Ras1-Mediated Modulation of Drosophila Homeotic Function in Cell and Segment Identity

Muriel Boube,* Corinne Benassayag,* Laurent Seroude† and David L. Cribbs*

**CBD-CNRS, 31062 Toulouse Cédex, France and †Division of Biology, California Institute of Technology, Pasadena, California 91125*

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ABSTRACT

Mutations of the Drosophila homeotic *proboscipedia* gene (*pb*; the Hox-A2/B2 homologue) provoke dose-sensitive defects. These were used to search for dose-sensitive dominant modifiers of *pb* function. Two identified interacting genes were the proto-oncogene *Ras1* and its functional antagonist *Gap1*, prominent intermediaries in known signal transduction pathways. $Ras1^+$ is a positive modifier of *pb* activity both in normal and ectopic cell contexts, while the *Ras1*-antagonist *Gap1* has an opposite effect. A general role for *Ras1* in homeotic function is likely, since $Ras1^+$ activity also modulates functions of the homeotic loci *Sex combs reduced* and *Ultrabithorax*. Our data suggest that the modulation occurs by a mechanism independent of transcriptional control of the homeotic loci themselves, or of the *Ras1/Gap1* genes. Taken together our data support a role for *Ras1*-mediated cell signaling in the homeotic control of segmental differentiation.

MAKING a fruit fly requires crucial contributions from homeotic "selector" genes. These selector genes direct the development of the structures unique to each segment: legs, wings, halteres, mouthparts. The mutation of a homeotic locus leads to the replacement, sometimes spectacular, of one body part by an inappropriate one. The homeodomain-containing transcription factors encoded by the homeotic genes regulate the expression of groups of target "realisator" genes that confer unique identities to the segmental units composing the embryonic and adult body (LAWRENCE and MORATA 1994). The majority of known homeotic loci in *Drosophila melanogaster* are located in the Antennapedia and Bithorax Complexes and correspond to vertebrate homologues making up the Hox complexes (KENYON 1994). In light of the striking evolutionary conservation of the homeotic/Hox genes and complexes, it is believed that these genes' functions are also highly conserved. This presumption, when tested, has been borne out (MALICKI *et al.* 1990; MCGINNIS *et al.* 1990; ZHAO *et al.* 1993; POPPERL *et al.* 1995).

Homeotic gene functions are required in specific regions of the embryo, larva, and pupa as seen by the localized effect of loss-of-function mutations. Gene expression is generally found to be spatially restricted to the region requiring homeotic function. Selector function is clearly seen through the action of homeotic gain-of-function mutations that direct the formation of normal structures in inappropriate localities. Proper gene function leads to the formation of a complex, differentiated structure comprising numerous cell types correctly proportioned and positioned. Ectopic expression may lead to the fabrication of the same structure elsewhere.

How does the function of a single gene direct the development of a complex structure? Much of the literature concerning homeotic gene function has focused on their selector functions via transcriptional control in the cell nucleus (BOTAS 1993; GEHRING *et al.* 1994). Several studies in mosaic animals concluded that homeotic function is cell autonomous: the action of these transcriptional regulators in a cell depends only on the genotype of that cell (GARCIA-BELLIDO and LEWIS 1976; MORATA *et al.* 1983; MERRILL *et al.* 1987). Still, this emphasis on the hierarchical regulation of downstream target genes, and on elements of protein structure (notably the homeodomain) involved in such regulation, is likely incomplete given the complexity of structures such as legs and wings formed through homeotic control. A small number of observations support a role for cell-cell communication in homeotic function. For example, one direct transcriptional target of the homeotic *Ultrabithorax* (*Ubx*) locus is the *decapentaplegic* (*dpp*) gene encoding a conserved TGF-β related growth factor (CAPOVILLA *et al.* 1994). *Ubx* activates *dpp* expression in the visceral mesoderm (IMMERGLUCK *et al.* 1990); the secreted DPP protein then modulates activity of the homeotic *labial* gene in cells of the adjacent endoderm (IMMERGLUCK *et al.* 1990). Thus at least one homeotic gene, *Ubx*, can send a signal and one other, *labial*, is capable of responding to it. Second, nonautonomous behavior of mitotic $Antp^-$ clones in mosaic animals was reported a number of years ago (STRUHL 1981). This observed nonautonomy suggests intercellular communication within the imaginal disc cells generating the appendage.

We present evidence here that homeotic function is

Corresponding author: David Cribbs, Centre de Biologie du Développement-CNRS, 118 route de Narbonne, Bâtiment 4R3, 31062 Toulouse Cedex, France. E-mail: cribbs@cict.fr

modulated by functions of the proto-oncogene *Ras1*, likely by cell signaling pathways. Our principal model is the *proboscipedia* locus (*pb*: homologous to Hox-A2/-B2), required for adult mouthparts development. In the absence of *pb* function, the adult labial palps are transformed to prothoracic legs and the maxillary palps reduced to vestigial stumps (KAUFMAN 1978; PULTZ *et al.* 1988; CRIBBS *et al.* 1992). The homeotic gain-of-function *pb* phenotype is a transformation of the adult antennae to maxillary palps (CRIBBS *et al.* 1995). Both the loss- and gain-of-function transformations are dose-sensitive. In searching for dose-sensitive modifiers of *pb* function, we found that altering activity of the proto-oncogene *Ras1* or of its antagonist *Gap1* can lead to changes in the attribution of specific cell identities within a segment, or of segmental identity. *Ras1* is centrally involved in the signal transduction pathways passing by the *sevenless* and *torso* receptor tyrosine kinases, modifying the nuclear activity of transcription factors via the balance of RAS1-GTP (active form) and RAS1-GDP (inactive form) (PERRIMON 1994; SIMON 1994). We present here the first evidence for a functional link between the *Ras1* proto-oncogene and homeotic function. The observed modulation of homeotic activity by *Ras1* is likely to be general, since we find that homeotic activities of the *Ubx* and *Sex combs reduced* (*Scr*) loci are also sensitive to *Ras1* activity levels.

MATERIALS AND METHODS

Fly culture and phenotypic analysis: All stocks and crosses were maintained at 25° on standard yeast-agar-cormeal molasses medium. Phenotypes were initially regarded under a stereomicroscope; detailed analyses were performed by light microscopy (Zeiss Axiophot) after mounting dissected samples in Hoyer's medium or by scanning electron microscopy.

Deficiency screen: The collections of deficiency stocks for the second and third chromosomes (DK2 and DK3) were obtained from the Indiana University Drosophila Stock Center (IUDSC), Bloomington, Indiana. Females heterozygous for a given deficiency were mated with males carrying the HSPB:4d transgene. F_1 progeny bearing both the transgene and the deficiency were analyzed for phenotypic modifications specific to the combination.

