Nipped-B, a Drosophila Homologue of Chromosomal Adherins, Participates in Activation by Remote Enhancers in the *cut* and *Ultrabithorax* Genes

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ABSTRACT

How enhancers are able to activate promoters located several kilobases away is unknown. Activation by the wing margin enhancer in the *cut* gene, located 85 kb from the promoter, requires several genes that participate in the Notch receptor pathway in the wing margin, including *scalloped*, *vestigial*, *mastermind*, *Chip*, and the *Nipped* locus. Here we show that *Nipped* mutations disrupt one or more of four essential complementation groups: *l(2)41Ae*, *l(2)41Af*, *Nipped-A*, and *Nipped-B*. Heterozygous *Nipped* mutations modify Notch mutant phenotypes in the wing margin and other tissues, and magnify the effects that mutations in the *cis* regulatory region of *cut* have on *cut* expression. *Nipped-A* and *l(2)41Af* mutations further diminish activation by a wing margin enhancer partly impaired by a small deletion. In contrast, *Nipped-B* mutations do not diminish activation by the impaired enhancer, but increase the inhibitory effect of a *gypsy* transposon insertion between the enhancer and promoter. *Nipped-B* mutations also magnify the effect of a *gypsy* insertion in the *Ultrabithorax* gene. *Gypsy* binds the Suppressor of Hairy-wing insulator protein [Su(Hw)] that blocks enhancer-promoter communication. Increased insulation by Su(Hw) in *Nipped-B* mutants suggests that *Nipped-B* products structurally facilitate enhancer-promoter communication. Compatible with this idea, Nipped-B protein is homologous to a family of chromosomal adherins with broad roles in sister chromatid cohesion, chromosome condensation, and DNA repair.

INTERACTIONS between transcription activators and promoters can be accommodated by DNA looping when the activator and promoter are within several hundred base pairs of each other (reviewed in Ptashne 1986, 1988). Passive DNA looping is not always sufficient. For example, activation of the *Klebsiella pneumoniae nifH* promoter by the NifA protein requires binding of integration host factor (IHF) between NifA and the promoter (Santero *et al.* 1992). The NifA binding site is ~130 bp upstream of the transcription start site, and IHF bends the DNA to bring the activator into proximity of the promoter. Similarly, interactions between different proteins binding to the same eukaryotic enhancer are facilitated by formation or deformation of DNA bends by high mobility group (HMG) proteins such as LEF-1 (Giese *et al.* 1992, 1995) and HMG I(Y) (Falvo *et al.* 1995; Thanos and Maniatis 1995). Thus, IHF, LEF-1, and HMG I(Y) play architectural roles and help form structures that facilitate interactions between other proteins.

Many metazoan genes contain remote enhancers located several kilobases from the promoter. This implies that in addition to architectural factors such as HMG proteins that facilitate interactions over short distances, higher eukaryotes also have factors that act between enhancers and promoters to facilitate communication over many kilobases.

The Su(Hw) insulator protein encoded by the *suppressor of Hairy-wing* [*su(Hw)*] gene of Drosophila interferes with enhancer-promoter communication. Su(Hw) binds a DNA sequence in the *gypsy* transposon (Dorsett 1990; Spana and Corces 1990). When *gypsy* inserts into a gene, enhancer-promoter interactions are blocked in a Su(Hw)-dependent manner (Geyer *et al.* 1990; Holdridge and Dorsett 1991; Jack *et al.* 1991; Geyer and Corces 1992; Dorsett 1993; Cai and Levine 1995; Scott and Geyer 1995). Only the Su(Hw)-binding region of *gypsy* is required to block enhancers (Holdridge and Dorsett 1991; Geyer and Corces 1992). Enhancers located promoter-distal to Su(Hw) do not activate, while enhancers promoter-proximal to Su(Hw) function normally. Enhancers blocked by Su(Hw) can still activate a second promoter in the other direction (Cai and Levine 1995; Scott and Geyer 1995), indicating that Su(Hw) does not inactivate enhancers but interferes with their ability to communicate with the promoter.

Su(Hw) blocks virtually all enhancers. Where examined, the same region in Su(Hw) is required, despite a wide diversity in genes and enhancers (Harrison *et al.* 1993; Kim *et al.* 1996b). This implies that Su(Hw) blocks

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enhancers in different genes by the same mechanism, and therefore that different genes use related mechanisms to facilitate enhancer-promoter communication.

Gypsy insertions in *cut* block a remote wing margin enhancer located 85 kb upstream of the promoter (Jack *et al.* 1991; Dorsett 1993). Failure of this enhancer to activate *cut* results in a cut wing phenotype in which most of the cells that form the adult wing margin are missing. The severity of this phenotype is sensitive to small differences in Su(Hw) insulator activity (Dorsett 1993). This sensitivity was exploited to find mutations that reduce activation by the wing margin enhancer in the presence of a *gypsy* insertion (Morcillo *et al.* 1996, 1997). In addition to enhancer-binding activators, these screens could identify architectural factors that act between enhancers and promoters to facilitate communication.

Previously these screens have identified mutations in two known genes, *scalloped* (*sd*) and *mastermind* (*mam*), and a novel gene named *Chip* (Morcillo *et al.* 1996, 1997). Genetic and biochemical evidence indicates that Sd and Mam are enhancer-binding factors (Morcillo *et al.* 1996). Chip, consistent with a potential role in enhancer-promoter communication, is a ubiquitous chromosomal protein that supports activation by several enhancers (Morcillo *et al.* 1997).

Here we characterize *Nipped*, an essential locus isolated in the same screens that identified the other *cut* regulators. We find that *Nipped* includes multiple essential complementation groups that play distinct roles both in regulating *cut* and in Notch receptor signaling. The *Nipped-B* complementation group is particularly antagonistic to the *gypsy* insulator in *cut* and *Ultrabithorax*. Strikingly, Nipped-B protein is homologous to a family of chromosomal adherins that participate diversely in DNA repair, chromosome compaction, and sister chromatid cohesion. We postulate that Nipped-B protein functions architecturally between enhancers and promoters to facilitate enhancer-promoter interactions.

MATERIALS AND METHODS

Drosophila culture: Flies were raised on cornmeal, yeast, and molasses medium (Wirtz and Semey 1982) at 25°. Crosses were performed in glass shell vials with 5–10 males and 10–15 females. Parents were transferred every 3–4 days, and progeny were scored for 10 days of eclosion.

Genetic screens: The screens for mutations that enhance the cut wing phenotype of $ct^{L\text{-}32}$; $su(Hw)^{e2}$ flies were previously described (Morcillo *et al.* 1996, 1997). After backcrossing to the parental $ct^{L\text{-}32}$; $su(Hw)^{e2}$ stocks for several generations, all homozygous lethal mutations on chromosome 2 were balanced over *In(2LR)CyO, Cy, Df(2R)Kr4*, Kr^{B80}, $Dp(1;2)y^{+}$ in a *y w* mutant background.

Complementation tests and mapping of mutations: Lethal complementation tests were performed by crossing balanced mutants to each other and scoring for progeny lacking the balancer. Arthur Hilliker (University of Guelph) provided *l(2)41Ae, l(2)41Af,* and *l(2)41Ah* mutants and deficiencies in the 41A region. Complementation tests with known wing development mutations were performed by crossing the balanced lethal mutants to homozygous or balanced known mutants and scoring for progeny lacking balancers. All known mutants were previously described (Dorsett 1993; Morcillo *et al.* 1996, 1997) or were obtained from the Bloomington stock center at Indiana University. *Nipped* mutations were mapped by recombination using their lethal phenotypes and *P*-element markers as previously described (Morcillo *et al.* 1996).

Determination of *Nipped* mutant lethal phases: *Nipped* mutant lethal phases were determined by scoring larval mouthparts in the balanced stocks for the *yellow* (*y*) marker as previously described (Morcillo *et al.* 1996). Mutant mouthparts indicate that the larvae are homozygous or heteroallelic for the *Nipped* mutations, and wild-type mouthparts indicate presence of the balancer. Approximately 100 each of first, second, and third instar larvae were scored.

Quantification of the effects of *Nipped* mutations on cut wing and bithorax mutant phenotypes: *y w* $ct^{L\text{-}32}$; $su(Hw)^{e2}$ bx^{34e} flies heterozygous for *Nipped* mutations were generated by crossing balanced *Nipped* mutant chromosomes into a *y w* $ct^{L\text{-}32}$; $su(Hw)^{e2}$ bx^{34e} stock. The *Nipped* mutant chromosomes were marked with a *P* element containing a mini-*white* gene, allowing *Nipped* mutant progeny to be distinguished in the *y w* background (Morcillo *et al.* 1997). Effects of *Nipped* mutations on ct^{53d} were tested by crossing balanced *Nipped* mutant males to ct^{53d} females and scoring male progeny wing margins. Effects of *Nipped* mutations on heterozygous ct^{2s} were quantitated by crossing balanced *Nipped* mutant males to ct^{2s} females and scoring female progeny. Control progeny were generated by conducting the same crosses with *y w*, *Oregon R*, and *Nipped* parental stocks.

Cut wing margins were quantitated as previously described (Dorsett 1993). The scale ranged from 0 to 31 nicks, with 31 nicks per fly being given to any phenotype stronger than 30 nicks per fly. Bithorax mutant phenotypes were scored as described previously (Morcillo *et al.* 1996). The scores range from 0, which is wild-type phenotype, to 10, which is the phenotype displayed by homozygous bx^{34e} flies wild type for *su(Hw)*.

Genetic interaction experiments: Flies transheterozygous for a *Nipped* mutation and a mutation in another gene were generated by crossing flies with a balanced *Nipped* allele to flies with a balanced mutation in the other gene. Progeny lacking balancers were scored for margin nicks, wing size, wing vein, eye morphology, or bristle defects. Controls were generated by crossing balanced *Nipped* mutants to the *Nipped* mutant parental stock, a *y w* stock, or Oregon-R wild-type flies. *Su(H)* hypermorphs and *Abruptex* mutants were provided by Mark Fortini (University of Pennsylvania). Other mutant *Notch* alleles were provided by Michael Young (Rockefeller University).

For scanning electron microscopy, live flies were mounted on stubs using superglue, and the area surrounding the flies covered with conductive carbon paint. After air-drying overnight, the samples were dried in a vacuum dessicator, sputter coated with gold/platinum, and photographed in a scanning electron microscope at ×180 magnification for eyes and ×78 for bristles.

Reversion of the *l(2)02047 P*-element insertion: *l(2)02047/CyO;* ry^{506} females (stock obtained from the Bloomington stock center, Indiana University) were crossed to *CyO, HOP2/Bc Elp* males (stock obtained from William Gelbart, Harvard University). F_1 *CyO, HOP2/l(2)02047* males were backcrossed to *l(2)02047/CyO;* ry^{506} females. Excision events were recovered as *ry* mutant progeny with *Cy* wings, and *l(2)02047* revertant chromosomes were recovered from Cy^{+} progeny. Excision and revertant chromosomes were tested for the ability to complement *Nipped-B* mutations.

Rescue of the *l(2)02047 P* element from genomic DNA: Genomic DNA from homozygous *l(2)02047* second instar lar-

vae was digested with *Xba*I, religated, and used to transform *Escherichia coli* using the kanamycin resistance gene in the *P{RZ}* transposon (Mlodzik and Hiromi 1992).

Isolation of *Nipped* genomic DNA: A 2.5-kb *Xba*I-*Hin*dIII fragment of genomic DNA flanking the rescued *l(2)02047 P* element was used to probe Southern blots of an *Eco*RI digest of the DS08617 P1 phage (obtained from the University of Wisconsin collection) using the procedures previously described (Morcillo *et al.* 1997). Two neighboring *Eco*RI fragments of 8 and 5 kb in length hybridizing to the probe were subcloned into a pBluescript (SK+) plasmid vector.

RNA preparation and Northern blot hybridization: RNA isolation and Northern blot hybridization were performed as previously described (Dorsett *et al.* 1989). Single-stranded [^{32}P]RNA probes were prepared from several restriction fragments spanning the length of the 13-kb region containing the *l(2)02047 P*-insertion site. Northern blots were stripped as previously described (Dorsett *et al.* 1989) and reprobed with *rp49* antisense probes as a loading control.

***Nipped-B* cDNA cloning:** A third instar imaginal disc cDNA library in λgt10 (provided by Jaeseob Kim, University of Wisconsin) was screened as previously described (Morcillo *et al.* 1997) using the 2.5-kb *Xba*I-*Eco*RI fragment located ~4 kb from the *l(2)02047 P*-element insertion site as a probe. Six hybridizing plaques were plaque purified, DNA was prepared, and the *Eco*RI phage inserts were cloned into pBluescript (SK+) plasmid vector. Restriction maps revealed that five of the phage inserts are overlapping. The largest insert (clone 6-1) is 6.3 kb in length and contains the 3′ end of the open reading frame (ORF). It was sequenced in both directions by the DNA Sequencing Facility at Cornell University. An overlapping 3-kb insert (clone 3-1) containing the 5′ end of the ORF was sequenced using Sequenase (United States Biochemicals, Cleveland) according to the manufacturer's recommendations. Database searches were performed using NCBI Blast programs (Altschul *et al.* 1997), and other sequence analysis was performed using MacVector software. The *Nipped-B* cDNA sequence has been deposited in GenBank under accession no. AF114160.

RESULTS

To identify genes that may encode architectural factors that support activation by the remote wing margin enhancer in *cut*, we screened for mutations that diminish *cut* expression. Most wing margin cells are lost when the wing margin enhancer is blocked by a *gypsy* insertion. Intermediate phenotypes with nicks in the wing margin occur when the wing margin enhancer is partially blocked by *gypsy*. The screens exploited the intermediate phenotype produced by the $ct^{L\text{-}32}$ *gypsy* insertion (Figure 1) when partially suppressed by the leaky $su(Hw)^{e2}$ mutation (Morcillo *et al.* 1996, 1997). These flies display ~0.01 wing margin nicks per fly, indicating that *cut* gene activity in the wing margin is less than half wild type. We reasoned that partial loss of enhancer-binding proteins or architectural factors that facilitate communication with the promoter should decrease activation by the enhancer and increase the number of wing margin nicks. In the screens for dosage-sensitive modifiers, which are described elsewhere (Morcillo *et al.* 1996, 1997), mutagenized $ct^{L\text{-}32}$; $su(Hw)^{e2}$ males were mated to $ct^{L\text{-}32}$; $su(Hw)^{e2}$ females, and progeny with two or more wing margin nicks were tested for the presence of heritable mutations.

