of ap^{UGO35}/ap^{DG} transvection ($N \geq 320$). (B) z^1 suppresses the wing phenotype of ap^{56f}/ap^{DG}. z^a has little or no effect on the wings of ap^{56f}/ap^{DG} flies ($N \geq 114$).

gain-of-function mutation that leads to hyperaggregation of the Zeste protein and thus generally increases the strength of a transvection effect (PIRROTTA *et al.* 1987; CHEN *et al.* 1992; CHEN and PIRROTTA 1993a,b). z^a is a hypomorphic mutation, which generally disrupts transvection (GOLDBERG *et al.* 1989). The z^1 mutant had little or no effect on transvection in ap^{UGO35}/ap^{DG} flies, while the z^a mutation caused only a slight disruption of transvection in this genotype (Figure 6A). However, stronger effects were seen when the *zeste* mutants were crossed to a pair of *ap* alleles in which transvection is less robust. In ap^{56f}/ap^{DG} flies, suppression of the wing phenotype was observed in the hyperaggregating z^1 mutant background, while little or no effect was seen with z^a (Figure 6B). This finding is similar to what was previously observed for transvection at the *dpp* locus, where effects of *zeste* mutations were observed only in a sensitized background in which pairing had been partially disrupted by chromosomal rearrangements (GELBART and WU 1982).

Loss of enhancer blocking in $ap^{MM\text{-}Mcp}/ap^{f00451}$ *trans*-heterozygotes: As with other instances of transvection, the alleles described in this study can be divided into several classes: those that disrupt the *ap* wing enhancer (ap^{DG}), those that disrupt the *ap* promoter/coding region (ap^{UGO35}), those that disrupt enhancer–promoter communication ($ap^{MM\text{-}Mcp}$, ap^{f00451}), and those that disrupt both the enhancer and the promoter/coding region [*Df(2R)nap1*, *Df(2R)nap2*]. As expected, the two deficiencies that lack the *ap* enhancer and coding region fail to complement any of the other *ap* mutants. In contrast, complementation is expected and is observed when mutants that disrupt the enhancer are combined with mutants that disrupt the promoter/coding region or when mutations that disrupt enhancer/promoter communication (boundary insertions) are combined with either an enhancer or a promoter/coding region mutation. Complementation/transvection is not expected to occur between alleles in the same class. Contrary to this expectation, when the *Mcp* ($ap^{MM\text{-}Mcp}$) and *su(Hw)* (ap^{f00451}) insertions are combined, the flies had wings that were completely wild type, indicating that the two boundaries fail to block when *trans*-heterozygous (Figure 7, A–C).

It seemed possible that this effect might be similar to the phenomenon of insulator bypass, which is observed when two tandem copies of the *su(Hw)* insulator are placed in between an enhancer and a promoter in a transgenic enhancer-blocking assay (CAI and SHEN 2001; MURAVYOVA *et al.* 2001). In this case, the two *su(Hw)* insulators are thought to pair with one another in *cis*. This cancels out their enhancer-blocking activity, allowing the upstream enhancer to activate the downstream promoter. Supporting this idea that insulator bypass is responsible for the loss of enhancer blocking in $ap^{MM\text{-}Mcp}/ap^{f00451}$ *trans*-heterozygotes, insulator bypass has recently been observed when *Mcp* is substituted for one of the two su*(Hw)* elements in the transgenic assay (MELNIKOVA *et al.* 2004).

If the loss of boundary activity in $ap^{MM\text{-}Mcp}/ap^{f00451}$ flies is caused by insulator bypass due to pairing in *trans* of the *Mcp* and *su(Hw)* elements on the two chromosomes, one would also expect to observe bypass when the *su(Hw)* insert, ap^{f00451}, is in *trans* to the enhancer deletion that retains an intact *Mcp* element, $ap^{DG\text{-}Mcp}$. However, this is not the case. Instead, the wing phenotype of $ap^{DG\text{-}Mcp}/ap^{f00451}$ *trans*-heterozygotes is equivalent to that of homozygous ap^{f00451} or ap^{f00451}/Df(2R)nap1 flies (Figure 8, A and C). While this finding argues against a *trans Mcp/su(Hw)* insulator bypass mechanism in $ap^{MM\text{-}Mcp}/ap^{f00451}$ flies, it could be argued that the failure to observe suppression of the *ap* wing phenotype in $ap^{DG\text{-}Mcp}/ap^{f00451}$ *trans*-heterozygotes is due to the deletion of the enhancer in the $ap^{DG\text{-}Mcp}$ chromosome. To exclude this possibility, we tested whether transvection is observed in flies that are *trans*-heterozygous for the *su(Hw)* insert, ap^{f00451}, and the enhancer deletion lacking the *Mcp* element, ap^{DG}. As can be seen in Figure 8, B and D, *trans* activation is observed in ap^{f00451}/ap^{DG} flies. Taken together,