Flies stocks: All mutations, aberrations and abbreviations are either found in LINDSLEY and ZIMM (1992) or are as described herein. Transgenic HSPB lines were employed as follows: all interactions shown with HSPB were obtained with the HSPB:4d line, carrying a single insertion at 82A on chromosome 3R. To ensure that the interactions were due to PB expression from the transgene and not to an effect of the insertional mutation, interacting deficiencies were retested in combination with a second line, HSPB:2.5, carrying a transgene at 14A on the *X* chromosome (CRIBBS *et al.* 1995). The large deficiencies removing *Ras1* are described in LINDSLEY and ZIMM (1992), two *Ras1* point mutations, Ras^{e1B} and Ras^{e2F}, in SIMON *et al.* (1991), the null allele $Ras1^{C40b}$ in HOU *et al.* (1995), and the *Ras1* duplication Dp(3;3)M-S31-2, in KEMPHUES *et al.* (1983). The *Gap1* mutant allele used in this work was Gap^{BJ61}, an insertion of a *P*[ry^+, *lacZ*] enhancer trap element described in ROGGE *et al.* (1992). The gain of function allele Scr^{ScxP} was described by PATTATUCCI and KAUFMAN (1991). T(Y;3)$Antp^+$ carries a *Y* chromosome-linked duplication of the entire Antennapedia Complex. This chromosome, isolated by R. DENELL, is available from the IUDSC.

Immunolocalizations: Antibodies and staining reactions were essentially as described in RANDAZZO *et al.* (1991). Disc stainings were as described by PATTATUCCI and KAUFMAN (1991).

***In situ* hybridizations:** Hybridizations were performed essentially as described by TAUTZ and PFEIFLE (1989). *Ras1*- and *Gap1*-specific probes were prepared from plasmids containing 3.2-kb of genomic *Ras1* sequences (provided by J. BISHOP), and a 5.3-kb cDNA for *Gap1* (from U. GAUL).

RESULTS

Specific functional interactions at the molecular level *in vivo* have in many cases first been identified as dose-sensitive genetic interactions. Loss-of-function *proboscipedia* (*pb*) mutations can yield qualitatively distinct adult transformations (KAUFMAN 1978; PULTZ *et al.* 1988; CRIBBS *et al.* 1992). In the pb^+ condition, the adult mouthparts comprise normal labial palps. Partial loss-of-function leads to the partial replacement of labial tissue by aristae, the plumed distal antennal structures. The *pb* null condition results in complete replacement of the labial palps by prothoracic legs. Certain hypomorphic alleles show a marked dosage sensitivity, allowing for transformations of labium to antenna, or to leg, in closely related conditions. Conversely, gain-of-function achieved by ectopic PB expression in transgenic HSPB lines (carrying a chimeric gene composed of the Hsp70 promoter fused to an 8.6-kb *pb* mini-gene) leads to the transformation of antennae to maxillary palps (CRIBBS *et al.* 1995). As for the action of loss-of-function mutations on the labial palps, this homeotic transformation of the antennae is dose sensitive. A highly reproducible partial transformation of antennae to maxillary palps results from basal (uninduced) expression of a single HSPB copy. Two copies result in a nearly complete antenna-to-maxillary transformation. Similarly, dose-sensitive effects due to the ectopic expression of PB protein from HSPB are also detected in the wings, the eyes, the posterior head and the prothoracic legs.

To identify genes that interact with *pb* in directing normal development, we sought dominant mutations that synergistically modify HSPB developmental activity. This was accomplished by testing HSPB lines in combination with deletion mutations removing defined portions of the genome. In the heterozygous state such deletions reduce by about half the activity of all genes within that interval, but show no visible developmental defects. We sought deletions whose combination with HSPB modified the antenna-to-maxillary transformation, or led to novel phenotypes provoked neither by HSPB alone, nor by the heterozygous deletion. Among 110 autosomal deficiencies tested (representing about half the genome), 10 showed dose-sensitive interactions

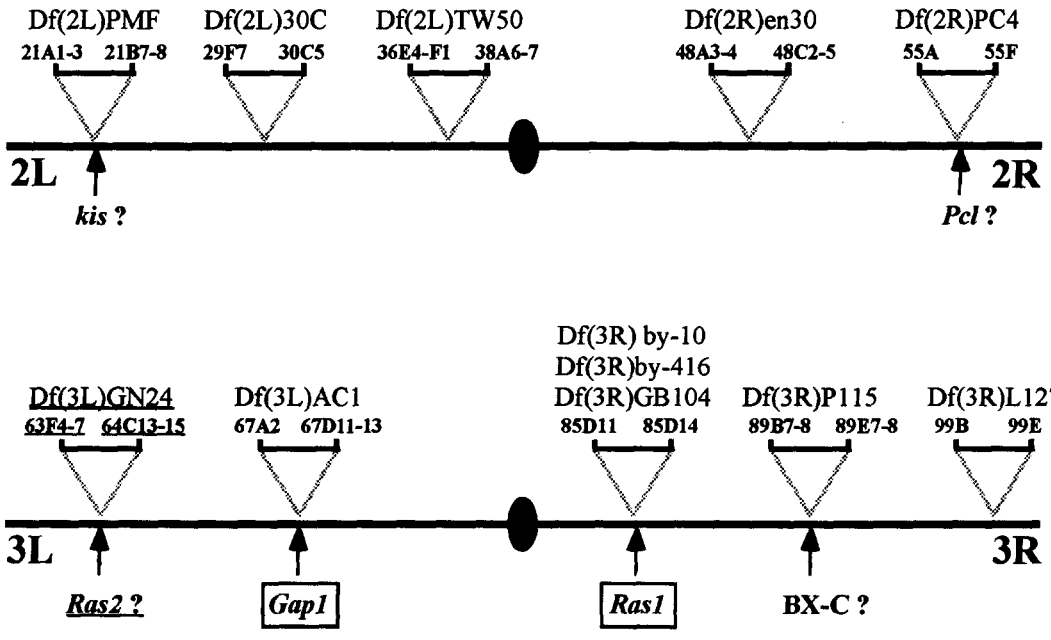

FIGURE 1.—Autosomal intervals containing dose-sensitive modifiers of HSPB. Chromosomes *2* and *3* are represented by the heavy lines, with a black oval for the centromere. Indicated above the lines are the names and extents of the deficiency mutations showing dose-sensitive interactions with HSPB. (The lone exception is for the Ras1 interval on chromosome *3*R, where the region indicated is delimited by three different interacting deficiencies.) Indicated below the lines are the identified (*Gap1, Ras1*) or suspected (*kis*?, *Pcl*?, *Ras2*?; BX-C?) interacting loci removed by these deficiencies.

with HSPB (summarized in Figure 1). Several of these regions harbor genes with functions obviously related to homeotic selector activities [for example, the Polycomb Group member *Polycomblike* (*Pcl*) and the Bithorax Complex]. Surprisingly, three of the identified genomic intervals contain the *Ras1*, *Gap1* and *Ras2* genes each known to play a role in signal transduction. This observation prompted us to examine homeotic function relative to *Ras1* and *Gap1* activity, since point mutations exist for these two genes.