Figure 1.—Lesions affecting the remote wing margin enhancer in the *cis* regulatory region of the Drosophila *cut* locus. The 2.7-kb fragment containing the *cut* wing margin enhancer (hatched box labeled "wm"; Jack *et al.* 1991) is ~85 kb upstream of the promoter (angled arrow) in the absence of a *gypsy* insertion (7.5 kb). The *gypsy* long terminal repeats (LTRs) are indicated by open boxes, and the Su(Hw) insulator protein-binding region by a filled circle. The extents of the ct^{2s} and ct^{53d} deletions are shown underneath by thick lines. ct^{2s} displays an extreme recessive cut wing phenotype (Mogila *et al.* 1992). ct^{53d} deletes ~0.5 kb and displays a weak recessive cut wing phenotype (Jack 1985). The $ct^{L\text{-}32}$ *gypsy* insertion blocks the wing margin enhancer and displays a strong recessive cut wing phenotype in the presence of wild-type *su(Hw)*.

Most mutations recovered in these screens are recessive lethal. Screening of ~30,000 progeny (~8,000 by EMS and ~22,000 by γ-ray mutagenesis) identified the *sd*, *mam*, and *Chip* genes (Morcillo *et al.* 1996). An additional screen of ~220,000 progeny by γ-ray mutagenesis was used to isolate additional *Chip* alleles (Morcillo *et al.* 1997). The larger screen also identified several additional *mam* alleles, three *vestigial* (*vg*) mutations, and ~30 alleles of a complex locus on chromosome 2 that we named *Nipped*. To understand the functions of *Nipped*, we characterized its multiple lethal complementation groups and their genetic interactions with *cut*.

***Nipped* mutations affect four lethal complementation groups near the chromosome 2 centromere:** The abilities of the *Nipped* mutant chromosomes to enhance the $ct^{L\text{-}32}$; $su(Hw)^{e2}$ cut wing phenotype are tightly linked with recessive lethal mutations. Dominant enhancement of the cut wing phenotype could not be separated from recessive lethality after multiple backcrosses to the parental $ct^{L\text{-}32}$; $su(Hw)^{e2}$ stock. As determined by segregation, several *Nipped* alleles are also translocations between chromosomes 2 and 3 (Table 1). As described below, where tested, the dominant effects of *Nipped* mutations on *cut* expression are mimicked by a deficiency, implying that the *Nipped* mutations are loss-of-function alleles.

Multiple *Nipped* mutations were mapped by recombination based on the recessive lethality to a position near the centromere on chromosome 2. Complementation tests with known deficiencies confirmed this location (Figure 2). All *Nipped* alleles are lethal over *Df(2R)M41A10* and *Df(2R)M41A8* and viable over *Df(2R)A″*. This places *Nipped* in the distal portion of 41A, near the heterochromatin-euchromatin boundary.

Three lethal complementation groups, *l(2)41Ae*, *l(2)41Af*, and *l(2)41Ah*, were previously identified in the

TABLE 1

Characterized mutant alleles of the *Nipped* locus

Lethal group[a]	Alleles[b]	Lethal phase[c]
l(2)41Ae	*34-14*[d]	Embryo
l(2)41Af	*45-72*[d]	Embryo
Nipped-A [*l(2)41Ah*$^-$]	*222.3, 323, T(2;3)394.2*[e]	Second–third instar molt
	226.1[f]*, 357.2*	Embryo / larval
	34-12[d]	Embryo
Nipped-B	*T(2;3)4, 292.1, T(2;3)359.1, 407*	Second–third instar molt
Nipped-C [*Nipped-A*$^-$ *Nipped-B*$^-$]	*160.1, T(2;3)138.2*[e]	Second–third instar molt
	25[g]	Embryo
Nipped-D [*l(2)41Af*$^-$ *Nipped-A*$^-$ *Nipped-B*$^-$]	*263.3, 341.1*	Embryo
Nipped-E [*l(2)41Ae*$^-$ *l(2)41Af*$^-$ *Nipped-A*$^-$ *Nipped-B*$^-$]	*43,299.1, 338*	Embryo

[a] *l(2)41Ae, l(2)41Af, Nipped-A* [*l(2)41Ah*], and *Nipped-B* complement each other for lethality; *Nipped-C* alleles fail to complement *Nipped-A* and *Nipped-B*; *Nipped-D* alleles fail to complement *l(2)41Af, Nipped-A,* and *Nipped-B*; *Nipped-E* alleles fail to complement *l(2)41Ae, l(2)41Af, Nipped-A,* and *Nipped-B* (see Figure 2).

[b] All alleles were produced by γ-ray mutagenesis unless indicated otherwise.

[c] Lethal phases, determined as described in the text, are those of the homozygous mutants unless indicated otherwise. All heteroallelic combinations of embryonic and larval lethal alleles tested ($Nipped\text{-}E^{43}$ / $Nipped\text{-}C^{160.1}$, $Nipped\text{-}D^{341.1}$ / $Nipped\text{-}B^{407}$, and $Nipped\text{-}A^{222.3}$ / $Nipped\text{-}E^{338}$) are larval lethal. All heteroallelic embryonic lethal combinations tested ($Nipped\text{-}E^{43}$ / $Nipped\text{-}E^{338}$, $Nipped\text{-}E^{43}$ / $Nipped\text{-}D^{341.1}$, and $Nipped\text{-}E^{338}$ / $Nipped\text{-}D^{341.1}$) are embryonic lethal.

[d] EMS-induced alleles provided by A. J. Hilliker (Hilliker 1976).

[e] Determined over the embryonic lethal $Nipped\text{-}E^{43}$ allele.

[f] Lethal over *Su(H)* null and gain-of-function alleles.

[g] Complements all *Nipped-A* alleles except *T(2;3)394.2* and *222.3*; semilethal over *34-12*.

portion of 41A containing *Nipped* (Figure 2; Hilliker 1976). The *Nipped* mutations were tested for which lethal complementation groups they disrupt by crossing them to each other and to representative alleles of the 41A lethals. Most *Nipped* mutations are lethal over each other. However, two smaller groups, *Nipped-A* and *Nipped-B*, complement each other and produce viable progeny. All other *Nipped* alleles are mutant for both *Nipped-A* and *Nipped-B. Nipped-B* mutations complement all three previously known 41A lethal mutations, and *Nipped-A* mutations complement all except *l(2)41Ah*. We conclude that *Nipped-B* is a unique complementation group and that *Nipped-A* is identical to *l(2)41Ah*.

The complementation tests divided several of the *Nipped* alleles mutant for both *Nipped-A* and *Nipped-B* into three classes: *Nipped-C*, *Nipped-D*, and *Nipped-E*. *Nipped-C* alleles fail to complement *Nipped-A* and *Nipped-B* mutations, but complement the *l(2)41Af* and *l(2)41Ae* mutations. *Nipped-D* mutations fail to complement *Nipped-A*, *Nipped-B*, and *l(2)41Af* mutations, but complement the *l(2)41Ae* mutation. *Nipped-E* mutations fail to complement all four lethal groups (Figure 2). Table 1 lists all the *Nipped-A* and *Nipped-B* mutations isolated in the screens and the *Nipped* alleles tested for complementation of all four lethal groups.

Only one of the characterized *Nipped* alleles displays ambiguous complementation behavior. $Nipped^{25}$ fails to complement all *Nipped-B* mutations but is lethal over some, but not all, *Nipped-A* alleles. $Nipped^{25}$ is lethal over $T(2;3)Nipped\text{-}A^{394.2}$ and $Nipped\text{-}A^{222.3}$, semilethal over $Nipped\text{-}A^{34\text{-}12}$, and viable over $l(2)41Af^{45\text{-}72}$ and $l(2)41Ae^{34\text{-}14}$. *Because* $Nipped^{25}$ is lethal over more than one *Nipped-A* allele, we classify it as a *Nipped-C* allele. Because it may be only weakly mutant for *Nipped-A*, we avoided use of $Nipped^{25}$.

***Nipped* mutant chromosomes do not contain mutations in known wing development genes:** It was important to confirm that the *Nipped* mutant chromosomes do not contain other mutations that affect wing development. Therefore, we tested all *Nipped* alleles for complementation of mutations in the known wing development genes on chromosome 2, including *apterous* (*ap*), *vg*, *wingless* (*wg*), *Suppressor of Hairless* [*Su(H)*], *mam*, and *Chip*. Only one *Nipped* mutant chromosome has a second mutation in a known gene. $Nipped\text{-}A^{226.1}$ is lethal over the $Su(H)^8$ and $Su(H)^2$ null alleles, and the $Su(H)^{16}$ gain-of-function allele. Like gain-of-function *Su(H)* alleles and unlike *Su(H)* loss-of-function mutations (Fortini and Artavanis-Tsakonas 1994), $Nipped\text{-}A^{226.1}$ suppresses the lethality caused by negative complementation between two *Abruptex* (Ax^{e2} and Ax^{9B2}) alleles of *Notch* (not shown). Other *Nipped* mutations do not suppress the *Ax* negative complementation (not shown), leading us to conclude that the $Nipped\text{-}A^{226.1}$ chromosome also contains a *Su(H)* gain-of-function mutation. We therefore avoided use of $Nipped\text{-}A^{226.1}$ in genetic experiments.

***Nipped* products are essential during embryonic and larval development:** The complementation tests do not distinguish whether the *Nipped* locus consists of multiple genes or whether it is a single transcription unit that

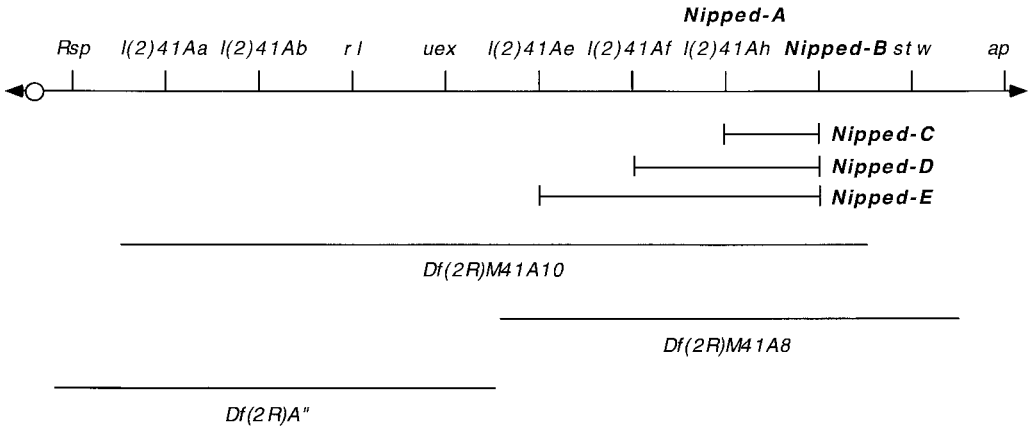

Figure 2.—Genetic map of the 41A region and the *Nipped* locus. The chromosome 2 centromere is indicated by an open circle. All *Nipped* mutations are lethal over *Df(2R)M41A10* and *Df(2R)M41A8*, and viable over the *Df(2R)A"* deficiency, placing them near the heterochromatin-euchromatin boundary. *Nipped-A* mutations complement all lethal mutations except *l(2)41Ah* (Hilliker 1976) and *Nipped-C, D,* and *E* mutations. *Nipped-B* mutations complement all except *Nipped-C, D,* and *E* mutations. *Nipped-C* alleles complement *l(2)41Ae* and *l(2)41Af* mutations (Hilliker 1976), and fail to complement *Nipped-A* [*l(2)41Ah*] and *Nipped-B* mutations. *Nipped-D* alleles complement *l(2)41Ae* mutations, and fail to complement *l(2)41Af*, *Nipped-A,* and *Nipped-B* mutations. *Nipped-E* mutations are lethal over *l(2)41Ae*, *l(2)41Af*, *Nipped-A,* and *Nipped-B* mutations.

produces multiple products. However, they demonstrate that *Nipped* has multiple individual functions essential for viability. *Nipped* is required for viability prior to expression of *cut* in the wing margin, which begins late in third instar larval development.

Three of the five *Nipped-A* alleles, including the 2;3 translocation, are lethal at the second to third instar larval transition (Table 1). *Nipped-A*$^{222.3}$ / *Nipped-E*^{338} heterozygotes also die at this stage. Two *Nipped-A* alleles, *Nipped-A*$^{357.2}$ and *Nipped-A*$^{226.1}$ (which also contains a *Su(H)* mutation), are primarily embryonic lethal, but produce a few larval escapers. The *Nipped-A*$^{34\text{-}12}$ allele is embryonic lethal, but this chromosome has been balanced for many years, and it may have acquired other lethals.

All four *Nipped-B* alleles, including the two translocations, are lethal at the second to third instar molt (Table 1). Two of the three *Nipped-C* alleles, including the translocation, are lethal at the same stage, as are *Nipped-C*$^{160.1}$ / *Nipped-E*^{43} heterozygotes. Because *Nipped-C* alleles are mutant for both *Nipped-A* and *Nipped-B*, this confirms that the second to third instar molt is the primary lethal phase for both *Nipped-A* and *Nipped-B*. As shown below for *Nipped-B*, it is possible that maternally supplied product allows survival to this stage.

All *Nipped-D* and *Nipped-E* alleles are homozygous embryonic lethal as are the two *Nipped-D* / *Nipped-E* combinations tested (Table 1). Because both *Nipped-D* and *Nipped-E* alleles are mutant for *l(2)41Af*, this indicates that embryogenesis is the lethal phase for the *l(2)41Af* complementation group. We are uncertain of the *l(2)41Ae* lethal phase because the only *Nipped* alleles mutant for *l(2)41Ae* are the *Nipped-E* alleles, which are also mutant for *l(2)41Af*. Although the *l(2)41Ae* allele is embryonic lethal, this chromosome has been balanced for many years and may have acquired additional lethals.