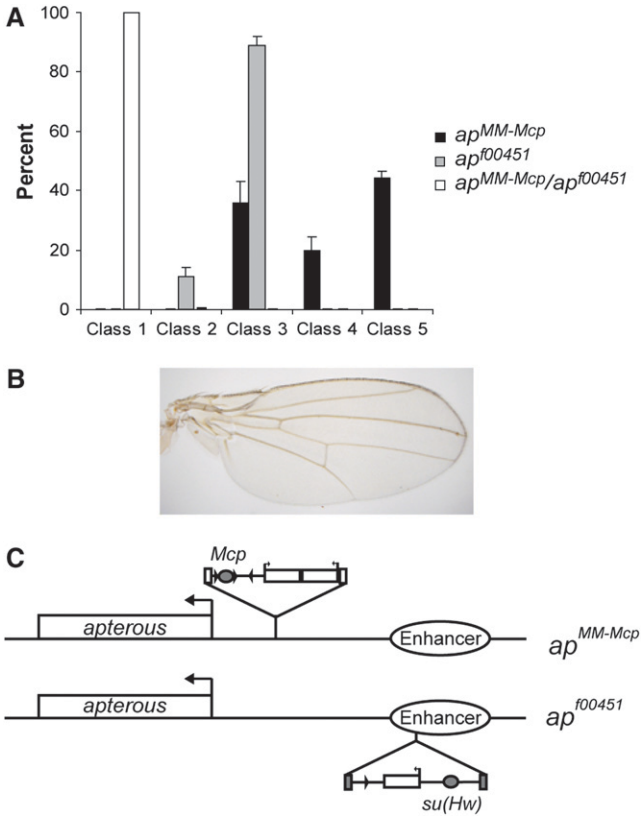

FIGURE 7.—Loss of enhancer blocking in flies *trans*-heterozygous for *su(Hw)/Mcp* boundary inserts. (A) $ap^{MM\text{-}Mcp}/ap^{f00451}$ flies have almost completely wild-type wings, suggesting that the enhancer-blocking activities of the *Mcp* and *su(Hw)* boundaries are compromised in these flies ($N \geq 362$). (B) A representative (class 1) wing from an $ap^{MM\text{-}Mcp}/ap^{f00451}$ fly. (C) A diagram depicting the $ap^{MM\text{-}Mcp}/ap^{f00451}$ chromosomes.

these results suggest that the loss of enhancer-blocking $ap^{MM\text{-}Mcp}/ap^{f00451}$ flies is unlikely to be due to a mechanism involving the pairing of *Mcp* and *su(Hw)* in *trans* and insulator bypass.

$ap^{MM\text{-}Mcp}$ was also crossed to $ap^{DG\text{-}Mcp}$. As was the case with $ap^{DG\text{-}Mcp}/ap^{f00451}$, the wing defects of the $ap^{MM\text{-}Mcp}/ap^{DG\text{-}Mcp}$ were as severe as the homozygous boundary inserts, indicating that the one remaining wing enhancer remained blocked (Figure 8A). In contrast, when the *Mcp* insert, $ap^{MM\text{-}Mcp}$, is crossed to the same enhancer deletion lacking the *Mcp* element, ap^{DG}, transvection was observed (Figure 8B). This demonstrates that, in the absence of a *trans* boundary, the wing enhancer in *cis* to *Mcp* can partially bypass the *Mcp* boundary to activate the *ap* gene in *trans* (Figure 8E).