***Ras1* modulates dominant HSPB homeotic activity:** Three overlapping deficiencies in the 85D region on chromosome *3*R [Df(3R)*by*-10, Df(3R)*by*-416 and Df(3R)GB104] gave dose-sensitive phenotypic interactions with HSPB, namely, a fully penetrant reduction of the segmental Ant to Mx transformation (Figure 2, B and C). Associated phenotypes were observed in the wings, where the distal portions of longitudinal veins L4 and L5 were often partially deleted. In contrast, the partially overlapping Df(3R)*by*-62 did not interact. These data allowed us to situate an interacting locus in the interval 85D11-14. The *Ras1* locus, isolated as a dose-sensitive modifier of *sevenless* receptor tyrosine kinase activity (described in SIMON *et al.* 1991) is situated at 85D8-14 on chromosome *3*R. The *Ras1* gene encodes a GTP-binding protein with a GTPase activity (NEUMAN *et al.* 1984), whose known functions are employed in signal transduction pathways. In light of the genetic localisation of a dose-sensitive modifier to the same interval, we tested the effects of combining *Ras1* point mutations with HSPB (the two *Ras1* missense alleles e2F and e1B were employed). These yielded wing phenotypes similar to those for the deletion mutations removing *Ras1* with HSPB (see Figure 3, A–C). Thus *Ras1* interacts with HSPB in controlling certain cell identities. In contrast, we did not detect a reliable reduction in the HSPB-induced Ant to Mx transformation with $Ras1^{e2F}$ or $Ras1^{e1B}$. We thus cannot formally exclude the possibility that a second gene in this interval also interacts with *pb*.

A very weak modification of the wings was detected in animals carrying HSPB in heterozygous combination with Df(3L)AC1, a deletion removing the interval 67A2; 67D11-13. This region contains the *Gap1* gene encoding a functional antagonist of *Ras*1 signaling. GAP1 protein activates the GTPase activity of RAS1-GTP, favoring conversion to the inactive GDP-bound form (GAUL *et al.* 1992). Having confirmed the phenotypic interaction between HSPB and $Ras1^-$, the effects of *Gap1* mutations on HSPB were examined. The allele employed, $Gap1^{BJ61}$, is a *P* insertion described as a strong or null mutation (ROGGE *et al.* 1992) that we refer to hereafter as $Gap1^-$.

Heterozygous combinations of $Gap1^-$ with HSPB gave only a very weak effect. Importantly, though, the $Gap1^-$ HSPB/$Gap1^-$ genotype led to a marked enhancement of the Ant to Mx transformation (Figure 2D). Since *Gap1* is generally viewed as a specific antagonist of $Ras1^+$ function, this supported our interpretation that *Ras1* is the relevant locus in the 85D11-14 interval that modifies both homeotic segmental transformation and wing vein formation. We therefore compared the Ant to Mx transformation in $Gap1^-$ HSPB/$Gap1^-$ adults with $Gap1^-$ HSPB/$Gap1^-$ Ras^{e1B} (Figure 2, D and E). Among the adults eclosing in this sensitized context, we observed a reduction of the Ant to Mx transformation. This reduction due to a *Ras1* point mutation confirms that both *Ras1* and *Gap1* mutations can modify the segmen-

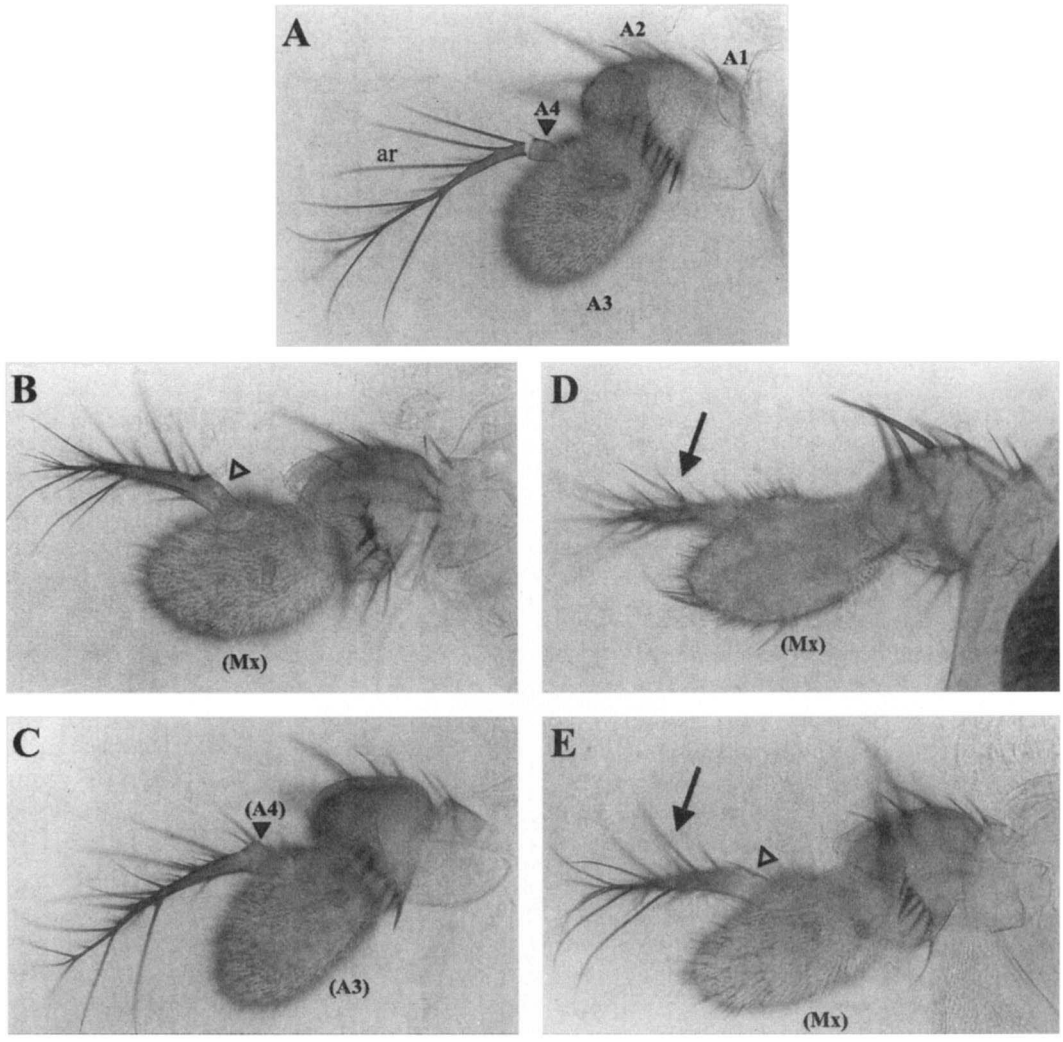

FIGURE 2.—Mutations of *Ras1* and *Gap1* modify the HSPB-induced antenna-to-maxillary palps transformation. (A) Wild-type antenna comprising, from proximal to distal, the antennal segments A1, A2, A3, A4 and the distal arista (ar). (B) An antenna of a HSPB:4d/+ fly showing a partial transformation of the antennal segment A3 (Mx) and of A4 and the arista (open arrowhead) toward maxillary palps (Ant to Mx). (C) An antenna of a HSPB/Df(3R)*by*-416 ($Ras1^{-}$/+) fly. The deletion of one *Ras*1 copy leads to a partial reversion of the homeotic Ant to Mx transformation indicated by A3 and the arrowhead pointing to A4. (D) An antenna of a $Gap1^{-}$ HSPB/$Gap1^{-}$ fly. The transformation of antenna toward maxillary palps is more complete in the presence of the *Gap1* mutation, as seen by the reduction of the arista and its replacement by a more horizontal maxillary palp (Mx). The effect of the homozygous $Gap1^{-}$ condition on the HSPB phenotype is opposite to that of a heterozygous deletion of the *Ras1* interval in C. (E) Antenna of $Gap1^{-}$ HSPB/$Gap1^{-}$ Ras^{e1B} fly. The heterozygous presence of a *Ras1* point mutation reduces the *Gap1*-conferred enhancement of the HSPB-induced Ant to Mx transformation.