***Nipped* mutations magnify the effects of a *gypsy* transposon insertion in *cut*:** To ascertain the roles of the *Nipped* locus in regulating *cut*, heterozygous *Nipped* alleles were compared for their abilities to alter expression of different mutations in the *cis* regulatory region of *cut* (Figure 1; Table 2). The goal was to determine if any of the lethal groups in *Nipped* specifically magnify insulation by *gypsy* and Su(Hw). We quantitatively compared the abilities of several *Nipped* mutations to magnify the partially suppressed *ct*$^{L\text{-}32}$*; su(Hw)*^{e2} cut wing phenotype. To ensure accuracy, we avoided the *Nipped* alleles that have a second mutation in the same chromosome (*Nipped-A*$^{226.1}$), an ambiguous complementation pattern (*Nipped-C*^{25}), or an atypical lethal phase (*Nipped-A*$^{357.2}$ and *Nipped-A*$^{34\text{-}12}$). Because the *su(Hw)*^{e2} mutation could be lost from chromosome 3 during the balancing crosses, we also could not unambiguously test the translocation alleles [*T(2;3)Nipped-A*$^{394.2}$, *T(2;3)Nipped-B*^{4}, *T(2;3)Nipped-B*$^{359.1}$, and *T(2;3)Nipped-C*$^{138.2}$] with the *gypsy* insertion. These constraints allowed us to compare two of the five *Nipped-A* alleles (*Nipped-A*^{323} and *Nipped-A*$^{222.3}$) and two of the four *Nipped-B* alleles (*Nipped-B*$^{292.1}$ and *Nipped-B*^{407}). Of the *Nipped* mutations that affect multiple lethal groups, we were able to test one of the three *Nipped-C* alleles (*Nipped-C*$^{160.1}$), both of the *Nipped-D* alleles (*Nipped-D*$^{341.1}$ and *Nipped-D*$^{263.3}$), and all three *Nipped-E* alleles (*Nipped-E*$^{299.1}$, *Nipped-E*^{43}, and *Nipped-E*^{338}).

As expected, all *Nipped* alleles isolated in the screens dominantly increase the severity of the *ct*$^{L\text{-}32}$*; su(Hw)*^{e2} cut wing phenotype (Table 2). However, the two *Nipped-B* mutations give ~3- to 12-fold more wing margin nicks (1.2 and 4.8 nicks per fly) than the strongest *Nipped-A* mutation (0.4 nicks per fly). It is unlikely that the *Nipped-A* alleles are weaker mutations than the *Nipped-B* alleles because, as described below, these *Nipped-A* alleles have stronger effects than the *Nipped-B* alleles on other *cut* mutations. The *l(2)41Ae*$^{34\text{-}14}$ and *l(2)41Af*$^{45\text{-}72}$ mutations have no detectable effects on the *ct*$^{L\text{-}32}$*; su(Hw)*^{e2} phenotype, which explains why mutations disrupting only these lethal groups were not isolated in

TABLE 2

Interactions between *cut* mutations and heterozygous *Nipped* mutations

	Wing margin phenotype[a]		
Nipped allele	$ct^{L\text{-}32}/Y$; $su(Hw)^{e2}$	ct^{53d}/Y	$ct^{2s}/+$
$Nipped^{+}$[b]	<0.02	7.23 ± 0.10	<0.01
$l(2)41Ae^{34\text{-}14}$	<0.02	7.73 ± 0.17	0.05 ± 0.02
$l(2)41Af^{45\text{-}72}$	<0.02	20.32 ± 0.51	0.09 ± 0.05
$Nipped\text{-}A^{34\text{-}12}$		7.78 ± 0.14	
$Nipped\text{-}A^{323}$	0.41 ± 0.20	8.52 ± 0.12	<0.01
$Nipped\text{-}A^{222.3}$	0.16 ± 0.09	11.07 ± 0.21	0.25 ± 0.05
$T(2;3)Nipped\text{-}A^{394.2}$		12.70 ± 0.24	0.94 ± 0.12
$T(2;3)Nipped\text{-}B^{4}$		6.17 ± 0.12	0.02 ± 0.02
$T(2;3)Nipped\text{-}B^{359.1}$		7.34 ± 0.13	0.03 ± 0.02
$Nipped\text{-}B^{292.1}$	1.20 ± 0.34	7.63 ± 0.09	0.09 ± 0.03
$Nipped\text{-}B^{407}$	4.76 ± 0.82	7.98 ± 0.12	0.05 ± 0.02
$Nipped\text{-}C^{160.1}$	0.31 ± 0.14	6.75 ± 0.09	0.08 ± 0.03
$T(2;3)Nipped\text{-}C^{138.2}$		17.75 ± 0.28	0.06 ± 0.02
$Nipped\text{-}D^{341.1}$	0.91 ± 0.24	18.69 ± 0.34	0.48 ± 0.06
$Nipped\text{-}D^{263.3}$	0.74 ± 0.25	24.63 ± 0.48	0.76 ± 0.08
$Nipped\text{-}E^{299.1}$	1.90 ± 0.55	16.60 ± 0.49	0.45 ± 0.08
$Nipped\text{-}E^{43}$	1.09 ± 0.29	17.77 ± 0.23	0.43 ± 0.05
$Nipped\text{-}E^{338}$	0.48 ± 0.17	23.06 ± 0.34	0.68 ± 0.07
Df(2R)M41A8		>30	

[a] Wing margin nicks per fly; error values are standard errors. The number of flies scored ranged from 20 to 39 for $ct^{L\text{-}32}/Y$; $su(Hw)^{e2}$, 68 to 303 for ct^{53d}/Y, and 64 to 211 for $ct^{2s}/+$. See Figure 4 for sample phenotypes with ct^{53d}.

[b] From Oregon R; all $Nipped^{+}$ alleles tested gave virtually the same phenotypes.

our screens. None of the *Nipped-C*, *D*, and *E* alleles, which disrupt multiple lethal groups, magnify the $ct^{L\text{-}32}$; $su(Hw)^{e2}$ cut wing phenotype more than the strongest *Nipped-B* allele. Indeed, only one, $Nipped\text{-}E^{299.1}$ (1.9 nicks per fly), has a slightly larger effect than the weaker *Nipped-B* allele (1.2 nicks per fly). We deduce, therefore, that disruption of the *Nipped-B* lethal group causes most of the magnification of the *gypsy* insertion phenotype by the *Nipped-C*, *D*, and *E* alleles. The weaker effects of the $Nipped\text{-}C^{160.1}$ and $Nipped\text{-}E^{338}$ alleles relative to both *Nipped-B* mutations suggest that they may not be fully mutant for *Nipped-B*. Because the *Nipped* mutations were recently isolated in the same genetic background, do not contain mutations in the known wing development genes, and were crossed to the same $ct^{L\text{-}32}$; $su(Hw)^{e2}$ stock, the differences between the *Nipped* alleles are unlikely to be genetic background effects.

***Nipped-B* mutations amplify the effect of a *gypsy* insertion in *Ultrabithorax*:** To examine the possibility that *Nipped* mutations may also magnify the effect that *gypsy* insertions have on other genes, we tested to see if heterozygous *Nipped* mutations increase the effect of a *gypsy* insertion in *Ultrabithorax* (*Ubx*). The bx^{34e} *gypsy* insertion is in a transcribed region (Peifer and Bender 1986), but blocks activation by remote enhancers located in the *abx*/*bx* region, ~50 kb downstream of the *Ubx* promoter (Simon *et al.* 1990; Qian *et al.* 1993). Like *gypsy* insertions in *cut*, bx^{34e} is partially suppressed by the $su(Hw)^{e2}$ mutation (Dorsett 1993), providing a sensitive intermediate phenotype made more severe by partial loss of *Chip* activity (Morcillo *et al.* 1996).

The effects of *Nipped* mutations on $su(Hw)^{e2}$ bx^{34e} were compared with their $Nipped^{+}$ siblings. Neither of the *Nipped-A* mutations tested significantly alters the bithorax phenotype (Table 3; Figure 3). In contrast, both *Nipped-B* alleles dramatically increase the severity of the mutant phenotype three- to fourfold. Furthermore, the *Nipped-C* allele, both of the *Nipped-D* alleles, and two of the three *Nipped-E* alleles significantly amplify the mutant phenotype. Although the $Nipped\text{-}E^{338}$ allele has little effect, this *Nipped-E* allele also has the weakest effect on the *gypsy* insertion in *cut* (Table 2). None of the *Nipped-C*, *D*, or *E* alleles is more effective than the *Nipped-B* alleles, indicating that *Nipped-B* is responsible for the increased severity of the bithorax phenotype.

We conclude that relative to the other *Nipped* lethal complementation groups, mutations in *Nipped-B* more strongly intensify the effects of the *gypsy* insertions in both *cut* and *Ubx*. However, we do not think that *Nipped-B* regulates expression of *su(Hw)* or *gypsy*. As described below, *Nipped-B* mutants display weak cut wing phenotypes in the absence of *gypsy* insertions.

***Nipped* mutations amplify the effects of a deletion in the *cut* wing margin enhancer:** Although *Nipped-B* has greater effects on the *gypsy* insertions in *cut* and *Ubx* than other *Nipped* lethal groups, it was feasible that *Nipped-B*

TABLE 3

Interactions between the *bx^{34e} gypsy* insertion in *Ultrabithorax* and heterozygous *Nipped* mutations

	su(Hw)^{e2} bx^{34e} bithorax phenotype[a]	
Nipped allele	+ / +	*Nipped* / +
Nipped-A^{323}	2.3 ± 0.1	2.6 ± 0.2
Nipped-A$^{222.3}$	2.6 ± 0.2	2.9 ± 0.2
Nipped-B$^{292.1}$	2.5 ± 0.2	7.6 ± 0.1
Nipped-B^{407}	2.2 ± 0.2	8.0 ± 0.1
Nipped-C$^{160.1}$	1.9 ± 0.1	7.6 ± 0.1
Nipped-D$^{341.1}$	1.7 ± 0.1	5.5 ± 0.4
Nipped-D$^{263.3}$	1.9 ± 0.2	4.4 ± 0.3
Nipped-E$^{299.1}$	2.0 ± 0.2	4.2 ± 0.2
Nipped-E^{43}	2.1 ± 0.2	5.9 ± 0.3
Nipped-E^{338}	1.9 ± 0.1	2.6 ± 0.3

[a] Phenotypes of female siblings; 0 indicates wild-type phenotype, 10 indicates maximal bithorax mutant phenotype; errors are standard errors. The number of flies scored ranged from 20 to 39. *su(Hw)^{e2}* partially suppresses the *bx^{34e} gypsy* insertion so that *su(Hw)^{e2} bx^{34e}* flies display a phenotype between 2 and 2.5 in the absence of other mutations. See Figure 3 for sample phenotypes. Virtually identical results were obtained with the male siblings.

products might simply be more limiting for *cut* expression than other *Nipped* products. If so, then *Nipped-B* mutations should also have stronger effects on other types of *cut* mutants. To test this we quantitatively compared heterozygous *Nipped* alleles for their ability to magnify the severity of the partial cut wing phenotype of *ct^{53d}*, a 0.5-kb deletion in the wing margin enhancer (Figure 1). Hemizygous *ct^{53d}* males display ~7 nicks per fly in *Nipped^{+}* backgrounds (Table 2; Figure 4). This partial phenotype presumably results from changes in the quantity or composition of activation complexes that form on the enhancer.

In contrast to their strong effects on the *cut gypsy* insertion, the *Nipped-B$^{292.1}$* and *Nipped-B^{407}* mutations do not magnify the *ct^{53d}* mutant phenotype (Table 2). The two *Nipped-B* translocations also do not magnify the *ct^{53d}* mutant phenotype. In contrast, the *l(2)41Af$^{45-72}$* mutation and three of four *Nipped-A* mutations increase the severity of the *ct^{53d}* cut wing phenotype (Table 2; Figure 4). The *l(2)41Ae$^{34-14}$* mutation has little effect on *ct^{53d}* (Table 2; Figure 4). These results indicate that opposite to what is observed with the *ct^{L-32} gypsy* insertion, *Nipped-A* or *l(2)41Af* products are more limiting than *Nipped-B* products for *cut* expression in *ct^{53d}* mutants.

Comparison of the strongest *Nipped-C*, *D*, and *E* alleles suggests that the effects of the individual *Nipped* lethal complementation groups on *ct^{53d}* are additive and confirms that *l(2)41Ae* has little or no effect (Table 2; Figure 4). We postulate that the *Nipped* mutations are hypomorphic and that the alleles with the strongest effects are the most mutant. Confirming this idea, *Df(2R)M41A8*, which deletes all four lethal groups (Figure 2), has the strongest effect, increasing the number of nicks more than 4-fold (Table 2). *T(2;3)Nipped-A$^{394.2}$* is the strongest *Nipped-A* allele, giving a 1.8-fold increase in the number of wing margin nicks over the controls. *T(2;3)Nipped-C$^{138.2}$*, which disrupts both *Nipped-A* and *Nipped-B*, increases the number of nicks ~2.5-fold over the controls. *Nipped-D$^{263.3}$*, which is mutant for *l(2)41Af*, *Nipped-A*, and *Nipped-B*, has a stronger effect, increasing the number of nicks ~3.5-fold. *Nipped-E^{338}*, which is mutant for all four lethal groups, has a similar effect as *Nipped-D$^{263.3}$*, confirming that *l(2)41Ae* has little or no effect on *ct^{53d}*. We conclude, therefore, that the effects of *Nipped-A* and *l(2)41Af* mutations on the *ct^{53d}* enhancer deletion are additive.