This suggested an alternative model for explaining why the $ap^{MM\text{-}Mcp}/ap^{f00451}$ *trans*-heterozygotes have wild-type wings (Figure 9A). *Mcp* can block an enhancer in *cis* when hemizygous (Figure 2, A and B), in *cis* and in *trans* when homozygous (Figure 2, A and B; Figure 8A), and in *trans* when over an *ap* promoter deletion (Figure 4, A and E; compare $ap^{DG\text{-}Mcp}/ap^{UGO35}$ and ap^{DG}/ap^{UGO35}). However, *Mcp* largely fails to block an enhancer that is in *cis* to the boundary from acting in *trans* (Figure 8, B, D, and E; Figure 9A). Thus, the fact that $ap^{MM\text{-}Mcp}/ap^{f00451}$ flies have wild-type wings can be explained by the additive effects of activation of *ap* by proximal enhancer elements on the ap^{f00451} chromosome and *trans* activation of *ap* by the enhancer on the $ap^{MM\text{-}Mcp}$ chromosome (Figure 9A). As with the other instances of *ap* transvection involving a boundary element (Figure 2C; Figure 3G), it remains formally impossible to distinguish between enhancer action in *trans* and disruption of boundary activity [possibly due to some sort of structural or conformational perturbation of the boundary caused by homolog pairing (MORRIS *et al.* 1998)], leading to activation of *ap* by the wing enhancer in *cis*.

As with $ap^{MM\text{-}Mcp}/ap^{DG}$, ap^{f00451}/ap^{DG} flies also exhibited a partial bypass of the *su(Hw)* boundary in *trans* (Figure 8B). And, while both the *Mcp* and *su(Hw)* boundary elements can be bypassed by a *cis*-linked enhancer in *trans*, the *trans* enhancer bypass does not occur when there is a second (paired) boundary on the other homolog (*i.e.*, in $ap^{MM\text{-}Mcp}$ and ap^{f00451} homozygotes; Figures 2A and 3A). The fact that $ap^{MM\text{-}Mcp}/ap^{f00451}$ does not exhibit enhancer blocking must mean that *Mcp* and *su(Hw)* are incapable of pairing with one another, that pairing between *Mcp* and *su(Hw)* is rendered impossible by local structural constraints, or that the insertions of *Mcp* and *su(Hw)* demarcate different chromosomal domains (see DISCUSSION; Figure 9, B and C).

DISCUSSION

A number of recent studies have underscored the importance of intra- and interchromsomal interactions in regulating gene expression in diverse organisms from yeast to humans (MÜLLER *et al.* 1999; WU and MORRIS 1999; CARTER *et al.* 2002; DEKKER *et al.* 2002; TOLHUIS *et al.* 2002; BANTIGNIES *et al.* 2003; MURRELL *et al.* 2004; OSBORNE *et al.* 2004; RONSHAUGEN and LEVINE 2004; SPILIANAKIS and FLAVELL 2004; SPILIANAKIS *et al.* 2005; CLEARD *et al.* 2006; LING *et al.* 2006; VAZQUEZ *et al.* 2006). Drosophila is a particularly good system for studying *trans* interactions, as the majority of the genome remains paired not only during meiosis, but also in somatic cells (STEVENS 1908; METZ 1916). To date, a number of pairing-dependent genetic effects have been found in Drosophila. These *trans* effects fall into two classes. First, there are homology-dependent *trans* interactions [such as transvection, bw^{D}, and *trans* silencing by heterochromatin (HENIKOFF and DREESEN 1989; WU and MORRIS 1999; DUNCAN 2002)], which rely on pairing between homologous chromosomes and are disrupted by inversions or translocations that abolish homolog pairing in the region. Second, there are sequence-specific *trans* interactions, in which relatively short sequences can mediate pairing between distant loci [for example, *Mcp* and *su(Hw)* can confer long-

distance pairing to PREs (Sigrist and Pirrotta 1997; Müller *et al.* 1999; Vazquez *et al.* 2006)].

Transvection at the *apterous* locus: Here we present evidence for transvection at the Drosophila *apterous* locus. While interallelic complementation at *ap* has been previously reported (Shtorch *et al.* 1995), the *ap* alleles were not molecularly characterized. Consequently, it was not clear whether the complementation between these alleles involved *trans*-regulatory interactions or occurred at the level of the mutant *ap* gene products. We have observed *trans*-regulatory interactions with several different classes of *ap* mutations.