tal transformation that results from ectopic PB expression. Further, $Gap1^{-}$ homozygotes possess additional wing vein tissue (Figure 3D), a defect aggravated by the presence of HSPB ($Gap1^{-}$ HSPB/$Gap1^{-}$; see Figure 3E). This mutant phenotype was reduced by a *Ras1* point mutation ($Gap1^{-}$ HSPB/$Gap1^{-}$ Ras^{e1B}; see Figure 3F). These data support the interpretation that the *Ras1* locus modifies ectopic *pb* homeotic activities in both segmental transformation and wing vein formation. We note, however, that this interpretation is based on our explicit (and as yet unproven) presumption that pathways with common components are employed in both wing and antennal development. As shown in the next section, the sensitive genetic interactions identified in the antennae and wings reflect interactions that can also be detected in the mouthparts, the normal site of *pb* function.

***Ras1* function modulates normal *pb* activity in the mouthparts:** Although the modified wings and the Ant to Max homeotic transformation offer sensitized contexts to screen for genetic interactors with *pb,* neither is a normal site for *pb* function. We therefore examined whether similar effects for *Ras1* and *Gap1* could be detected in a normal lieu of pb^{+} function, the labial palps.

Double mutant combinations were constructed placing appropriate *pb* hypomorphic mutations in combination with either the null allele $Ras1^{C40b}$ ($Ras1^{-}$) or with $Gap1^{-}$. One test employed the *pb* alleles pb^{4} and pb^{5}, an

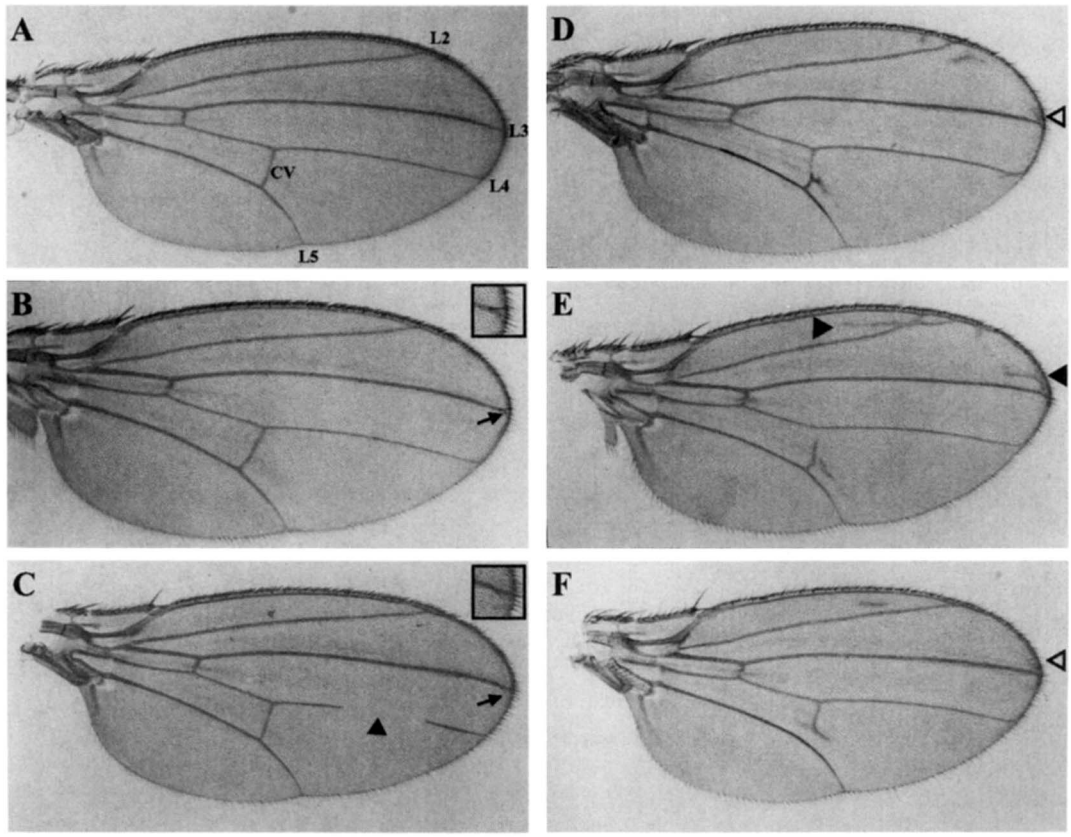

FIGURE 3.—HSPB interacts with *Ras1* and *Gap1* in the wings. (A) Wild-type wing. The longitudinal veins L2, L3, L4 and L5 and the posterior crossvein between L4 and L5 (cv) are indicated. (B) A HSPB/+ wing. Most of these wings are wild type, but a small proportion (~10% of female wings and ~2% for males) possess an ectopic bristle on the distal end of the vein L3 (arrow and inset enlargement). (C) Wing of a HSPB/Ras^{e1B} fly. Whereas HSPB/+ and Ras^{e1B}/+wings are nearly wild type, their combination provokes a partially penetrant truncation of wing veins L4 (and L5, not shown) and augments the frequency of the ectopic L3 bristle (~90% for females and ~50% for males; see arrow and inset enlargement). (D) A $Gap1^{-}$ wing showing ectopic vein tissue near the junction of L2 with the wing margin, thickening of the L3 vein extremity (open arrowhead), and modification of the crossvein. (E) Wing of a $Gap1^{-}$ HSPB/$Gap1^{-}$ fly. The HSPB transgene enhances the effects of *Gap1*, yielding ectopic veins that are more prominent, particularly adjacent to the distal end of L3 (closed arrowhead). (F) Wing of a $Gap1^{-}$ HSPB/$Gap1^{-}$ Ras^{e1B} fly. As for the Ant to Mx transformation (Figure 1E), the Ras^{e1B} mutation diminishes the effects of *Gap1* on HSPB-induced phenotypes in the wing.

intermediate strength hypomorph and a protein null, respectively. We compared pb^{5} $Ras1^{C40b}/pb^{4}$ and pb^{5} $Ras1^{+}/pb^{4}$ animals. The pb^{5}/pb^{4} combination leads to a mixed transformation of the distal labium to leg/antennal appendages. The prothoracic or T1 leg tissue can be distinguished from labial or aristal tissue by the appearance of leg-specific bracted bristles, distal claws and associated sense organs, and the male-specific sex comb. On examining adult pb^{5}/pb^{4} males with two (control) or only one functional copy of $Ras1^{+}$, the labial palps of the latter showed generally more severe mutant phenotypes including the appearance of prothorax-specific sex comb teeth and distal claws (Figure 4, A and B). These results thus clearly support a role for $Ras1^{+}$ activity in wild-type *pb* homeotic function in the adult mouthparts.