It was possible that the effects of *Nipped* mutations on *ct^{53d}* may depend on the particular sequences deleted from the wing margin enhancer. *ct^{2s}* is a larger deletion that removes virtually all of the enhancer (Figure 1; Mogila *et al.* 1992). Homozygous *ct^{2s}* females display an extreme cut wing phenotype, while heterozygous females have a wild-type phenotype. We previously observed that loss-of-function mutations in *sd*, which encodes a protein that binds the enhancer (Morcillo *et*

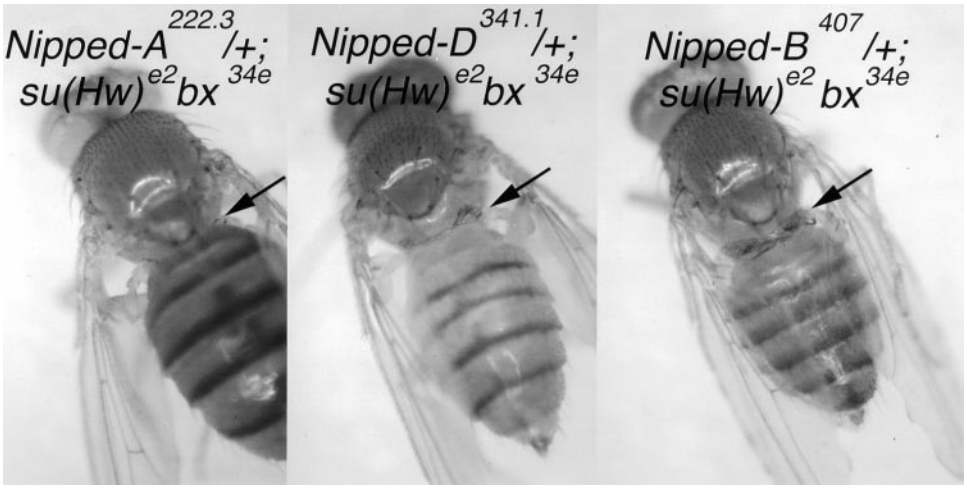

Figure 3.—Dominant enhancement of the *su(Hw)^{e2} bx^{34e}* bithorax phenotype by some *Nipped* mutations. Dorsal views of representative flies with the indicated genotypes are shown. *Nipped-A$^{222.3}$* / +; *su(Hw)^{e2} bx^{34e}* (left) displays a weak bithorax phenotype indistinguishable from *Nipped^{+}* controls (not shown). The arrow points to extra dorsal bristles. *Nipped-D$^{341.1}$* / + (middle) and *Nipped-B^{407}* / + (right) are strongly enhanced, with increases in thoracic cuticle and bristles between the thorax and abdomen (arrows). See Table 3 for quantitated phenotypes with these and other *Nipped* alleles.

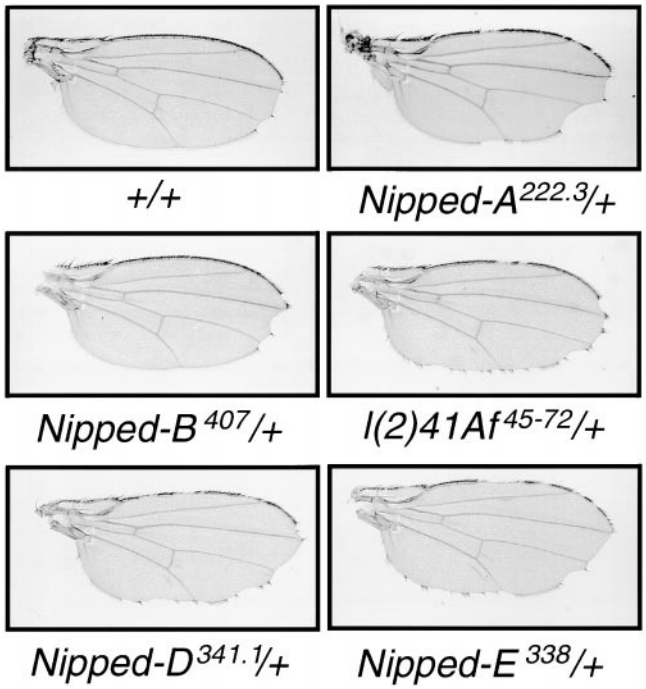

Figure 4.—Dominant enhancement of the ct^{53d} partial cut wing phenotype by some *Nipped* mutations. Representative wings from ct^{53d} males with the indicated genotypes are shown. A control ct^{53d} wing with a few margin nicks is shown in the upper left (+/+). Little or no effect is observed with *Nipped-B*^{407} (middle left), while the number of margin nicks is increased with *Nipped-A*$^{222.3}$ (top right), *l(2)41Af*$^{45\text{-}72}$ (middle right), *Nipped-D*$^{341.1}$ (bottom left), and *Nipped-E*^{338} (bottom right). See Table 2 for quantitated phenotypes with these and other *Nipped* alleles.

al. 1996), and null alleles of *mam*, which encodes a protein that binds chromosomes in the vicinity of *cut* (Bettler *et al.* 1996), both dominantly enhance heterozygous ct^{2s} females to produce cut wing phenotypes (Morcillo *et al.* 1996). Presumably, these phenotypes occur because reducing the amount of enhancer-binding protein reduces activation of *cut* transcription by the solo wild-type enhancer present in ct^{2s} heterozygotes.

All four *Nipped-B* alleles exhibit weak but significant cut wing phenotypes in the presence of heterozygous ct^{2s} (0.02–0.09 nicks per fly; Table 2). Importantly, this verifies that *Nipped-B* regulates *cut* expression in the absence of a *gypsy* insertion. However, two of the three heterozygous *Nipped-A* mutations tested display more severe cut wing phenotypes (0.25 and 0.94 nicks per fly; Table 2) in the presence of heterozygous ct^{2s}, indicating that *Nipped-A* is more limiting for *cut* expression than *Nipped-B*. *Nipped-A*^{323} does not show a phenotype with ct^{2s}, but this allele also has a weaker effect on ct^{53d}, suggesting that it is a hypomorph. The *Nipped-C*$^{160.1}$ mutation, which has no detectable effect on ct^{53d}, also displays a weak cut wing phenotype with heterozygous ct^{2s} (0.08 nicks per fly; Table 2), suggesting that it is weakly mutant for *Nipped-A*. Surprisingly, *T(2;3)Nipped-C*$^{138.2}$, which has a strong effect on ct^{53d}, displays a weak cut wing phenotype in combination with ct^{2s} (0.06 nicks per fly; Table 2). This is the only *Nipped* allele affecting multiple lethal groups, however, in which the effect on ct^{53d} does not correlate with the effect on ct^{2s}. Thus, both *Nipped-D* and all three *Nipped-E* alleles magnify the effect of the ct^{53d} lesion and display strong cut wing phenotypes with heterozygous ct^{2s} (0.43–0.76 nicks per fly; Table 2). None of the *Nipped-D* and *E* alleles has stronger effects on heterozygous ct^{2s} than the strongest *Nipped-A* alleles, suggesting that most of the effect is the result of disrupting *Nipped-A*. Confirming this idea, *l(2)41Af*$^{45\text{-}72}$ has only a weak effect on ct^{2s} (Table 2). Combined, the results with ct^{53d} and ct^{2s} confirm that *Nipped-B* is more limiting for *cut* expression than the other *Nipped* products only when there is a *gypsy* insulator insertion between the enhancer and promoter.

***Nipped* mutations do not cause bithorax phenotypes with heterozygous deletions in *Ubx*:** The observation that several *Nipped* mutations cause cut wing phenotypes in combination with heterozygous ct^{2s} led us to consider the possibility that some *Nipped* mutations might also cause bithorax phenotypes in combination with heterozygous deletions in *Ubx*. However, none of the *Nipped* mutations tested, including *Nipped-A*$^{222.3}$, *Nipped-A*^{323}, *Nipped-B*$^{292.1}$, *Nipped-B*^{407}, *Nipped-D*$^{341.1}$, and *Nipped-E*^{338}, resulted in mutant phenotypes in combination with heterozygous $bx^{34ePartRev}$ and pbx^{2}, both of which cause strong bithorax phenotypes (8 to 10) as homozygotes.

***Nipped* displays dosage-sensitive interactions with other *cut* regulators:** To further define the roles of the different *Nipped* lethal groups in regulating *cut*, we compared dosage-sensitive interactions between *Nipped* mutations and other wing development mutations. These include mutations in *ap*, which defines the dorsal-ventral boundary at which the margin will form (Diaz-Benjumea and Cohen 1993; Blair *et al.* 1994), and *Su(H)*, *vg*, and *wg*, which act at the margin prior to and during the time of *cut* expression (reviewed in Cohen 1996).

None of the *Nipped* mutations displays a mutant phenotype when combined with heterozygous loss-of-function alleles of *Su(H)* ($Su(H)^{2}$ and $Su(H)^{8}$ (not shown), or *ap* or *wg* (Table 4). In contrast, *l(2)41Af*$^{45-72}$ and certain *Nipped-D* and *Nipped-E* alleles exhibit significant mutant phenotypes when transheterozygous with vg^{1}, with up to ~1 wing margin nick per fly (Table 4). *Nipped-B* alleles display little or no mutant phenotype with vg^{1}, while some *Nipped-A* and *Nipped-C* alleles display a weak cut wing phenotype (~0.1–0.2 nicks per fly). Therefore, *l(2)41Af* and *Nipped-A* display dosage-sensitive interactions with *vg* and *Nipped-B* does not.

Several *Nipped* mutations also display dosage-sensitive interactions with *mam* and *Chip* mutations. The strongest *Nipped-A* mutations result in 2.5–3 nicks per fly when transheterozygous with $mam^{g2.1}$, and 1–2 nicks per fly when transheterozygous with $Chip^{e5.5}$ (Table 4). In contrast, *Nipped-B* mutations display fewer nicks, from 0.2

TABLE 4

Interactions between heterozygous *Nipped* and wing development mutations

Nipped allele	Wing margin phenotype[a]				
	wg^{CX4}/+	ap^{56f}/+	vg^{1}/+	$mam^{g2.1}$/+	$Chip^{e5.5}$/+
$Nipped^{+}$[b]	<0.02	<0.01	<0.01	0.12 ± 0.05	0.08 ± 0.04
$l(2)41Af^{45\text{-}72}$	<0.02	<0.02	0.27 ± 0.11	0.88 ± 0.16	0.38 ± 0.14
$Nipped\text{-}A^{34\text{-}12}$	<0.02	<0.02	0.01 ± 0.01	0.39 ± 0.07	0.02 ± 0.02
$Nipped\text{-}A^{323}$	<0.02	<0.01	0.06 ± 0.03	0.60 ± 0.11	<0.02
$Nipped\text{-}A^{222.3}$	<0.02	<0.01	0.16 ± 0.05	3.09 ± 0.19	1.11 ± 0.14
$T(2;3)Nipped\text{-}A^{394.2}$			0.06 ± 0.04	2.49 ± 0.25	1.80 ± 0.22
$T(2;3)Nipped\text{-}B^{4}$			<0.02	0.34 ± 0.09	0.15 ± 0.06
$T(2;3)Nipped\text{-}B^{359.1}$			<0.02	0.18 ± 0.07	<0.02
$Nipped\text{-}B^{292.1}$	<0.02	<0.02	<0.02	0.50 ± 0.10	0.05 ± 0.02
$Nipped\text{-}B^{407}$	<0.02	<0.02	0.01 ± 0.01	0.21 ± 0.09	
$Nipped\text{-}C^{160.1}$			0.03 ± 0.02	1.66 ± 0.15	0.03 ± 0.02
$T(2;3)Nipped\text{-}C^{138.2}$			0.13 ± 0.05	0.72 ± 0.11	2.04 ± 0.19
$Nipped\text{-}D^{341.1}$	<0.02	<0.01	1.01 ± 0.16	3.56 ± 0.3	6.83 ± 0.22
$Nipped\text{-}D^{263.3}$			0.12 ± 0.05	3.53 ± 0.25	2.13 ± 0.24
$Nipped\text{-}E^{299.1}$		<0.02	0.08 ± 0.04	2.15 ± 0.17	2.68 ± 0.29
$Nipped\text{-}E^{43}$	<0.02	<0.02	0.31 ± 0.07	4.99 ± 0.24	5.52 ± 0.18
$Nipped\text{-}E^{338}$	<0.02	<0.02	1.05 ± 0.13	5.89 ± 0.30	6.22 ± 0.24

[a] Wing margin nicks per female heterozygous for both the indicated *Nipped* and wing development mutations; error values are standard errors.
[b] Wild-type *Nipped* alleles from *Nipped* mutant parental stocks.

to 0.5 nicks per fly with $mam^{g2.1}$, and <0.2 nicks per fly with $Chip^{e5.5}$. *Nipped-C* mutations, which are mutant for both *Nipped-A* and *Nipped-B*, display interactions with *mam* and *Chip* mutations similar to those displayed by *Nipped-A* mutations. *Nipped-D* and *Nipped-E* mutations have stronger interactions than the *Nipped-A* mutations, showing 3.5 to nearly 6 nicks per fly with heterozygous $mam^{g2.1}$, and 2 to nearly 7 nicks per fly with heterozygous $Chip^{e5.5}$ (Table 4). The stronger effects of *Nipped-D* and *Nipped-E* mutations are likely the result of disrupting both *Nipped-A* and *l(2)41Af*. The $l(2)41Af^{45\text{-}72}$ mutation displays ~1 nick per fly with $mam^{g2.1}$ and ~0.4 nicks per fly with $Chip^{e5.5}$.

The dosage-sensitive interactions between *Nipped* mutations and the *vg*, *mam*, and *Chip* mutations have noteworthy parallels in the interactions between *Nipped* mutations and the ct^{53d} enhancer deletion. In both cases, *l(2)41Af* and *Nipped-A* show stronger interactions than *Nipped-B* mutations, and the effects of *Nipped-A* and *l(2)41Af* are additive. The cut wing phenotypes exhibited by flies heterozygous for both *Nipped* and *mam* mutations, or by *Nipped* and *Chip* transheterozygotes, are similar in strength to those displayed by flies transheterozygous for *Chip* and *mam*, *Chip*, and *sd*, or *sd* and *mam* mutations (Morcillo *et al.* 1996). These results indicate that *l(2)41Af* and *Nipped-A* cooperate closely with *Chip*, *mam*, and *vg* to regulate *cut* expression in the wing margin, and further confirm that *Nipped-B* plays a unique role.

***Nipped* mutations modify *Notch* mutant phenotypes:** The genes that display dosage-sensitive interactions with *Nipped* function downstream of *Notch* in the wing margin, leading us to consider the possibility that *Nipped* may function widely in Notch receptor signaling. We therefore tested the ability of *Nipped* mutations to modify the phenotypes displayed by various *Notch* receptor mutants.