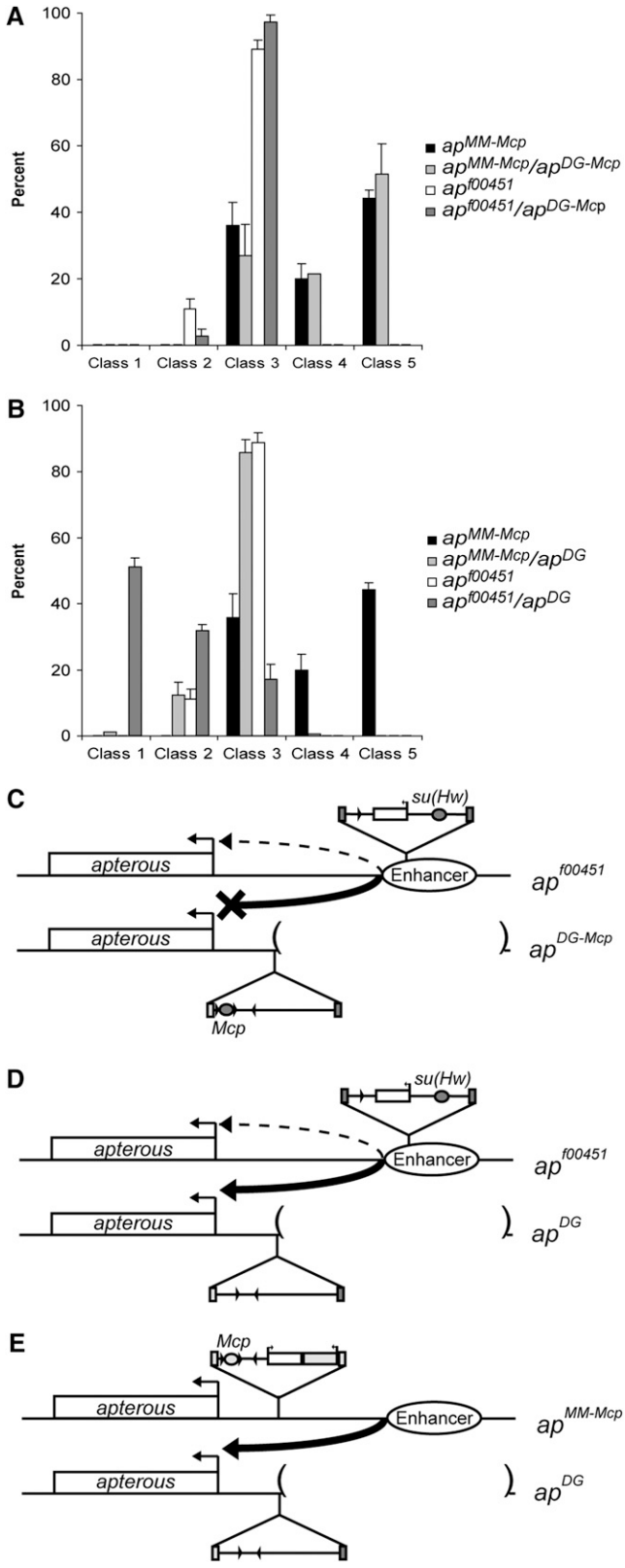

Figure 8.—Enhancer bypass of the *Mcp* and *su(Hw)* bounaries in *trans*. (A) When the *ap* boundary inserts ($ap^{MM\text{-}Mcp}$ and ap^{f00451}) are crossed to $ap^{DG\text{-}Mcp}$, the blocking of the wing enhancer observed is comparable to the homozygous boundary insert ($N \geq 220$). (B) The *ap* wing enhancer is able to partially bypass both the *Mcp* ($ap^{MM\text{-}Mcp}$) and the *su(Hw)* (ap^{f00451}) boundaries in *trans* when the boundary inserts are crossed to the *ap* wing-enhancer deletion ($N \geq 362$). (C) The *ap* wing enhancer remains blocked when *ap* boundary insertions, such as ap^{f00451}, are crossed to $ap^{DG\text{-}Mcp}$ (a deletion of the *ap* wing enhancer that still contains the *Mcp* boundary). Presumably, the unblocked, *ap* proximal portions of the wing enhancer are still able to activate *ap* (dashed arrow). (D) A model for the partial bypass of the *su(Hw)* boundary element of ap^{f00451} in *trans* by the *cis*-linked *ap* wing enhancer. (E) A model for the partial bypass of the *Mcp* boundary element of $ap^{MM\text{-}Mcp}$ in *trans* by the *cis*-linked *ap* wing enhancer.