Adults homozygous for pb^{4} and $Gap1^{-}$ pb^{4} were also examined in the hypothesis that the $Gap1^{-}$ condition would lead to increased pb^{+} activity. The pb^{4} genotype leads to a reliable partial transformation of the labial palps to antennal aristae (Figure 4C). In the $Gap1^{-}$ pb^{4} double mutant the labial to antennal transformation was consistently altered toward wild type (Figure 4D). This indicates that reduced *Gap1* activity augments pb^{+} function in the distal labium.

Taken together these data indicate that *Ras1*/*Gap1* functions modulate *pb* activity in a variety of cell types, including the mouthparts and the antennae. $Ras1^{+}$ acts as a positive modulator of pb^{+} activity, and $Gap1^{+}$ exerts an opposite effect. This is true for gain-of-function PB phenotypes in diverse contexts including the antennae (Figure 2), wings (Figure 3) and legs (not shown). Importantly, it is also true for normal *pb* functions in the mouthparts (Figure 4).

***Ras1* activity modulates the homeotic activities of *Scr* and *Ubx*:** To test whether the modification of *pb* homeotic activity by *Ras1* might be more general, we examined the functional relationship between *Ras1* and the homeotic *Sex combs reduced* (*Scr*) and *Ultrabithorax* (*Ubx*) loci.

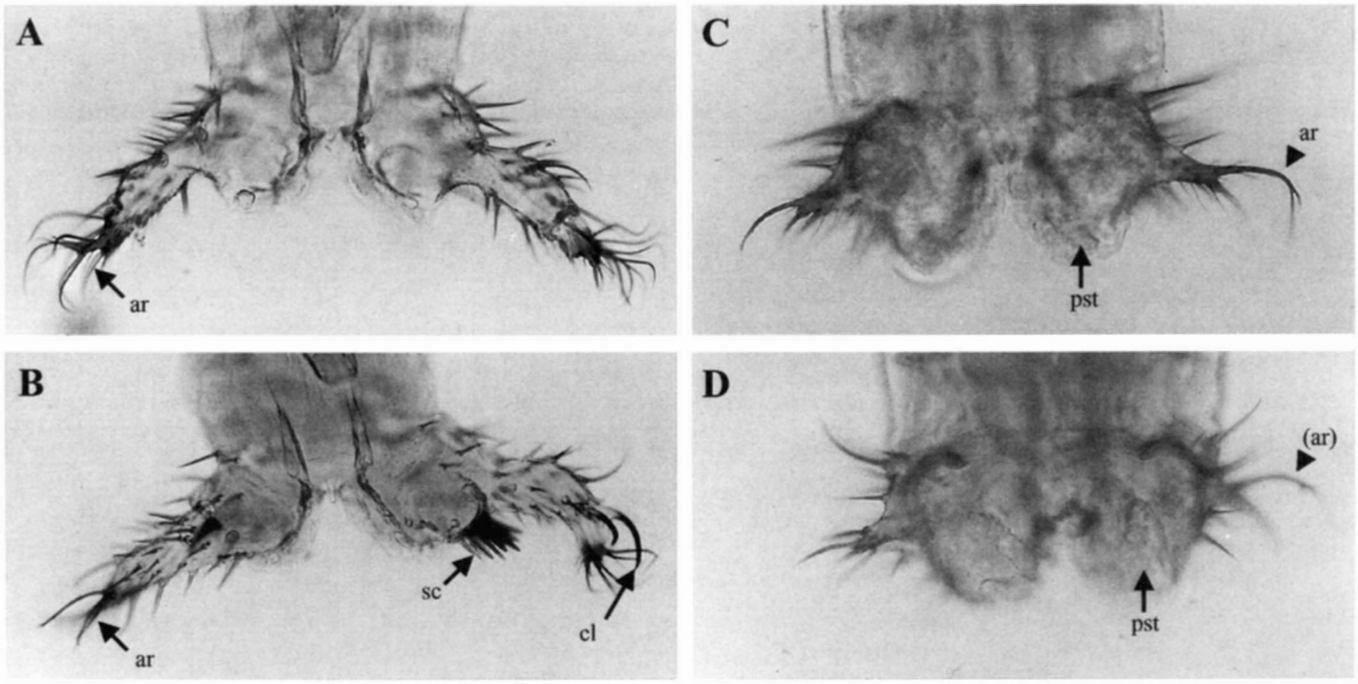

FIGURE 4.—Interactions between *Ras1* and *pb* in the adult mouthparts, the normal site of *pb* function. The pb^4 and pb^5 alleles cause labial transformations to arista and prothoracic leg, respectively. (A) Labial palps of a pb^4/pb^5 fly, showing a mixed transformation of the labium to leg and aristal (ar) structures. (B) Labial palps of a pb^4/pb^5 Ras^{C40b} fly. In the $Ras1^-$ heterozygotes, leg-specific structures such as sex comb teeth (sc) and distal claws (cl) appear. These structures are observed rarely if at all in the control genotype. [For the distal claw, 10% (9/94) of pb^5 $Ras1^{C40b}/pb^4$ males, compared to 0% (0/63) for the control class. Twenty-two percent (21/94) carried at least one sex comb tooth compared to 11% (7/63) for the control class. Each result is significant (chi-square P values < 0.05)]. (C and D) Interaction of *Gap1* and *pb* in the adult mouthparts. (C) Labial palps from flies of genotype *thr* pb^4 are partially transformed to arista (ar; indicated by a closed arrowhead) due to pb^4. The form of the ectopic arista seen here is modified by the *thread* (*thr*) mutation. Residual labial pseudotrachea are present (pst). (D) The addition of the $Gap1^-$ mutation (genotype $Gap1^-$ *thr* pb^4) leads to a reduction of the aristal transformation accompanied by improved pseudotracheal form (pst; not discernible in this focal plane).

Scr: Normal male flies carry a sex comb on the most proximal tarsal segment of the prothoracic (T1) leg, composed of "teeth" that are specialized bristles. Prothoracic identity, including the presence of the single, properly placed sex comb, depends on the homeotic *Sex combs reduced* locus (PATTATUCCI and KAUFMAN 1991; PATTATUCCI *et al.* 1991). Scr^+ function is haploinsufficient (hence dose-sensitive), as most simply visualized by the sex comb. Whereas normal males possess a sex comb with ~12 teeth, in Scr^-/Scr^+ heterozygotes this comb is reduced to approximately six teeth (Figure 5A). Because quantitative modulations of the sex comb were not readily detected on modifying *Ras1* activity, we sought a more sensitized background in which to examine potential interactions of *Scr* with *Ras1*.