The hypomorphic nd^{1} mutation alters the intracellular domain of the Notch receptor (Xu *et al.* 1990) and displays a wing margin phenotype similar to that displayed by ct^{53d}, with a few nicks per wing (Figure 5). Heterozygous $Nipped\text{-}A^{222.3}$ (Figure 5) and $Nipped\text{-}A^{323}$ (not shown) enhance this phenotype to give several more nicks. $Nipped\text{-}B^{407}$ has no effect (Figure 5), while $Nipped\text{-}B^{292.1}$ may slightly reduce the number of nicks (not shown). Strikingly, heterozygous $Nipped\text{-}D^{341.1}$ and $Nipped\text{-}E^{338}$ strongly enhance the nd^{1} phenotype to give strap-like wings (Figure 5). The strong effect of *Nipped-D* and *Nipped-E* mutations is likely the result of disrupting *l(2)41Af* because $l(2)41Af^{45\text{-}72}$ exhibits a similar phenotype, while $l(2)41Ae^{34\text{-}14}$ has no effect (Figure 5). Because loss of *cut* expression does not cause loss of wing blade, we conclude that *l(2)41Af* has functions in wing development beyond regulation of *cut*.

The ability of heterozygous *l(2)41Af* mutations to give strap-like wings with nd^{1} is similar to the effects of heterozygous *vg* (Rabinow and Birchler 1990; Figure 5), $Chip^{e5.5}$ (Figure 5), and *Su(H)* gain-of-function mutations (Fortini and Artavanis-Tsakonas 1994; Figure 5). Chip protein interacts with Apterous protein and is required for Apterous activity in the wing (Morcillo *et al.* 1997; Milan *et al.* 1998; Shoresh *et al.* 1998; P.

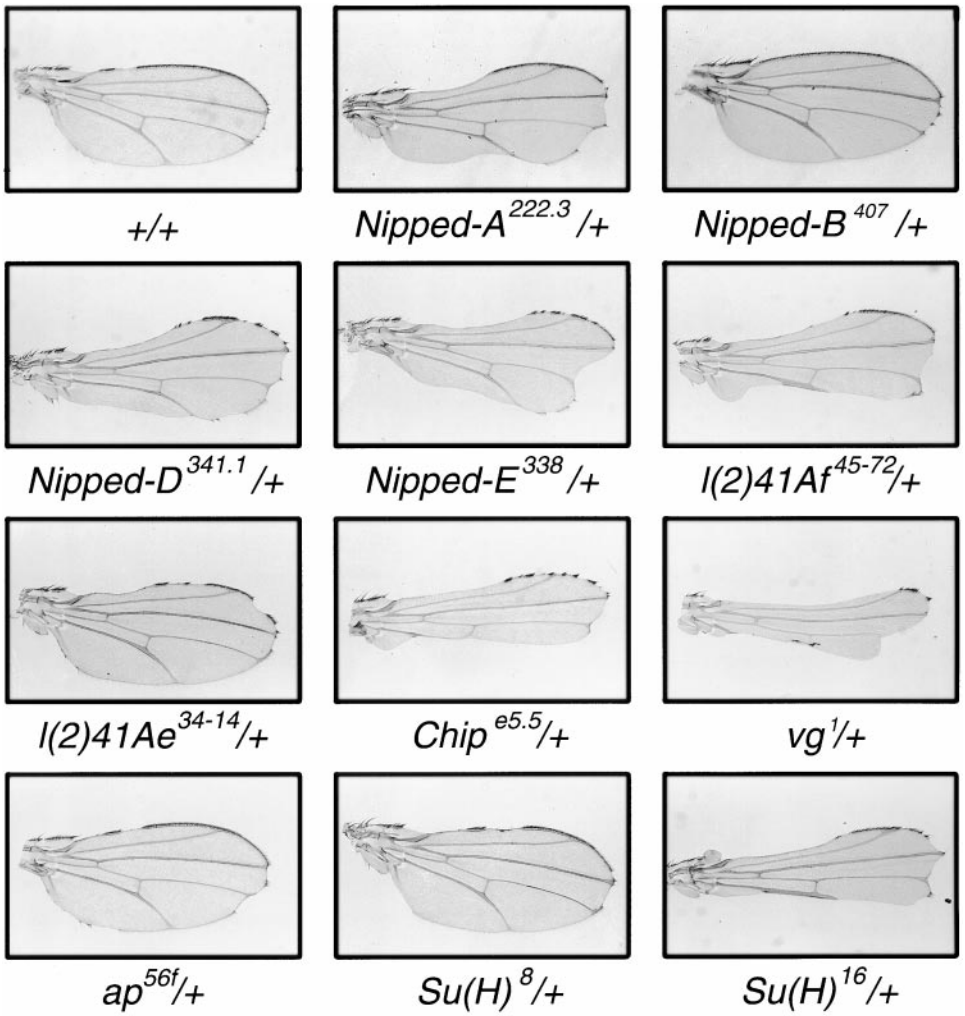

Figure 5.—Dominant effects of *Nipped* mutations on the nd^1 *Notch* mutant phenotype. Representative wings from nd^1 males with the indicated genotypes are shown. A control nd^1 wing with margin nicks is shown on the left in the top row (+/+). Little or no effect is observed with $l(2)41Ae^{34\text{-}14}$ (third row, left), ap^{56f} (bottom left), and the $Su(H)^8$ loss-of-function allele (bottom row, middle). Slight suppression of the mutant phenotype is observed with $Nipped\text{-}B^{407}$ (top right). Enhancement of the mutant phenotype is observed with $Nipped\text{-}A^{222.3}$ (top center), $Nipped\text{-}D^{341.1}$ (second row, left), $Nipped\text{-}E^{338}$ (second row, center), $l(2)41Af^{45\text{-}72}$, $Chip^{e5.5}$ (third row, center), vg^1 (third row, right; Rabinow and Birchler 1990), and the $Su(H)^{16}$ gain-of-function allele (bottom right; Fortini and Artavanis-Tsakonas 1994).

Morcillo and D. Dorsett, unpublished results). Apterous activity is required for expression of Serrate protein (Diaz-Benjumea and Cohen 1995), which serves as the dorsal ligand for Notch at the wing margin (Couso *et al.* 1995; Diaz-Benjumea and Cohen 1995; Kim *et al.* 1995; De Celis *et al.* 1996; Neumann and Cohen 1996). However, heterozygous ap^{56f}, in contrast to $Chip^{e5.5}$, has no detectable effect on the nd^1 phenotype (Figure 5), indicating that Chip has roles in wing development beyond mediating Apterous activity and regulation of *cut*.

Nipped mutations also modify the vein-shortening phenotype of the Ax^{E2} gain-of-function allele of *Notch*, which encodes a Notch receptor with a lesion in epidermal growth factor (EGF)-like repeat 29 in the extracellular portion (Hartley *et al.* 1987; Kelley *et al.* 1987). Heterozygous $Nipped\text{-}A^{323}$ (not shown) and $Nipped\text{-}A^{222.3}$ (Figure 6) weakly and moderately suppress the vein phenotype, while $Nipped\text{-}B^{407}$ (Figure 6) and $Nipped\text{-}B^{292.1}$ (not shown) have no detectable effect. $Nipped\text{-}D^{341.1}$ (Figure 6), $Nipped\text{-}E^{43}$ (not shown), and $Nipped\text{-}E^{338}$ (Figure 6) strongly suppress the vein-shortening phenotype. The strong suppression is presumably the result of disrupting *l(2)41Af* because $l(2)41Af^{45\text{-}72}$ strongly suppresses the Ax^{E2} mutant phenotype, while $l(2)41Ae^{34\text{-}14}$ has no effect (Figure 6). The effects of *Nipped-A* and *l(2)41Af* mutations on Ax^{E2} are similar to those of *Su(H)* gain-of-function mutations (Figure 6). *vg* (Rabinow and Birchler 1990) and *Chip* mutations have no visible effects on the Ax^{E2} vein phenotype (Figure 6).

The phenotypes displayed by the *split* (*spl*) allele of *Notch*, which encodes an amino acid substitution in EGF-like repeat number 14 (Hartley *et al.* 1987; Kelley *et al.* 1987), are also affected by *Nipped* mutations. *spl* displays small rough eyes, occasional twinned bristles, and missing bristles (Figures 7 and 8). $l(2)41Ae^{34\text{-}14}$ has no detectable effects on the *spl* eye phenotype (Figure 7). $T(2;3)Nipped\text{-}A^{394.2}$ (not shown) and $Nipped\text{-}A^{222.3}$ (Figure 7) also have no detectable effect on the rough eye phenotype, while $Nipped\text{-}A^{323}$ slightly suppresses (not shown), making the eyes slightly larger and less rough. $Nipped\text{-}B^{407}$ (not shown), $Nipped\text{-}B^{292.1}$, and $l(2)41Af^{45\text{-}72}$ also suppress the rough eye phenotype (Figure 7). $Nipped\text{-}E^{338}$, $Nipped\text{-}D^{263.3}$ (not shown), and $Nipped\text{-}D^{341.1}$, which disrupt both *Nipped-B* and *l(2)41Af*, have similar effects to the *Nipped-B* alleles (Figure 7). The *Nipped-A* and *Nipped-B* alleles have little or no effect on the thoracic bristle

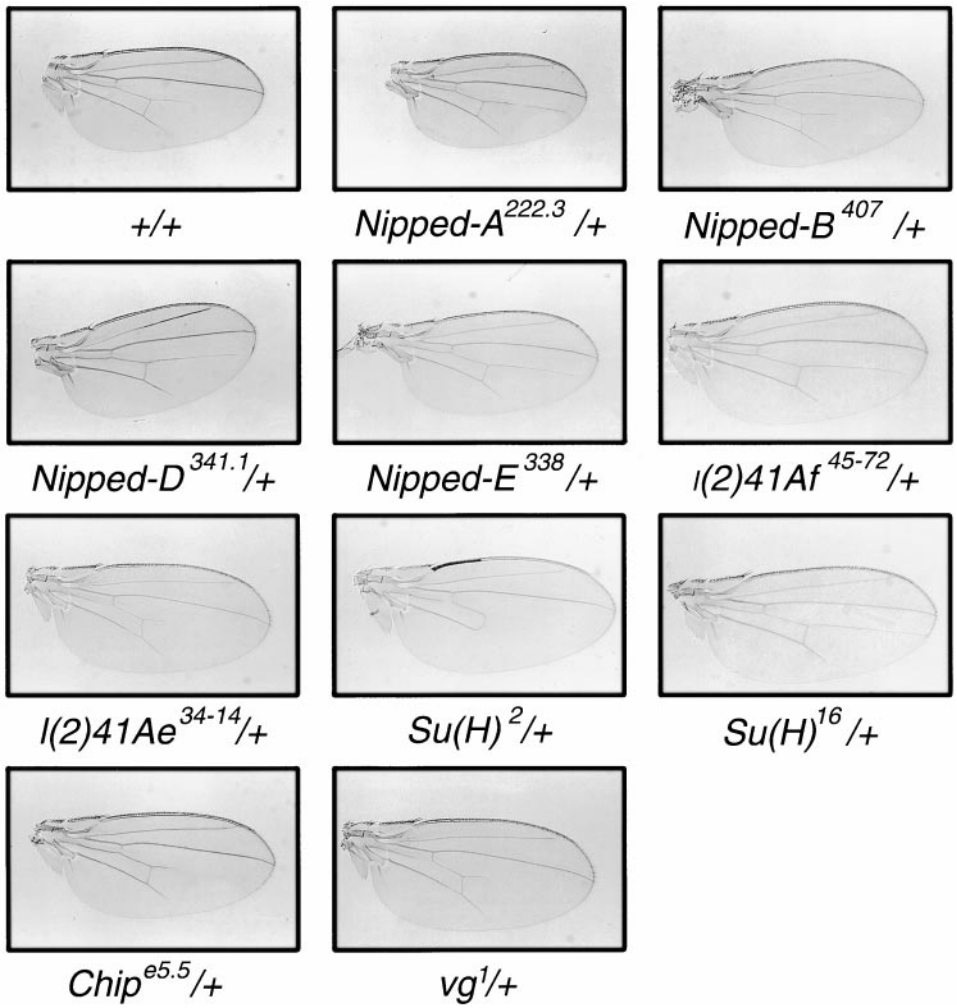

Figure 6.—Dominant suppression of the Ax^{E2} *Notch* mutant phenotype by some *Nipped* mutations. Representative wings from Ax^{E2} males with the indicated mutations are shown. An Ax^{E2} control wing with shortened veins is shown on the left in the top row (+/+). Little or no effect is observed with *Nipped-B*^{407} (top row, right), *l(2)41Ae*$^{34\text{-}14}$ (third row, left), *Chip*$^{e5.5}$ (bottom row, left), and *vg*^{1} (bottom row, center; Rabinow and Birchler 1990). Lengthening of the veins (suppression) is observed with *Nipped-A*$^{222.3}$ (top row, center), *Nipped-D*$^{341.1}$ (second row, left), *Nipped-E*^{338} (second row, center), *l(2)41Af*$^{45\text{-}72}$ (second row, right), and the *Su(H)*^{16} gain-of-function mutation (third row, right). Shortening of the veins (enhancement) is observed with the *Su(H)*^{2} loss-of-function allele (third row, center).

phenotypes of *spl* (not shown and Figure 8), but *Nipped-D*$^{263.3}$ (not shown), *Nipped-D*$^{341.1}$ (Figure 8), *Nipped-E*^{43} (not shown), and *Nipped-E*^{338} (Figure 8), all of which disrupt *l(2)41Af*, make the thoracic bristles sparser, thinner, and shorter. Because the *l(2)41Af*$^{45\text{-}72}$ chromosome contains a *Pin* mutation that affects bristle morphology, we could not evaluate the effect of this allele on bristles. We conclude that *Nipped-B* and *l(2)41Af* mutations suppress the *spl* eye phenotype, and infer that *l(2)41Af* mutations enhance the thoracic bristle phenotype of the *spl* allele of *Notch*.