The first type is the transvection seen in *trans* combinations between mutations that disrupt enhancers and mutations that disrupt the promoter. At the *ap* locus, this is illustrated by the ap^{DG}/ap^{UGO35} combination (Figure 4, B, F, and G). Interestingly, the transvection observed between ap^{DG} and ap^{UGO35} is sufficient to express *ap* at or near wild-type levels, as >90% of the wings are completely wild type. *ap* mutants are recessive, so there is likely a range of *ap* activity that is sufficient to produce wild-type wings (on the basis of the haplosufficiency of *ap* and the fact that the ap^{2xE} allele generated in parallel to ap^{DG}, which has a duplication of the wing enhancer, has wild-type wings, this range is likely to extend from at least 0.5 to 2 times normal levels).

It is unknown to what extent Dipterans have learned to exploit this interesting feature of their genomes for normal gene regulation. For example, it is unlikely that *trans* regulation occurs at the endogenous *y* locus in wild-type flies, as the enhancers appear to be strongly tethered in *cis* by the promoter. Instead, *trans* regulation is observed only at *y* when the enhancers are freed by deletion of the *cis* promoter (Morris *et al.* 1999a,b, 2004; Lee and Wu 2006). *ap* is clearly different from *y* in this respect as we also observe relatively strong *trans* regulation when the enhancer deletion, ap^{DG}, is combined with presumed *ap*-coding region mutations that are likely to retain an intact promoter (Figure 5C). Since the suppression of these coding region mutants by ap^{DG} is not as strong as that observed with the promoter deletion ap^{UGO35}, *cis* interactions between the upstream wing enhancer and the promoter of the mutant gene must compete with the ap^{DG} promoter in *trans*.

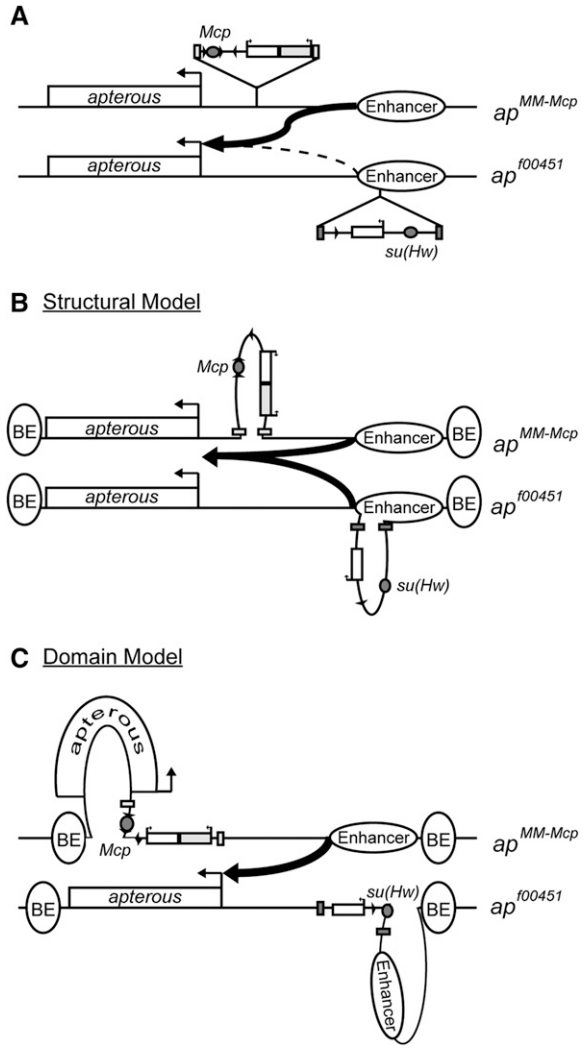

FIGURE 9.—Possible models for enhancer bypass of the *Mcp* and *su(Hw)* boundaries in *trans*. (A) A model depicting *trans* activation of the *ap* gene on the ap^{f00451} chromosome by the *ap* wing enhancer on the $ap^{MM\text{-}Mcp}$ chromosome (see Figure 8, B and D). The additive effects of this *trans* activation (solid arrow) and the activation of *ap* by the unblocked, *ap* proximal portions of the *ap* wing enhancer on the ap^{f00451} chromosome (dashed arrow) could account for the wild-type wings observed in $ap^{MM\text{-}Mcp}/ap^{f00451}$ *trans*-heterozygotes. (B) A model depicting how homology-driven chromosome pairing between the structurally dissimilar alleles $ap^{MM\text{-}Mcp}$ and ap^{f00451} could cause looping out and inactivation of the boundary elements, presumably due to conformational stress. (C) A model depicting pairing between the boundaries present in the $ap^{MM\text{-}Mcp}$ and ap^{f00451} inserts and two hypothetical boundaries on either side of the *ap* regulatory region. Such pairing would function to demarcate two distinct chromosomal domains, each of which would exhibit enhancer blocking when homozygous or hemizygous, but could lead to interallelic complementation when *trans*-heterozygous.