Another locus affecting sex comb formation is *sex combs distal* (*scd*), an *X*-linked gene represented by the single viable mutant allele (LINDSLEY and ZIMM 1992). About 70% of mutant *scd*; Scr^+ males carry a small distal sex comb on the second tarsal segment of the T1 leg (Figure 5C). Reducing Scr^+ function by half in *scd*; Scr^-/Scr^+ males abolishes the more distal sex comb (though the single remaining sex comb contains approximately eight teeth instead of six; Figure 5B). Conversely, increasing Scr^+ function by introduction of the chromosomal duplication Dp(Y;3)$Antp^+$ leads to the fully penetrant appearance of a second sex comb containing on average four teeth (Figure 5D). Dp(Y;3)$Antp^+$ comprises a duplication of the entire Antennapedia Complex, but the observed enhancement is attributable to Scr^+ alone since it is reversed by an *Scr* point mutation (not shown). An effect similar to that of the duplication was obtained with the gain-of-function allele Scr^{ScxP} (not shown). These data show that the fabrication of a second more distal sex comb depends on Scr^+ function.

The effects of *Ras1*/*Gap1* activities on the formation of a distal sex comb were then examined. As for Scr^+, *scd* males with increased dosage of *Ras*1$^+$showed a prominent second sex comb with full penetrance, as seen for *scd*; $Ras1^+$/Dp(3;3)M-S31-2 (carrying three functional copies of *Ras1*; Figure 5E). The same effect is observed on reducing activity of the *Ras1* antagonist *Gap1*, as seen for *scd*; $Gap1^-$ (Figure 5F). The effect of Dp(3;3)M-S31-2 containing a supplementary $Ras1^+$ copy is reversed when placed in combination with the *Ras1* point mutation $Ras1^{e1B}$ (not shown), and is thus specifically attributable to $Ras1^+$. The formation of a more distal sex comb depends on the state of both Scr^+ and $Ras1^+$ activities.

Ubx: Normal haltere development is sensitive to *Ubx*

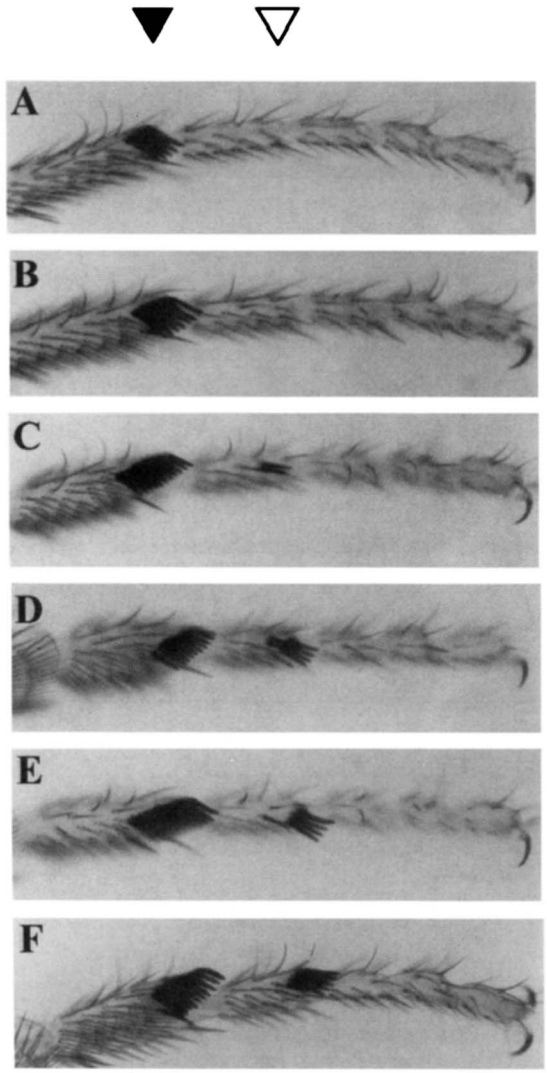

FIGURE 5.—Interaction between *Sex combs reduced* (*Scr*) and *Ras1/Gap1*. These tests were carried out in a sensitized genetic context that yields an enhanced phenotypic effect, by placing all mutant combinations in a background carrying the *X* chromosome-linked *sex combs distal* (*scd*) mutation. (A) The prothoracic or T1 leg of an $Scr^4/+$ adult male. The sex comb on the first tarsal segment is reduced by about half by the haploinsufficient *Scr* null mutation. (B) The T1 leg of an *scd;* $Scr^4/+$ male. The *scd* allele partially restores the sex comb teeth removed in the $Scr^-/+$ phenotype. (C) The T1 leg of an *scd;* $+/+$ male fly. The *scd* mutation provokes a small, more distal sex comb on the second tarsal segment. This comb contains two teeth on average, and is detected in ~70% of adult males. (D) The T1 leg of *scd;* T(Y;3)$Antp^+/+$ fly. The *scd* phenotype is enhanced (penetrance is raised from 70 to 100%, and the number of ectopic sex comb teeth is increased from two to about four) by a duplication of the Antennapedia Complex ($Antp^+$). This effect is due to increased *Scr* locus activity alone since it is reversed by placing the duplication in combination with the Scr^4 point mutation. (E) The T1 leg of an *scd;* Dp(3;3)M-S31-2(Dp($Ras1^+$))/+ male. The duplication of the $Ras1^+$ gene enhances the distal sex comb phenotype. (F) A T1 leg of a *scd;* $Gap1^{BJ61}/Gap1^{BJ61}$ male. The reduction of *Gap1* function has the same effect on the *scd* phenotype as does increasing $Ras1^+$ function by a duplication. As for *Scr*, the effect of the chromosomal duplication is reversed by a point mutation of the *Ras1* locus.