The dominant effects of *Nipped* mutations on the *nd*^{1}, Ax^{E2}, and *spl Notch* mutant phenotypes indicate that *l(2)41Af* influences Notch receptor signaling, or *Notch* expression during development of the wing margin, wing veins, eye, and thoracic bristles. *Nipped-A* influences *Notch* phenotypes in both the wing veins and margin, while *Nipped-B* has influences primarily in the eye. The effects of *l(2)41Af* mutations in the wing margin, wing vein, and eye are similar to those gain-of-function mutations in *Su(H)*. Similarly, the effects of *Nipped-A* mutations in the wing veins and wing margin, and the effect of *Nipped-B* mutations in the eye also mimic the effects of *Su(H)* gain-of-function mutations. Su(H) protein is a direct mediator of Notch signaling (Fortini and Artavanis-Tsakonas 1994) and a direct activator of *vg* (Kim *et al.* 1996a). We postulate that *l(2)41Af* mutations, *Nipped-A* mutations, and Su(H) gain-of-function mutant proteins downregulate Notch signaling in the wing margin. Su(H) gain-of-function mutant proteins may recruit transcription repressors, similar to the wild-type mammalian homologues of Su(H) (Kao *et al.* 1998; Taniguchi *et al.* 1998).

Nipped-B protein is homologous to chromosomal adherins, which have cohesin-like activities: The *in vivo* observations indicate that the role of *Nipped-B* in regulation of *cut* and in Notch signaling differs significantly from those of the other *Nipped* lethal groups. Most importantly, *Nipped-B* is particularly antagonistic to the insulator activity of Su(Hw). We hypothesized, therefore, that *Nipped-B* products may participate in enhancer-promoter communication in *cut* and *Ubx*. To explore this idea we cloned and sequenced *Nipped-B* cDNAs.

Complementation tests revealed that the *l(2)02047* *P*-element insertion (Berkeley Drosophila Genome

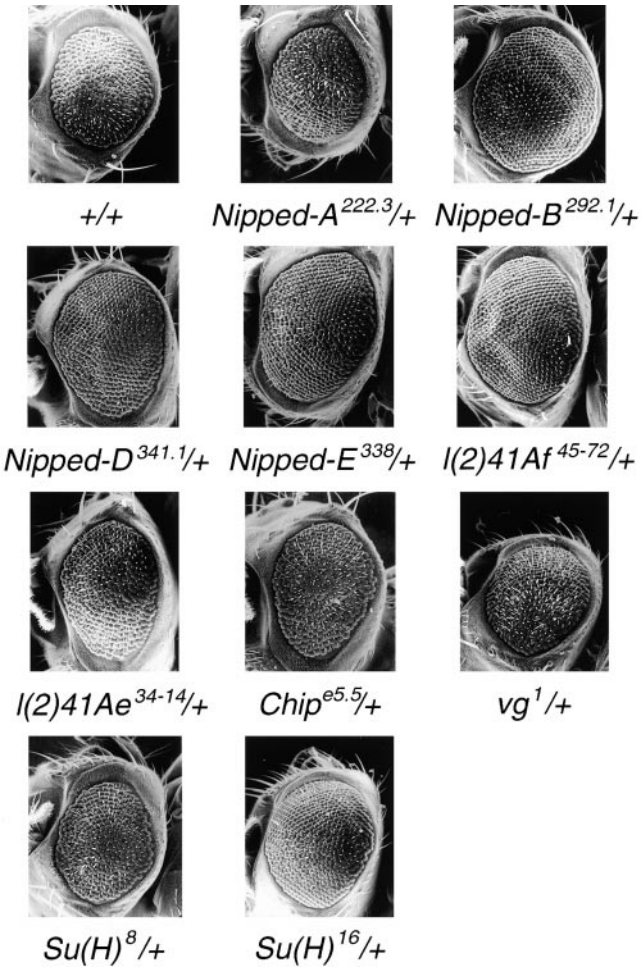

Figure 7.—Dominant effects of some *Nipped* mutations on the *spl Notch* mutant eye phenotype. Representative eyes of *spl* mutant males with the indicated mutations are shown. All photographs are at the same magnification. In a wild-type background (+/+), *spl* mutant eyes are small and rough (top row, left). Little or no effect is observed with *Nipped-A*$^{222.3}$ (top row, middle), *l(2)41Ae*$^{34\text{-}14}$ (third row, left), *Chip*$^{e5.5}$ (third row, center), *vg*^{1} (third row, right), and the *Su(H)*^{8} loss-of-function allele (bottom left). Larger, less rough eyes are observed with *Nipped-B*$^{292.1}$ (top right), *Nipped-D*$^{341.1}$ (second row, left), *Nipped-E*^{338} (second row, center), *l(2)41Af*$^{45\text{-}72}$ (second row, right), and the *Su(H)*^{16} gain-of-function allele (bottom row, center).

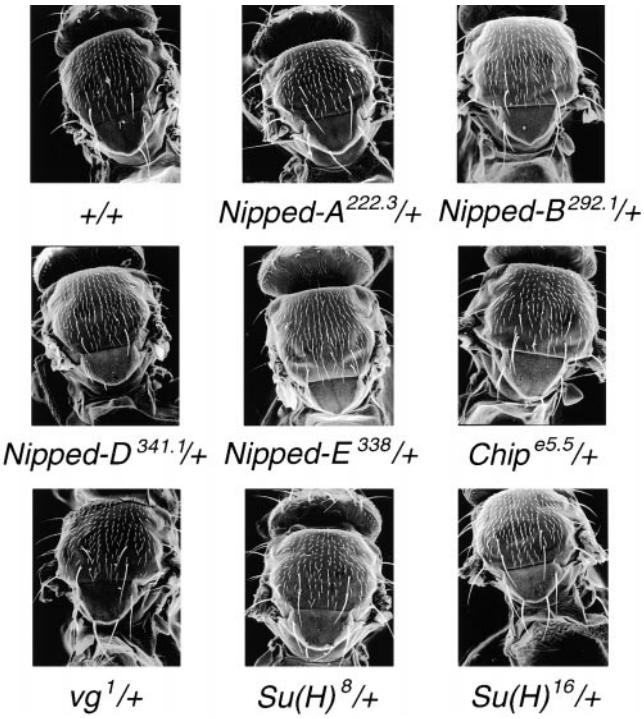

Figure 8.—Dominant effects of some *Nipped* mutations on the *spl Notch* mutant bristle phenotype. Dorsal views of representative thoraces from *spl* mutant males with the indicated mutations are shown. In a wild-type background (+/+, top left), *spl* displays missing and occasional twinned bristles. Little or no effect is observed with *Nipped-A*$^{222.3}$ (top middle), *Nipped-B*$^{292.1}$ (top right), *Chip*$^{e5.5}$ (middle row, right), *vg*^{1} (bottom left), the *Su(H)*^{8} loss-of-function allele (bottom center), and the *Su(H)*^{16} gain-of-function (bottom right). Loss of scutellar bristles and shorter, thinner bristles are observed with *Nipped-D*$^{341.1}$ (middle row, left) and *Nipped-E*^{338} (middle row, center).

Project) is allelic to *Nipped-B*. *l(2)02047* flies are lethal over all *Nipped-B* alleles, and viable over *l(2)41Ae*$^{34\text{-}14}$, *l(2)41Af*$^{45\text{-}72}$, and *Nipped-A* mutations. Nine of 13 induced excisions of the *P* element reverted the *Nipped-B* mutation in *l(2)02047*, confirming that the *P* insertion is the *Nipped-B* mutation.

We rescued the *P* insertion from genomic DNA and cloned a 13-kb genomic region surrounding the insertion site (Figure 9A). In this region, only a 2.5-kb *Eco*RI-*Xba*I fragment located ~4 kb from the insertion site (Figure 9A) detects transcripts in Northern blots. This probe hybridizes to 7- and 4-kb transcripts oriented toward the *P*-insertion site (Figure 9, B and C). The 7-kb transcript is undetectable in homozygous *l(2)02047* second instar larvae, while the relative levels of the 4-kb transcript appear to increase (Figure 9B). The 7-kb transcript is reduced in size in homozygous *T(2;3)Nipped-B*$^{359.1}$ second instar larvae, while the 4-kb transcript is unaffected (Figure 9B). The 7-kb transcript is not affected by two *Nipped-A* mutants, but the levels are reduced to ~50 and 30% wild-type levels in homozygous *Nipped-C*$^{160.1}$ and *Nipped-B*^{407} mutants (Figure 9B). Alterations in the size or reductions in the levels of the 7-kb transcript in multiple *Nipped-B* mutants demonstrate that the 7-kb transcript is a *Nipped-B* mRNA. These results also confirm that *Nipped-B*^{407} is a hypomorph. *Nipped-B* mRNA is expressed at all stages of development, but the highest levels are present in newly laid embryos, indicating that it is maternally loaded (Figure 9C).

The probe detecting the *Nipped-B* mRNA was used to isolate overlapping cDNA clones from an imaginal disc library. The probe sequence hybridizes near the middle of the cDNAs (Figure 9A). One clone (6-1) contains a long ORF starting at the 5′ end, while an overlapping clone (3-1) contains the putative initiation codon. Combined, the clones give a complete ORF of 6159 nucleo-

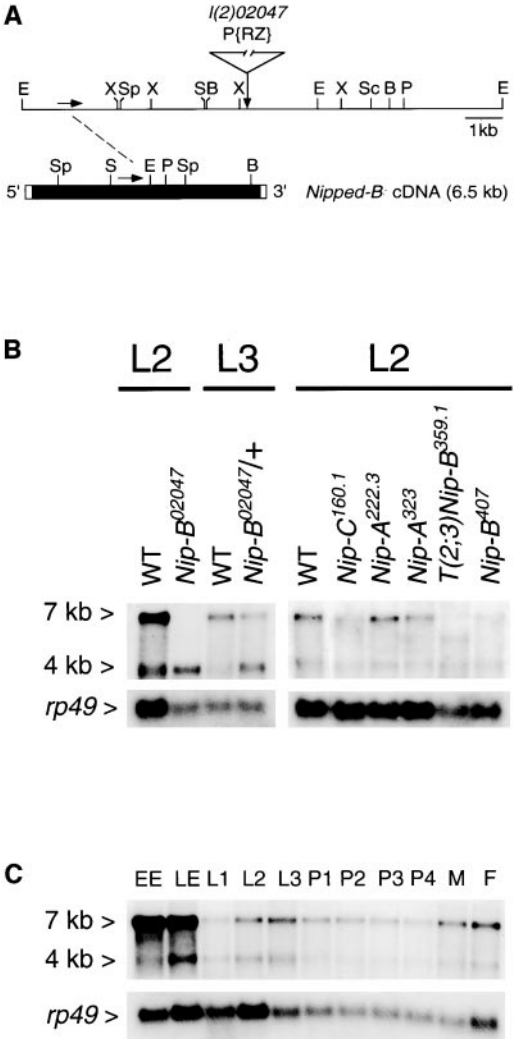

Figure 9.—The *Nipped-B*^{02047} *P*-element insertion [*l(2)02047*] and *Nipped-B* transcript. (A) Restriction maps of the *l(2)02047 P*-insertion site and *Nipped-B* cDNA. As described in the text, excisions of the *P{RZ}* element revert the *Nipped-B* mutation. At the top is a restriction map of the 13-kb genomic region surrounding the *P*-insertion site (E, *Eco*RI; X, *Xba*I; Sp, *Spe*I; S, *Sal*I; B, *Bam*HI; Sc, *Sac*I; P, *Pst*I). The 2.5-kb *Eco*RI-*Xba*I genomic fragment, marked with an arrow indicating the direction of transcription, is the only fragment in the 13-kb region that detects transcripts (7 and 4 kb) in Northern blots. None of the other fragments in the 13-kb genomic region hybridizes to transcripts. The *Eco*RI-*Xba*I genomic fragment was therefore used as a probe to isolate cDNAs. As indicated by the dashed line, the *Sal*I-*Eco*RI fragment in the cDNA, marked with an arrow showing the direction of transcription, is the only fragment in the cDNA that hybridizes to the *Eco*RI-*Xba*I genomic fragment. Because the genomic DNA has not been sequenced, the extent of sequence overlap between the *Eco*RI-*Xba*I genomic fragment and the *Sal*I-*Eco*RI cDNA fragment is unknown, but the *Eco*RI site at the upstream end of the *Eco*RI-*Xba*I genomic fragment is not the same *Eco*RI site present at the downstream end of the *Sal*I-*Eco*RI cDNA fragment. Indeed, none of the restriction sites shown for the cDNA are present in the 13-kb cloned genomic DNA. Because the *Eco*RI-*Xba*I genomic fragment is the only genomic fragment that hybridizes to the cDNA, and because it only hybridizes to the *Sal*I-*Eco*RI fragment in the middle of the cDNA, it is clear that the 5′ end of the cDNA comes from an uncloned genomic region to the left of the *P*-insertion site, and that the 3′ end of the cDNA comes from an uncloned genomic position to the right of the *P*-insertion site. Therefore, we conclude that the *P*-insertion site is in an intron. The filled region of the cDNA indicates the ORF. (B) Northern blots of total RNA isolated from second (L2) and third (L3) instar larvae. The blots were hybridized with antisense [^{32}P]RNA probe prepared from the 2.5-kb genomic *Eco*RI-*Xba*I fragment (top) and then stripped and probed with antisense *rp49* probe as a loading control (bottom). On the left, the lanes contained 10 μg of RNA from wild-type second and third instar larvae (WT), homozygous *l(2)024047* second instar larvae (*Nip-B*^{02047}), and heterozygous *l(2)02047* third instar larvae (*Nip-B*^{02047}/+). On the right, the lanes contained 10 μg of RNA from wild-type second instar larvae and second instar larvae homozygous for the indicated *Nipped* alleles: *Nip-C*$^{160.1}$, *Nip-A*$^{222.3}$, *Nip-A*^{323}, *T(2;3)Nip-B*$^{359.1}$, and *Nip-B*^{407}. The 7-kb transcript is absent or altered in size in *Nip-B*^{02047} mutants and reduced in size in *T(2;3)Nip-B*$^{359.1}$ mutants. Quantification with a phosphorimager indicates that the 7-kb transcript is reduced to ~50% wild-type levels in *Nip-C*$^{160.1}$ homozygotes and ~30% wild type in *Nip-B*^{407} homozygotes. (C) Developmental Northern of *Nipped-B* mRNA. Probes were as described above. The lanes contained 10 μg of total cellular RNA isolated from wild-type flies at different developmental stages: EE, 0 to 30 min after egg laying; LE, 30 min to 16 hr after egg laying; L1–L3, first to third instar larvae; P1–P4, first to fourth day of pupation; M, 0- to 1-day-old adult males; F, 0- to 1-day-old females.

tides, encoding a protein of 2053 amino acids. Because the 2.5-kb *Eco*RI-*Xba*I genomic probe fragment is the only fragment in the cloned genomic region that hybridizes to the cDNA, and because it only hybridizes to a fragment near the middle of the cDNA, we can deduce that the 5′ end of the cDNA comes from an uncloned genomic region on one side of the *P*-insertion site in *l(2)02047* and that the 3′ end of the cDNA comes from an uncloned region on the other side of the *P*-insertion site. Thus, we conclude that the *P*-insertion site is in an intron.