The second type of *trans*-regulatory interaction observed at *ap* is the transvection effects observed with boundary elements. We identified two different boundary insertions in the *ap* regulatory region. $ap^{MM\text{-}Mcp}$ is an insertion of the *Mcp*-containing Flipper 2 transposon 403 bp upstream of the *ap* transcriptional start site between the wing enhancer and the *ap* promoter (Figure 1A). Although the *Mcp* element in this transgene contains both a boundary element and a PRE, our results indicate that the wing defects seen in homozygous or hemizygous $ap^{MM\text{-}Mcp}$ flies are due to the enhancer-blocking activity of the boundary and not due to silencing by the *Mcp* PRE (Figure 1, B, F, and G; Figure 2, A and B). In the absence of an *Mcp* boundary insertion that lacks the PRE, the possibility remains that the *Mcp* PRE contributes to the *ap* wing phenotype. However, if this is the case, it is likely that the role of the PRE is a modulatory one, as the *bxd* PRE alone is not sufficient to cause wing defects (Figure 1B). ap^{f00451} is a *su(Hw)*-containing *piggyBac* element and is also inserted between *ap* enhancer elements and the *ap* promoter (Figure 3).

One version of this boundary-element-induced transvection is that seen in the interallelic complementation between the boundary insertions and the *ap* promoter deletion, ap^{UGO35}. This *trans*-regulatory interaction is observed with both the *Mcp* and *su(Hw)* elements. The *Mcp* insert, $ap^{MM\text{-}Mcp}$, has a strong *ap* wing phenotype, but when it is combined with the promoter deletion, ap^{UGO35}, the wing defects are partially suppressed (Figure 2, A and C). The fact that full suppression is not observed in this combination, while it is observed when the enhancer deletion is combined with the promoter deletion, indicates that the *Mcp* element must be capable of partially blocking *trans* interactions between the ap^{UGO35} wing enhancers and the $ap^{MM\text{-}Mcp}$ promoter. This suggestion is substantiated by a comparison of the wing phenotypes in combinations between ap^{UG035} and the enhancer deletion with ($ap^{DG\text{-}Mcp}$) and without (ap^{DG}) the *Mcp* element. While nearly full suppression is observed in the latter case, the suppression of the wing defects in $ap^{DG\text{-}Mcp}/ap^{UGO35}$ flies is comparatively modest (Figure 4, A, B, E, and F). This difference can be attributed to the ability of the *Mcp* element to block the *ap* enhancers in *trans* from activating the *ap* promoter in *cis* to the boundary. On the other hand, a comparison of the wing phenotype of the $ap^{DG\text{-}Mcp}/ap^{UGO35}$ *trans* combination (Figure 4A) with flies that are either hemizygous or homozygous for the *Mcp* insertion, $ap^{MM\text{-}Mcp}$ (Figure 2A), reveals that the enhancer-blocking activity of this boundary element is stronger when the enhancer and promoter are in *cis* than when they are in a *trans* configuration.

The other version of boundary-element-induced transvection that we observed is the *trans* combination between the boundary insertions and the *ap* wing-enhancer deletion, ap^{DG}. This combination was tested for the *Mcp* and *su(Hw)* inserts and in both cases the wing phenotype of the enhancer deletion was suppressed (Figure 8, B, D, and E). Since the extent of suppression in both cases is considerably less than seen when the enhancer deletion ap^{DG} is combined with the promoter deletion ap^{UGO35}, it would appear that the

boundary in *cis* to the enhancer is able to partially block its interactions with the *ap* promoter in *trans*. As noted above, the converse is also true: boundary elements in *trans* to the enhancer are able to partially block interactions with the *ap* promoter in *cis*.