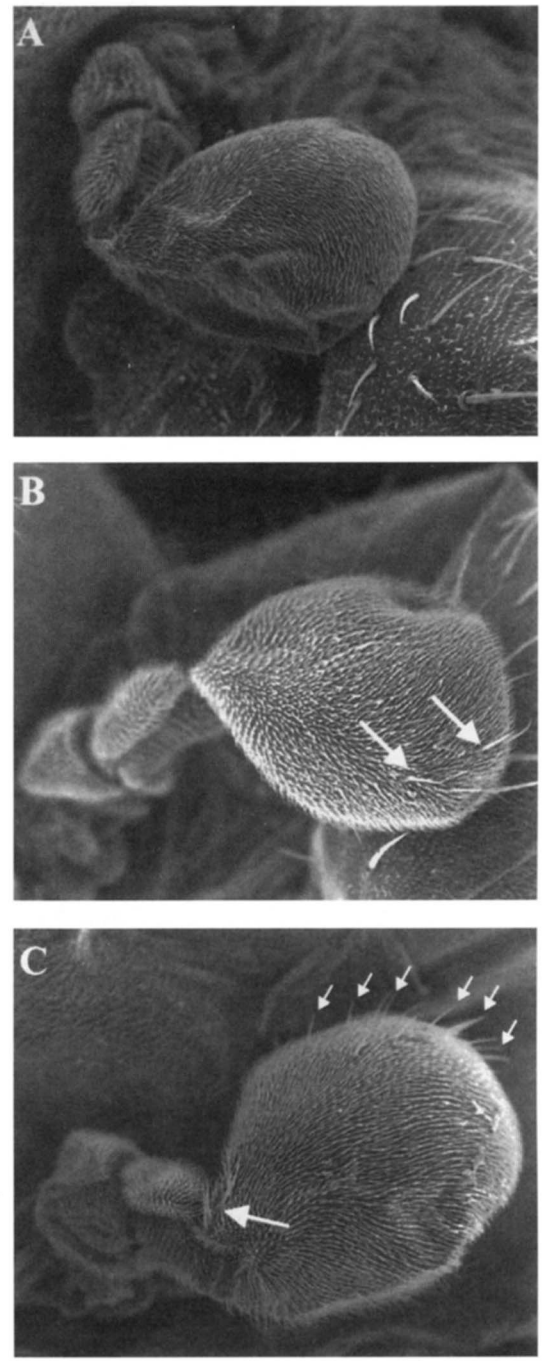

FIGURE 6.—The haploinsufficient *Ubx* haltere to wing transformation is modified by the $Gap1^-$ condition. (A) A wild-type haltere. (B) A haltere from a $Ubx^{109}/+$ fly. The *Ubx* haploinsufficient phenotype described as a partial haltere to wing transformation is characterized by the appearance of bristles (indicated with arrow) forming and apparent anterior wing margin on the enlarged appendage. (C) Haltere of $Gap1^- Ubx^{109}/Gap1^-$ fly. The $Gap1^-$ mutant enhances the haploinsufficient *Ubx* loss of function phenotype obtained with this *Ubx* null allele, with additional wing margin bristles (including more proximally) on the further enlarged appendage. All images are magnified 250×.

dose, as shown by the haploinsufficient transformation toward wing in Ubx^-/Ubx^+ animals (Figure 6B). In contrast to HSPB, we did not detect dose-sensitive (hetero-

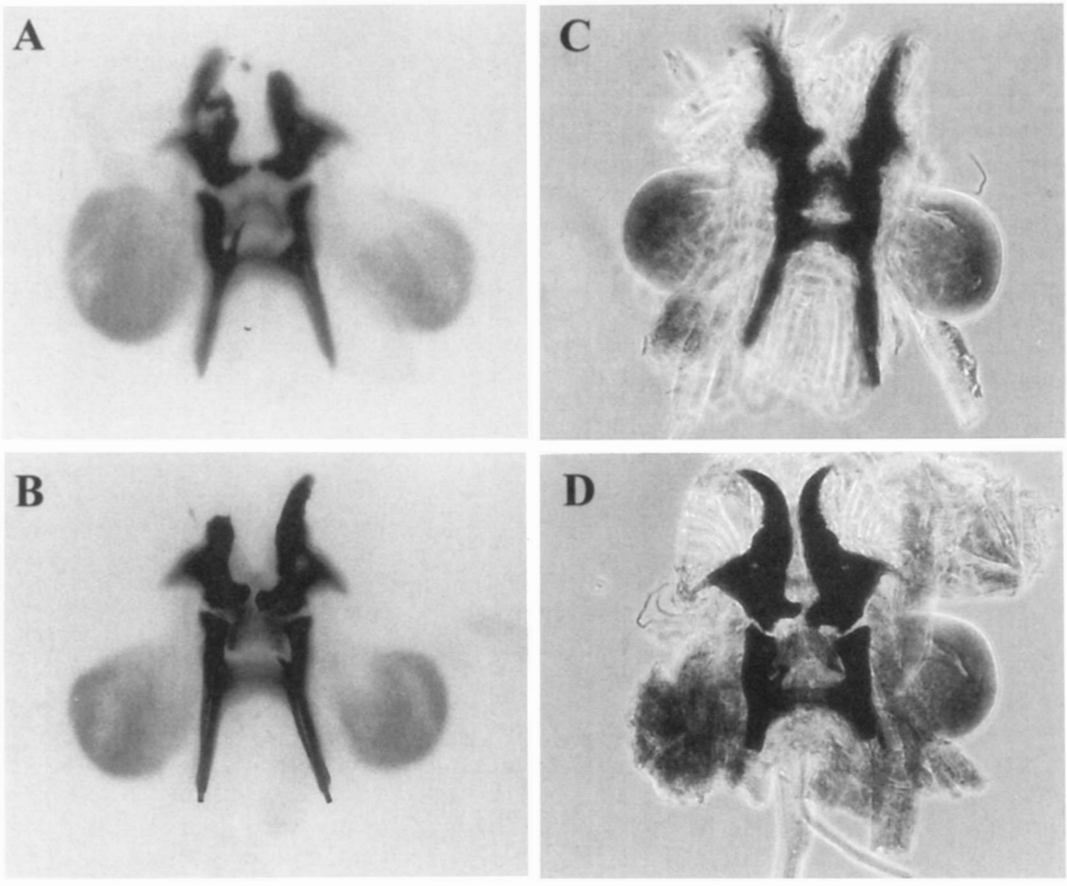

FIGURE 7.—*pb* and *Gap1* expression in labial imaginal discs. (A and B) Immunostaining of PB protein employing antisera directed against the C-terminal region of the Proboscipedia protein. Shown are labial discs from wild type (A) or *Gap1*$^-$ (B) larvae. PB expression is not significantly modified in the mutant discs. (C and D) *In situ* hybridization to labial imaginal discs, using a *Gap1*-specific probe. Labial discs were from wild type (C), or *pb*$^-$ third instar (D) larvae. No alteration of *Gap1* expression was detected in the mutant discs. Similar results were obtained for hybridizations employing a *Ras1* probe (not shown). All magnifications are 200×.

zygous) interactions between *Ubx* and the *Ras1* deletion chromosomes Df(3R)*by*-10 and Df(3R)*by*-416, nor with the *Gap1* deletion chromosome Df(3L)AC-1. However, the haploinsufficient haltere to wing phenotype of Ubx^{109}/+ was clearly aggravated in *Gap1*$^-$ Ubx^{109}/*Gap1*$^-$ adults (Figure 6, B and C). This effect of *Gap1* on *Ubx* function is partially reversed in *Gap1*$^-$ Ubx^{109}/*Gap1*$^-$ Ras^{1B} individuals (not shown), again supporting mutually antagonistic roles for *Ras1* and *Gap1* activities in *Ubx* function. Interestingly, this observation suggests that *Gap1*$^+$ interacts oppositely with *Ubx* compared to *pb* and *Scr* (above), since *Ubx*$^+$ function is favored rather than opposed by *Gap1*$^+$ activity.