Database searches reveal homologues of the Nipped-B protein in fungi, worms, and mammals. Only short expressed-sequence tags (ESTs) of *Caenorhabditis elegans*, mouse, and humans were identified. The human ESTs are from a variety of tissue-specific libraries, suggesting that the human homologues are widely expressed. The combined human ESTs, which do not represent a complete sequence, encode 411 amino acids. Residues 2–232 of the partial human protein overlap Nipped-B residues 1744–1994 with 34% identity and 52% similarity. In order of decreasing homology, the 2157-amino acid Rad9 protein of *Coprinus cinereus*, the 1583-amino acid Mis4 protein of *Schizosaccharomyces pombe*, and the 1493-amino acid Scc2 protein of *Saccharomyces cerevisiae*, are more distantly related. Rad9 residues 669–2071 display 21% identity and 41% similarity to Nipped-B residues 576–1887, Mis4 residues 780–1492 have 19% identity and 41% similarity to Nipped-B residues 1110–1818, and Scc2 residues 697–1291 display 19% identity with 39% similarity to Nipped-B residues 1103–1704. The fungal homologues show similar levels of homology between themselves, but it is evident that there is a large conserved domain among all the proteins (Figure 10).

Consistent with the idea that Nipped-B plays an architectural role in enhancer-promoter communication, the fungal homologues of Nipped-B all participate in regu-

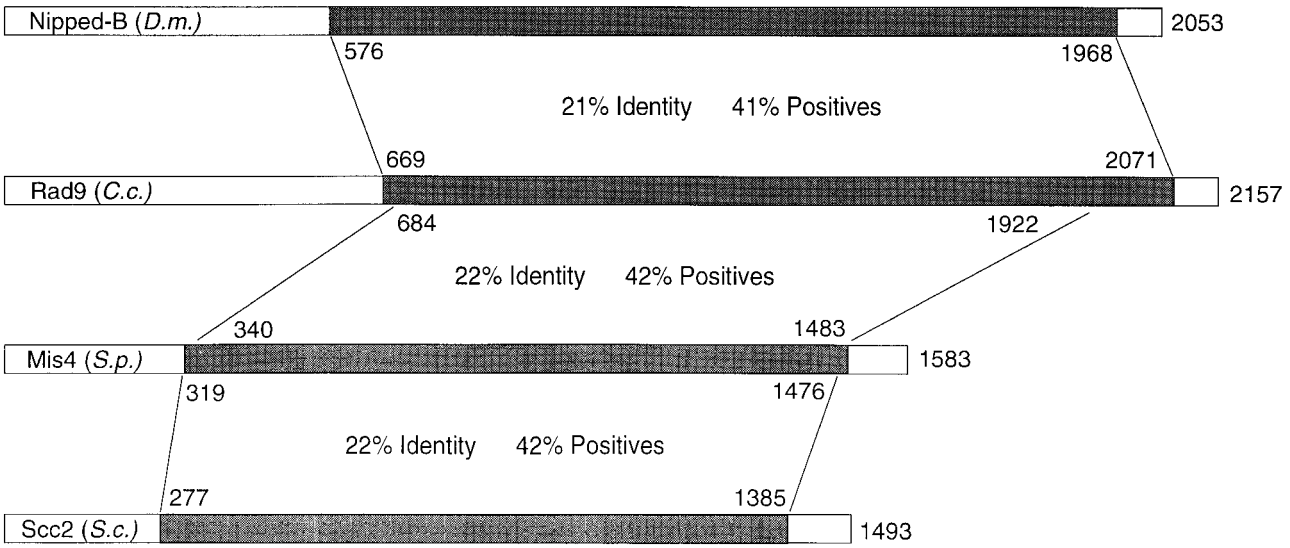

Figure 10.—The Nipped-B protein shares a central core of homology with fungal chromosomal adherins. The proteins are diagrammed with the regions of homology in gray and the lengths of the protein on the right. Comparisons were made using the Blastp program (www.ncbi.nlm.nih.gov/gorf/wblast2.cgi/; Altschul *et al.* 1997) with the BLOSUM62 matrix, a gap open penalty of 11, a gap extension penalty of 1, gap x_dropoff of 50, expect value of 10.0, a wordsize of 3, and no filtering. Nipped-B protein is compared with the *Coprinus cinereus* (*C.c.*) Rad9 protein, which is compared with the *Schizosaccharomyces pombe* (*S.p.*) Mis4 protein, which is compared with the *Saccharomyces cerevisiae* (*S.c.*) Scc2 protein. The numbers near the ends of the lines that connect the homologous regions indicate the amino acid endpoints of the regions of homology detected by the alignment program, and the numbers between the proteins indicate the percentage of identical residues in the homologous regions and the percentage of residues that are either identical or similar (positives).

lating chromosome structure, with roles in DNA repair (Valentine *et al.* 1995; Furuya *et al.* 1998), meiotic chromosome condensation (Seitz *et al.* 1996), or sister chromatid cohesion (Michaelis *et al.* 1997; Furuya *et al.* 1998). It has been proposed that these three fungal proteins define a new class of chromosomal proteins and have been named adherins to distinguish them from the cohesins that have similar functions (Furuya *et al.* 1998).

DISCUSSION

The Su(Hw) insulator protein that binds *gypsy* transposon DNA only blocks activation by an enhancer when the *gypsy* insertion is between the enhancer and promoter, suggesting that Su(Hw) is antagonistic to architectural factors that act between enhancers and promoters to facilitate enhancer-promoter communication. To identify putative architectural factors, we screened for mutations that reduce activation by a remote wing margin enhancer in the Drosophila *cut* gene partially blocked by a mutant Su(Hw) protein. To date this screen has identified five genetic loci that participate in the Notch receptor signaling pathway and promote *cut* expression in the wing margin: *sd*, *mam*, *Chip*, *vg*, and *Nipped*.

We have previously provided evidence that some genes identified in the screen directly regulate *cut*. Sd protein is a wing margin enhancer-binding protein (Morcillo *et al.* 1996), and Mam binds chromosomes in the vicinity of *cut*, suggesting that it is also a direct regulator (Bettler *et al.* 1996). Recently, it has been found that Vg protein interacts with Sd to regulate gene expression in the wing (Simmonds *et al.* 1998). Thus, Sd likely recruits Vg to the *cut* wing margin enhancer.

The screen for *cut* regulators can also identify factors that act broadly to regulate gene expression. Chip is a ubiquitously expressed chromosomal protein required for maximal activation by several remote enhancers in diverse genes, including *Ubx* and *even-skipped* (Morcillo *et al.* 1996, 1997). In addition to its broad activities, the *in vivo* antagonism of Chip to the Su(Hw) insulator suggests that it may play a role in enhancer-promoter communication (Morcillo *et al.* 1996). Chip and its mammalian homologues, Nli (Ldb1/Clim-2) and Ldb2 (Clim-1), interact with themselves and various LIM and homeodomain proteins to promote formation of homo- and heterotypic transcription factor complexes (Agulnick *et al.* 1996; Jurata *et al.* 1996, 1998; Bach *et al.* 1997; Jurata and Gill 1997; Morcillo *et al.* 1997; Wadman *et al.* 1997; Breen *et al.* 1998; E. Torigoi, C. Rosen, and D. Dorsett, unpublished observations). Thus, Chip and its homologues can act as cross-linking proteins that mediate interactions between diverse DNA-binding proteins. We speculate that in addition to helping form complexes on enhancers, promoting interactions between proteins that bind to DNA between enhancers and promoters could create chromatin loops that bring enhancers and promoters closer together.

Here we present genetic characterization of the

Nipped mutations isolated in the screen for *cut* regulators and identification of a *Nipped* gene product. Although the data do not yet distinguish whether the heterochromatic *Nipped* locus is a single complex transcription unit or a cluster of distinct genes, we can draw several conclusions about *Nipped* functions and their roles relative to the other *cut* regulators. To summarize, *Nipped* mutations define three separable essential functions that regulate *cut* in the wing margin, provided by the *Nipped-A*, *Nipped-B*, and *l(2)41Af* lethal complementation groups. Dosage-sensitive genetic interactions indicate that *Nipped-A* and *l(2)41Af* cooperate closely with *mam* and *vg* in the regulation of *cut*. Similar to *mam* and unlike *sd* and *vg*, *Nipped-A* and *l(2)41Af* also modulate Notch receptor signaling or expression in multiple tissues. *Nipped-B* has the most unique function. Like *Chip*, *Nipped-B* regulates both *cut* and *Ubx* and is antagonistic to insulation by Su(Hw). Together, the antagonism to Su(Hw) and the homology to chromosomal adherins lead us to propose that Nipped-B protein performs an architectural role in enhancer-promoter communication.

***Nipped-B* is antagonistic to Su(Hw) insulator activity:** The primary evidence that *Nipped-B* is antagonistic to Su(Hw) insulator activity is that *Nipped-B* activity is only strongly limiting for *cut* expression when there is a *gypsy* insertion between the wing margin enhancer and promoter. Strikingly, in contrast to mutations disrupting any of the other *cut* regulators (Jack and Delotto 1992; Morcillo *et al.* 1996; R. A. Rollins and D. Dorsett, unpublished observations), including *sd*, *mam*, *Chip*, *vg*, *Nipped-A*, and *l(2)41Af*, heterozygous *Nipped-B* mutations do not detectably reduce activation by the partially crippled wing margin enhancer in ct^{53d}. Compared with *sd*, *mam* (Morcillo *et al.* 1996), or *Nipped-A* mutations, heterozygous *Nipped-B* mutations also only slightly reduce activation of *cut* expression by the solo wild-type wing margin enhancer present in ct^{2s} heterozygotes. Therefore, with both ct^{53d} and ct^{2s}, *Nipped-B* products are less limiting for wing margin enhancer activity than are *Nipped-A* products. Remarkably, the opposite is true when there is a *gypsy* insulator insertion in *cut*. Heterozygous *Nipped-B* mutations are severalfold more effective than *Nipped-A* mutations in magnifying the effect of the Su(Hw) insulator in $ct^{L\text{-}32}$; $su(Hw)^{e2}$ flies. Furthermore, of the known *cut* regulators, only *Chip* (Morcillo *et al.* 1996) and *Nipped-B* mutations magnify the effect of the Su(Hw) insulator in $su(Hw)^{e2}$ bx^{34e} flies. The antagonism between *Nipped-B* and Su(Hw) is unlikely to be specific to the $Su(Hw)^{e2}$ protein. $Su(Hw)^{e2}$ has an amino acid substitution in a zinc finger that reduces DNA-binding activity but contains a wild-type enhancer-blocking domain (Harrison *et al.* 1993; Kim *et al.* 1996b). Moreover, *Nipped-B* mutations also reduce *cut* expression in the absence of a *gypsy* insertion, indicating that the increased effectiveness of $Su(Hw)^{e2}$ in *Nipped-B* mutants reflects a change in *cut* regulation rather than a change in $Su(Hw)^{e2}$ protein activity.

Does *Nipped-B* directly regulate *cut*? The available data are insufficient to determine with absolute certainty whether or not *Nipped-B* directly regulates *cut*. However, direct regulation provides the simplest explanation for several observations. First, as shown above, the ability of *Nipped-B* mutations to exacerbate different *cut* mutant phenotypes differs from all other *cut* regulators such as *sd*, *vg*, and *mam*. Therefore, *Nipped-B* does not regulate *cut* indirectly by altering expression of any of the other known *cut* regulators. Moreover, the effects of the $Nipped\text{-}B^{407}$ mutation on *cut* and *Ubx* mutant phenotypes are dominant, although $Nipped\text{-}B^{407}$ only partially reduces *Nipped-B* mRNA levels. A partial loss of *Nipped-B* activity is unlikely to cause a similar or greater loss of activity of another *cut* regulator. Therefore, in light of the observation that *Nipped-B* mutations magnify insulation by *gypsy* insertions in both *cut* and *Ubx*, we strongly favor the idea that *Nipped-B* products directly support enhancer-promoter communication in *cut* and *Ubx*. Because *Nipped-B* is essential and *Nipped-B* mRNA is expressed at all developmental stages, it may play a similar role in other genes.

Nipped-B protein homologues have diverse roles in chromosome structure: The hypothesis that Nipped-B protein plays an architectural role to facilitate enhancer-promoter interactions in *cut* and *Ubx* is supported by the diverse effects that the fungal adherin homologues of the Nipped-B protein have on chromosome structure and function. The Rad9 protein of Coprinus was identified in a screen for radiation-sensitive mutants (Valentine *et al.* 1995). *rad9* mutants were subsequently observed to display defects in synaptonemal complex formation and chromosome condensation during meiosis (Valentine *et al.* 1995; Seitz *et al.* 1996). Mutations in the *Scc2* gene of budding yeast were identified as lethal temperature-sensitive mutants that display defects in sister chromatid cohesion during mitosis (Michaelis *et al.* 1997). In *scc2* mutants, sister chromatids separate prematurely, just after formation of the bipolar spindle. Mutations in the *Mis4* gene of fission yeast were identified as temperature-sensitive lethal mutants that missegregate minichromosomes (Takahashi *et al.* 1994). *mis4* mutants also missegregate regular chromosomes and are radiation sensitive (Furuya *et al.* 1998). The Mis4 protein is required during S phase and associates with chromosomes during the entire cell cycle (Furuya *et al.* 1998). These diverse mutant phenotypes indicate that adherins play fundamental roles in chromosome structure.