Since these results demonstrate that the *Mcp* and *su(Hw)* boundaries can act not only in *cis* but also in *trans*, one might predict either that no interallelic complementation would be observed when two different boundary inserts are combined or that the phenotype would actually become even stronger because of the ability of boundaries to inhibit regulatory interactions in *trans*. Surprisingly, however, neither of these expectations holds. Instead, flies *trans*-heterozygous for the *Mcp* insert $ap^{MM\text{-}Mcp}$, and the *su(Hw)* insert ap^{f00451} have completely wild-type wings (Figure 7, A–C). One mechanism that could account for this unexpected result is insulator bypass. Studies on the *su(Hw)* insulator have shown that enhancer-blocking activity is neutralized when there are two copies of this element in tandem between the enhancer and the promoter (Cai and Shen 2001; Muravyova *et al.* 2001). While bypass is thought to involve *su(Hw)*-pairing interactions, other insulators, including *Mcp*, can be substituted for one of the two *su(Hw)* elements (Melnikova *et al.* 2004). A strong prediction of the insulator bypass model is that interallelic complementation should also be observed when the *su(Hw)* element in ap^{f00451} is in *trans* to the enhancer deletion that retains an intact *Mcp* element, $ap^{DG\text{-}Mcp}$. However, this is not the case as the wing phenotype of $ap^{DG\text{-}Mcp}/ap^{f00451}$ *trans*-heterozygotes is the same as that of ap^{f00451} alone (Figure 8, A and C). This result indicates that the *Mcp* element is able to prevent *trans* activation of the *ap* promoter in *cis* by the wing enhancers on the ap^{f00451} chromosome. The ability to block enhancers on the *trans* chromosome from contacting the promoter in *cis* to a boundary element was also observed when $ap^{MM\text{-}Mcp}$ is combined with the *Mcp*-containing enhancer deletion $ap^{DG\text{-}Mcp}$ (Figure 8A).

Thus, the interallelic complementation observed in $ap^{MM\text{-}Mcp}/ap^{f00451}$ flies is not likely to be an instance of insulator bypass. Instead, it seems that the additive effects of the unblocked, *ap* proximal portion of the ap^{f00451} enhancer and *trans* activation by the enhancer on the $ap^{MM\text{-}Mcp}$ chromosome (similar to that observed in Figure 8, B, D, and E) can account for the wild-type wings of $ap^{MM\text{-}Mcp}/ap^{f00451}$ flies (Figure 9A).

Enhancer blocking by boundary elements and transvection: Including the studies reported here on boundary insertions in the *ap* locus, there are now several examples in which the blocking activity of a boundary element can be partially bypassed by interactions between enhancers on one chromosome and the target gene/promoter on the other chromosome (Peifer and Bender 1986; Geyer *et al.* 1990; Morris *et al.* 1998, 1999a; Golovnin *et al.* 2003). These findings raise the question of why boundary elements are more permissive for regulatory interactions in *trans* than they are for interactions in *cis*.

Answering this question depends upon how enhancers communicate with promoters and how boundaries block this communication. Two general models have been proposed to explain how enhancers interact with their target promoters (West and Fraser 2005). In the first model, the enhancer (or an activator molecule recruited by the enhancer) processively tracks along the chromosome (perhaps modifying the intervening chromatin) until it encounters the promoter. In this model, boundary elements function as roadblocks (or "promoter decoys"), stopping the tracking activator and/or the spread of active chromatin (West *et al.* 2002). As this model requires the enhancer to act in *cis*, it is difficult to reconcile it with the phenomenon of transvection, which depends upon regulatory interactions occurring in *trans*. In addition, if transvection is explained in this model by postulating that the tracking activator skips from one paired chromosome to the other, then it is hard to understand how a boundary element would ever be able to prevent an enhancer from activating a promoter since an activator molecule that can skip freely in *trans* should also be able to skip over a boundary in *cis*.

The second model, which is strongly supported by recent studies, hypothesizes that the sliding of the chromatin fiber against itself within a higher-order chromatin domain brings the enhancer and promoter into contact while looping out the intervening DNA (Carter *et al.* 2002; Tolhuis *et al.* 2002; Spilianakis and Flavell 2004; Petrascheck *et al.* 2005; Spilianakis *et al.* 2005; Lomvardas *et al.* 2006). This is more easily reconciled with transvection since the enhancer could interact with a promoter in *trans* by a similar sliding-looping mechanism as long as the chromatin fibers of the two chromosomes are paired. Indeed, chromosomal rearrangements that disrupt pairing also tend to disrupt transvection (Lewis 1954; Gelbart 1982; Leiserson *et al.* 1994; Wu and Morris 1999; Duncan 2002; Coulthard *et al.* 2005). In this model, boundary elements prevent enhancer–promoter contact by isolating the enhancer and the promoter from each other in topologically independent looped domains. It is thought that boundaries generate topologically independent looped domains through pairing interactions with the neighboring boundaries (or by interacting with some fixed structure such as the nuclear matrix) (reviewed in West *et al.* 2002). This mechanism is supported by studies on *su(Hw)*, *scs/scs′*, and several boundaries from the Drosophila BX-C (Sigrist and Pirrotta 1997; Müller *et al.* 1999; Gerasimova *et al.* 2000; Cai and Shen 2001; Muravyova *et al.* 2001; Bantignies *et al.* 2003; Blanton *et al.* 2003; Byrd and Corces 2003; Gruzdeva *et al.* 2005; Vazquez *et al.* 2006). For example, pairing between tandem *su(Hw)* insulators neutralizes their boundary function, enabling an upstream enhancer to activate a downstream promoter