***Ras1*/*Gap1* modulation of *pb* homeotic activity and transcriptional regulation of these genes:** *Ras1*$^+$ is formally an activator of *pb* while *Gap1*$^+$ is a negative modulator of *pb*$^+$ function in adult development. If the observed effect occurs at the level of *pb* transcriptional regulation, altering *Ras1* or *Gap1* activity should alter *pb* expression. We examined PB accumulation in the labial discs of homozygous *Gap1*$^-$ third instar larvae (from homozygous *Gap1*$^-$ mothers) by immunostaining with anti-PB sera. PB expression appeared normal in *Gap1*$^-$ embryos (not shown) and in the larval labial imaginal discs (Figure 7, A and B). Spatial expression of *pb* appears normal, as does PB accumulation within expressing cells. These data argue against a role of *Gap1* in modulating *pb* activity via transcriptional regulation. We were unable to examine PB expression in *Ras1*$^-$ imaginal discs, since *Ras1*$^-$ is embryonic-lethal and previous results indicate that the *Ras1*$^-$ condition is cell lethal in adult development (SIMON *et al.* 1991).

We also tested the inverse hypothesis, that the PB homeodomain protein could regulate the transcription of the *Ras1* and/or *Gap1* genes. We therefore examined the expression of Ras1 and Gap1 mRNAs in labial discs from *pb*$^+$ and *pb*$^-$ larvae by *in situ* hybridization. No change of expression was detected for *Gap1* in discs of mutant *pb* larvae (Figure 7, C and D). Ras1 expression was similarly indifferent to *pb* activity (data not shown).

Taken together, these results show that the activities of three homeotic selector loci can be altered by the activities of the *Ras1* and *Gap1* loci. Further, these observations suggest that the modulation occurs by a mechanism independent of transcriptional control, either of the homeotic loci themselves or of the *Ras1*/*Gap1* genes.

DISCUSSION

Homeotic mutations can lead to the replacement of one body part by another. In some cases the transformation is dramatic, yielding flies with four wings instead of two or with legs in place of antennae. This remarkable capacity implies a mechanism that permits the coordination of homeotic gene action within a segment,

yielding many different cell types in correct numbers and distributions. In the present work we have found that homeotic activities can be modulated by *Ras1*-mediated signal transduction. We observe *Ras1*-modulated changes in homeotic effects on cell identity (bristle to distal sex combs, wing trichomes to veins, veins to trichomes or veins to bristles). Only a small number of cell identities in precise contexts are changed by HSPB activity. This suggests that most cells are aware of their positions and correct associated fates, perhaps as a consequence of cell-cell communication. We have also observed *Ras1*-dependent modifications of segmental identity. These occur in a concerted fashion on groups of adjacent cells, again suggesting cell communication.

Here we have found that a *Ras1*-mediated activity modulates homeotic function of the *pb*, *Scr* and *Ubx* loci in their normal contexts. $Ras1^+$ acts as a positive modulator of *pb* and *Scr*, but as a negative modulator of *Ubx* in the halteres (as seen by reducing its inhibitor $Gap1^+$). Further, our data support the interpretation that transcriptional regulation of the homeotic genes is not involved. An artifactual "phenocopy" role of *Ras1* in cell proliferation leading to changes in cell differentiation can apparently be excluded. For example, the phenotypes associated with *pb* mutations in the mouthparts, or *Scr* mutations in the first leg, can be modified by *Ras1/Gap1* mutations in a fashion that appears to affect attribution of specific cell identities, without changing appendage size (Figures 4 and 5).

Existing models of *Ras1* activity involve the transduction of external signals through membrane-bound receptors, across the molecular switch *Ras1*, and subsequently via protein kinase cascades to modify specific nuclear transcription factors. *Ras1* might modify *pb* activity through known signal transduction pathways, employing protein kinases and phosphatases to modulate activity of the PB protein. Consistent with this possibility, the mutant phenotype in hypomorphic pb^4 adults is ameliorated in a $Gap1^-$ context (Figure 4, C and D), whereas no change occurred on combining $Gap1^-$ with the protein null allele pb^5 (not shown). Mutants of the known MAP kinase-associated protein kinases that act in *Ras1*-mediated signal transduction pathways were therefore tested for dose-sensitive interactions with HSPB. However, no modification of HSPB function was detected in combination with a deletion of the *Sos* gene encoding a nucleotide exchange factor (SIMON *et al.* 1991) nor with mutations of the protein kinase genes *raf* (DICKSON *et al.* 1996), D*sor* (TSUDA *et al.* 1993), *hemipterous* (GLISE *et al.* 1995), *rolled* or *Sevenmaker* ($rolled^{Sev}$) (BIGGS *et al.* 1994).

Though *Ras1* can modify homeotic selector functions, we have been unable to find any evidence that would place this interaction within the framework of known signal transduction pathways (PERRIMON 1994; HUNTER 1995). The absence of detectable interactions between known *Ras*-associated protein kinases and HSPB leaves open the possibility that *Ras1* modifies homeotic function by a new and as yet unknown mechanism. We feel that this is unlikely since we were able to detect interactions of *pb*, *Scr* and *Ubx* with both *Ras1* and *Gap1*, and in each case *Ras1* and its antagonist *Gap1* acted oppositely. This supports the interpretation that *Ras1* activity modulates homeotic activity by signal transduction in a manner related to the presently known pathways.

If signal transduction provides the connection between *Ras1/Gap1* and the homeotic functions, three potential explanations may rationalize the absence of a detectable interaction with known protein kinase genes. First, the conditions employed may simply have been insufficiently sensitive. [We note, however, that similar screens based on dose sensitivity readily revealed the MAP kinase gene *rolled* (DICKSON *et al.* 1996)]. A second possibility is that signal transduction through *Ras1* is directed toward the homeotic genes via as yet unidentified protein kinase cascades. A third possibility, not necessarily exclusive of the second, is that the homeotic activity can be modified by multiple *Ras1*-mediated protein kinase cascades. This view is potentially satisfying in light of the role that signaling seems likely to play, namely in permitting the integrated development of diverse cell types composing an appendage. It is also worth noting in this light that while *Ras1* and *Gap1* act oppositely on the homeotic functions examined here, they do not act with equal "weights:" the effects of *Ras1* could generally be detected in heterozygotes, whereas for *Gap1* clear effects were obtained only in the homozygous state.

An important problem will now be to identify putative protein kinases modifying homeotic gene function *in vivo*. Continued genetic screens for interacting loci will undoubtedly reveal new genes implicated in such pathways. Biochemical approaches should permit access to elements of this functional connection: for example, the use of site-directed mutagenesis to change homeotic protein coding sequences coupled with the establishment of transgenic lines, will permit functional tests of the hypothesis that the homeotic selector proteins themselves are the targets of protein kinases. Much further work will clearly be necessary to elucidate the molecular basis of the relations between *Ras1*-mediated signaling and homeotic function. Given the evolutionary conservation of these various molecules, these will be most interesting questions to address. The power of the genetic use of dose-sensitive modifiers in diverse genetic contexts should afford numerous means to address these questions, and the present work offers useful starting points in this direction.

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