Although we do not yet know if Nipped-B also participates in mitotic or meiotic chromosome structure, its homology to adherins suggests explanations for how Nipped-B could architecturally facilitate enhancer-promoter communication. It is tempting to speculate, for example, that the biochemical activity of Nipped-B is to

recognize and stabilize chromatin loops that hold distant chromosomal sites closer together. The chromatin loops could be created by other factors involved in enhancer-promoter interactions.

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LITERATURE CITED

Agulnick, A. D., M. Taira, J. J. Breen, T. Tanaka, I. B. Dawid *et al.*, 1996 Interactions of the LIM-domain binding factor Ldb1 with LIM homeodomain proteins. Nature **384:** 270–272.

Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang *et al.*, 1997 Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:** 3389–3402.

Bach, I., C. Carriere, H. P. Ostendorff, B. Andersen and M. G. Rosenfeld, 1997 A family of LIM domain-associated cofactors confer transcriptional synergism between LIM and Otx homeodomain proteins. Genes Dev. **11:** 1370–1380.

Bettler, D., S. Pearson and B. Yedvobnick, 1996 The nuclear protein encoded by the Drosophila neurogenic gene *mastermind* is widely expressed and associates with specific chromosomal regions. Genetics **143:** 859–875.

Blair, S. S., D. L. Brower, J. B. Thomas and M. Zavortink, 1994 The role of *apterous* in the control of dorsoventral compartmentalization and PS integrin gene expression in the developing wing of *Drosophila*. Development **120:** 1805–1815.

Breen, J. J., A. D. Agulnick, H. Westphal and I. B. Dawid, 1998 Interactions between LIM domains and the LIM domain-binding protein Ldb1. J. Biol. Chem. **273:** 4712–4717.

Cai, H., and M. Levine, 1995 Modulation of enhancer-promoter interactions by insulators in the *Drosophila* embryo. Nature **376:** 533–536.

Cohen, S. M., 1996 Controlling growth of the wing: *vestigial* integrates signals from the compartment boundaries. Bioessays **18:** 855–858.

Couso, J. P., E. Knust and A. Martinez-Arias, 1995 *Serrate* and *wingless* cooperate to induce *vestigial* gene expression and wing formation in Drosophila. Curr. Biol. **5:** 1437–1448.

De Celis, J. F., A. Garcia-Bellido and S. J. Bray, 1996 Activation and function of *Notch* at the dorsal-ventral boundary of the wing imaginal disc. Development **122:** 359–369.

Diaz-Benjumea, F. J., and S. M. Cohen, 1993 Interaction between dorsal and ventral cells in the imaginal disc directs wing development in Drosophila. Cell **75:** 741–752.

Diaz-Benjumea, F. J., and S. M. Cohen, 1995 Serrate signals through Notch to establish a Wingless-dependent organizer at the dorsal/ventral compartment boundary of the *Drosophila* wing. Development **121:** 4215–4225.

Dorsett, D., 1990 Potentiation of a polyadenylylation site by a downstream protein-DNA interaction. Proc. Natl. Acad. Sci. USA **87:** 4373–4377.

Dorsett, D., 1993 Distance-independent inactivation of an enhancer by the *suppressor of Hairy-wing* DNA-binding protein of Drosophila. Genetics **134:** 1135–1144.

Dorsett, D., G. A. Viglianti, B. J. Rutledge and M. Meselson, 1989 Alteration of *hsp82* gene expression by the *gypsy* transposon and suppressor genes in *Drosophila melanogaster*. Genes Dev. **3:** 454–468.

Falvo, J. V., D. Thanos and T. Maniatis, 1995 Reversal of intrinsic DNA bends in the IFNβ gene enhancer by transcription factors and the architectural protein HMG I(Y). Cell **83:** 1101–1111.

Fortini, M. E., and S. Artavanis-Tsakonas, 1994 The Suppressor of Hairless protein participates in Notch receptor signaling. Cell **79:** 273–282.

Furuya, K., K. Takahashi and M. Yanagida, 1998 Faithful anaphase is ensured by Mis4, a sister chromatid cohesion molecule required in S phase and not destroyed in G_1 phase. Genes Dev. **12:** 3408–3418.

Geyer, P. K., and V. G. Corces, 1992 DNA position-specific repression of transcription by a *Drosophila* zinc finger protein. Genes Dev. **6:** 1865–1873.

Geyer, P. K., M. M. Green and V. G. Corces, 1990 Tissue-specific transcriptional enhancers may act in trans on the gene located in the homologous chromosome: the molecular basis of transvection in *Drosophila*. EMBO J. **9:** 2247–2256.

Giese, K., J. Cox and R. Grosschedl, 1992 The HMG domain of lymphoid enhancer factor 1 bends DNA and facilitates assembly of functional nucleoprotein structures. Cell **69:** 185–195.

Giese, K., C. Kingsley, J. R. Kirshner and R. Grosschedl, 1995 Assembly and function of a TCRα enhancer complex is dependent on LEF-1-induced DNA bending and multiple protein-protein interactions. Genes Dev. **9:** 995–1008.

Harrison, D. A., D. A. Gdula, R. S. Coyne and V. G. Corces, 1993 A leucine zipper domain of the *suppressor of Hairy-wing* protein mediates its repressive effect on enhancer function. Genes Dev. **7:** 1966–1978.

Hartley, D., T. Xu and S. Artavanis-Tsakonas, 1987 The embryonic expression of the *Notch* locus of *Drosophila melanogaster* and the implications of point mutations in the extracellular EGF-like domains of the predicted protein. EMBO J. **6:** 3407–3417.

Hilliker, A. J., 1976 Genetic analysis of the centromeric heterochromatin of chromosome 2 of *Drosophila melanogaster*: deficiency mapping of EMS-induced lethal complementation groups. Genetics **83:** 765–782.

Holdridge, C., and D. Dorsett, 1991 Repression of *hsp70* heat shock gene transcription by the suppressor of Hairy-wing protein of *Drosophila melanogaster*. Mol. Cell. Biol. **11:** 1894–1900.

Jack, J. W., 1985 Molecular organization of the *cut* locus of *Drosophila melanogaster*. Cell **42:** 869–876.

Jack, J., and Y. Delotto, 1992 Effect of wing scalloping mutations on *cut* expression and sense organ differentiation in the Drosophila wing margin. Genetics **131:** 353–363.

Jack, J. W., D. Dorsett, Y. Delotto and S. Liu, 1991 Expression of the *cut* locus in the *Drosophila* wing margin is required for cell type specification and is regulated by a distant enhancer. Development **113:** 735–747.

Jurata, L. W., and G. N. Gill, 1997 Functional analysis of the nuclear LIM domain interactor, NLI. Mol. Cell. Biol. **17:** 5688–5698.

Jurata, L. W., D. A. Kenney and G. N. Gill, 1996 Nuclear LIM interactor, a rhombotin and LIM homeodomain interacting protein, is expressed early in neuronal development. Proc. Natl. Acad. Sci. USA **93:** 11693–11698.

Jurata, L. W., S. L. Pfaff and G. N. Gill, 1998 The nuclear LIM domain interactor NLI mediates homo- and heterodimerization of LIM domain transcription factors. J. Biol. Chem. **273:** 3152–3157.

Kao, H. Y., P. Ordentlich, N. Koyano-Nakagawa, Z. Tang, M. Downes *et al.*, 1998 A histone deacetylase corepressor complex regulates the Notch signal transduction pathway. Genes Dev. **12:** 2269–2277.

Kelley, M. R., S. Kidd, W. A. Deutsch and M. W. Young, 1987 Mutations altering the structure of the epidermal growth factor-like coding sequences at the *Drosophila Notch* locus. Cell **51:** 539–548.

Kim, J., K. D. Irvine and S. B. Carroll, 1995 Cell recognition, signal induction, and symmetrical gene activation at the dorsal-ventral boundary of the developing Drosophila wing. Cell **82:** 795–802.

Kim, J., A. Sebring, J. J. Esch, M. E. Kraus, K. Vorwerk *et al.*, 1996a Integration of positional signals and regulation of wing formation and identity by *Drosophila vestigial* gene. Nature **382:** 133–138.

Kim, J., B. Shen, C. Rosen and D. Dorsett, 1996b The DNA-binding and enhancer-blocking domains of the *Drosophila suppressor of Hairy-wing protein*. Mol. Cell. Biol. **16:** 3381–3392.

Michaelis, C., R. Ciosk and K. Nasmyth, 1997 Cohesins: chromo-

somal proteins that prevent premature separation of sister chromatids. Cell **91:** 35–45.

Milan, M., F. J. Diaz-Benjumea and S. M. Cohen, 1998 Beadex encodes an LMO protein that regulates Apterous LIM-homeodomain activity in *Drosophila* wing development: a model for LMO oncogene function. Genes Dev. **12:** 2912–2920.

Mlodzik, M., and Y. Hiromi, 1992 Enhancer trap method in Drosophila and its application to neurobiology. Methods Neurosci. **9:** 397–414.

Mogila, V. A., A. B. Ladvishenko, O. B. Simonova and T. I. Gerasimova, 1992 Intragenic suppression: stalker, a retrovirus-like transposable element, can compensate for a deficiency at the *cut* locus of *Drosophila melanogaster*. Genetica **86:** 305–311.

Morcillo, P., C. Rosen and D. Dorsett, 1996 Genes regulating the remote wing margin enhancer in the Drosophila *cut* locus. Genetics **144:** 1143–1154.

Morcillo, P., C. Rosen, M. K. Baylies and D. Dorsett, 1997 Chip, a widely expressed chromosomal protein required for segmentation and activity of a remote wing margin enhancer in *Drosophila*. Genes Dev. **11:** 2729–2740.

Neumann, C. J., and S. M. Cohen, 1996 A hierarchy of cross-regulation involving *Notch*, *wingless*, *vestigial* and *cut* organizes the dorsal/ventral axis of the *Drosophila* wing. Development **122:** 3477–3485.

Peifer, M., and W. Bender, 1986 The anterobithorax and bithorax mutations of the bithorax complex. EMBO J. **5:** 2293–2303.

Ptashne, M., 1986 Gene regulation by proteins acting nearby and at a distance. Nature **322:** 697–701.

Ptashne, M., 1988 How eukaryotic transcriptional activators work. Nature **335:** 683–689.

Qian, S., M. Capovilla and V. Pirrotta, 1993 Molecular mechanisms of pattern formation by the BRE enhancer of the *Ubx* gene. EMBO J. **12:** 3865–3877.

Rabinow, L., and J. A. Birchler, 1990 Interactions of *vestigial* and *scabrous* with the *Notch* locus of *Drosophila melanogaster*. Genetics **125:** 41–50.

Santero, E., T. R. Hoover, A. K. North, D. K. Berger, S. C. Porter *et al.*, 1992 Role of integration host factor in stimulating transcription from the σ^{54}-dependent *nifH* promoter. J. Mol. Biol. **227:** 602–620.

Scott, K. S., and P. K. Geyer, 1995 Effects of the *su(Hw)* insulator protein on the expression of the divergently transcribed *Drosophila yolk protein* genes. EMBO J. **14:** 6258–6267.

Seitz, L. C., K. Tang, W. J. Cummings and M. E. Zolan, 1996 The *rad9* gene of *Coprinus cinereus* encodes a proline-rich protein required for meiotic chromosome condensation and synapsis. Genetics **142:** 1105–1117.

Shoresh, M., S. Orgad, O. Shmueli, R. Werczberger, D. Gelbaum *et al.*, 1998 Overexpression *Beadex* mutations and loss-of-function *heldup-a* mutations in Drosophila affect the 3′ regulatory and coding components, respectively, of the *Dlmo* gene. Genetics **150:** 283–299.

Simmonds, A. J., X. Liu, K. H. Soanes, H. M. Krause, K. D. Irvine *et al.*, 1998 Molecular interactions between Vestigial and Scalloped promote wing formation in *Drosophila*. Genes Dev. **12:** 3815–3820.

Simon, J., M. Peifer, W. Bender and M. O'Connor, 1990 Regulatory elements of the bithorax complex that control expression along the anterior-posterior axis. EMBO J. **9:** 3945–3956.

Spana, C., and V. G. Corces, 1990 DNA bending is a determinant of binding specificity for a Drosophila zinc finger protein. Genes Dev. **4:** 1505–1515.

Takahashi, K., H. Yamada and M. Yanagida, 1994 Fission yeast minichromosome loss mutants *mis* cause lethal aneuploidy and replication abnormality. Mol. Biol. Cell **5:** 1145–1158.

Taniguchi, Y., T. Furukawa, T. Tun, H. Han and T. Honjo, 1998 LIM protein KyoT2 negatively regulates transcription by association with the RBP-J DNA-binding protein. Mol. Cell. Biol. **18:** 644–654.

Thanos, D., and T. Maniatis, 1995 Virus induction of human IFNβ gene expression requires the assembly of an enhanceosome. Cell **83:** 1091–1100.

Valentine, G., Y. J. Wallace, F. R. Turner and M. E. Zolan, 1995 Pathway analysis of radiation-sensitive meiotic mutants of *Coprinus cinereus*. Mol. Gen. Genet. **247:** 169–179.

Wadman, I. A., H. Osada, G. G. Grutz, A. D. Agulnick, H. Westphal *et al.*, 1997 The LIM-only protein Lmo2 is a bridging molecule assembling an erythroid, DNA-binding complex which includes the TAL1, E47, GATA-1 and Ldb1/NLI proteins. EMBO J. **16:** 3145–3157.

Wirtz, R. A., and H. G. Semey, 1982 The Drosophila kitchen-equipment, media preparation and supplies. Dros. Inf. Serv. **58:** 176–180.

Xu, T., I. Rebay, R. J. Fleming, T. N. Scottgale and S. Artavanis-Tsakonas, 1990 The Notch locus and the genetic circuitry involved in early Drosophila neurogenesis. Genes Dev. **4:** 464–475.

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