(Cai and Shen 2001; Muravyova *et al.* 2001). According to this model for enhancer blocking, the *Mcp* [or *su(Hw)*] boundary would isolate the *ap* wing enhancer from the *ap* promoter in *cis* through interactions with the hypothetical upstream and downstream boundaries that define the *ap* domain.

This mechanism for boundary function in *cis* still leaves open the question of why boundaries can be partially bypassed in *trans*. One possibility is that pairing interactions between boundaries occur not only in *cis* but also in *trans*. In this model, the arrangement of loop domains would be the same on each chromosome when they both contain the *Mcp* or *su(Hw)* boundary insert—there would be two loops, one containing the *ap* enhancer and the other containing the *ap* promoter. These loops would be generated by interactions between *Mcp* and the neighboring proximal and/or distal boundaries. The situation would be more complicated when one chromosome has the boundary element insertion and the other does not. In this case, the wild-type chromosome should have a single *ap* loop containing both the enhancer and the promoter, while the chromosome containing *Mcp* should have two loops, one containing the enhancer and the other the promoter. However, this arrangement of loops on the two chromosomes might be dynamically unstable if *trans*-boundary interactions also tend to stabilize *cis* contacts between the boundary elements that flank the *ap* locus. This dynamic instability could disrupt or weaken *cis* interactions between *Mcp* and the boundaries flanking the *ap* locus. In this case, the arrangement of loops on the *Mcp*-containing chromosome might switch back and forth from two to one, permitting a partial bypass of *Mcp* through *trans*-regulatory interactions.

While both the *Mcp* and *su(Hw)* boundary elements can be partially bypassed by interactions between the *ap* enhancer and promoter in *trans*, *trans* interactions do not occur when the same boundary insertion is present on both homologs. On the other hand, when the *Mcp* and *su(Hw)* boundary insertions are present in *trans* on the two chromosomes ($ap^{MM\text{-}Mcp}/ap^{f00451}$), this seems to abrogate their blocking activity. One explanation for this effect is that *Mcp* and *su(Hw)* are unable to interact with each other; however, it was previously demonstrated that *su(Hw)* and *Mcp* can pair with one another, possibly through the interaction of GAGA factor and Mod(mdg4) (Melnikova *et al.* 2004). Since the *Mcp* and *su(Hw)* boundary insertions are located at distant sites within the *ap* locus, another possibility is that the pairing of the two structurally dissimilar alleles in this arrangement results in conformational stress that precludes the formation of stable *Mcp/su(Hw)* interactions either with each other or with the hypothetical flanking *ap* boundaries (Morris *et al.* 1998). In this model (illustrated in Figure 9B), homologous pairing between sequences in the *ap* locus would loop out the transposons containing the *Mcp* and *su(Hw)* boundary elements, preventing them from blocking enhancer–promoter contacts. An alternative possibility is that boundary interactions occur only in pairwise combinations. Thus, instead of interacting simultaneously with the boundaries that flank the *ap* locus, *Mcp* and *su(Hw)* might be paired only with either the upstream or the downstream *ap* boundary at a given time. If the pairing of *Mcp* and *su(Hw)* with the flanking boundaries occurs independently [or if *Mcp* and *su(Hw)* differ in their pairing preferences], either of these distinct domains might be predicted to confer enhancer blocking to both homozygous or hemizygous flies. However, when these two alleles are crossed together, the domains in effect would be complementary, with one unblocked enhancer and one unblocked *ap* gene (Figure 9C). It may be possible to distinguish between these different models by generating new insertions into the *ap* locus in which the *Mcp* and *su(Hw)* boundaries are brought closer together and by substituting other boundary elements for *Mcp* or *su(Hw)*.

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