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Levels of homeotic protein function can determine developmental identity: evidence from low-level expression of the *Drosophila* homeotic gene *proboscipedia* under Hsp70 control

David L.Cribbs[1], Corinne Benassayag[1], Filippo M.Randazzo[2] and Thomas C.Kaufman[3]

Howard Hughes Medical Institute, Institute for Cellular and Molecular Biology, and Programs in Genetics and Cellular, Molecular and Developmental Biology, Department of Biology, Indiana University, Bloomington, IN 47405, USA, and [1]Centre de Biologie du Développement, CNRS, 118 route de Narbonne, Bâtiment 4R3, 31062 Toulouse Cedex, France
[2]Present address: HHMI, Beckman Center, Room B202, Stanford University Medical Center, Stanford, CA 94305-5428, USA

[3]Corresponding author

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The autonomous selector capacity of the homeotic *proboscipedia (pb)* gene of the *Drosophila* Antennapedia Complex was tested. We introduced into the germline a P element containing a transcriptional fusion of a mini-gene for *pb* (normally required for formation of the labial and maxillary palps of the mouthparts) and the Hsp70 promoter. Uninduced expression of this Hsp70:*pb* element (HSPB) directs a novel, fully penetrant dominant transformation of antennae toward maxillary palps. Gene dosage experiments varying the number of HSPB elements indicate that the extent of the dominant transformation depends on the level of PB protein. At the same time, expression from the transgene also rescues recessive *pb* mutations. Finally, HSPB function may override the dominant antennal transformations caused by *Antennapedia* (*Antp*) mutations in a dose-sensitive manner, directing a switch of the antennal disc-derived appendage from ectopic leg to ectopic maxillary palp. This switch correlated with strikingly reduced ANTP protein accumulation when PB concentrations exceeded a genetically defined threshold level. These observations support a crucial role for quantitative aspects of *pb* function in determining segmental identity, including cross-regulatory events involved in this determination.

Key words: Antennapedia Complex/proboscipedia/homeodomain/homeotic/development

Introduction

During early embryogenesis in *Drosophila* the body axes are established, then subdivided into a series of similar segments, or parasegments (Akam, 1987; Ingham, 1988). The homeotic genes then confer developmental identity on the newly established subdivisions, directing the differentiation of segment-specific structures specialized for feeding, locomotion or sensory functions. A majority of homeotic loci are clustered in the *Bithorax Complex* (*BX-C*; Lewis, 1978; Peifer *et al.*, 1987) and in the Antennapedia Complex (ANT-C); Kaufman *et al.*, 1980; Kaufman *et al.*, 1989). The homeotic genes of the BX-C and of the ANT-C, controlling the differentiation of abdominal and thoracic segments, or of the head and anterior thorax, respectively, are evolutionarily conserved and homologs have been found in a wide variety of other organisms. Strikingly, the homologous genes in the flour beetle *Tribolium castaneum*, in mice and humans, and even in the non-segmented worm *Caenorhabditis elegans*, are clustered and organized as seen in *Drosophila* (Beeman, 1987; Acampora *et al.*, 1989; Beeman *et al.*, 1989; Duboule and Dollé, 1989; Graham *et al.*, 1989; Salser and Kenyon, 1994). The remarkable extent to which these genes and proteins, their genomic organization and even expression patterns have been conserved during evolution (Duboule and Dolíe, 1989; Graham *et al.*, 1989) raises many fundamental questions concerning the basis of this multi-tiered conservation, and the capabilities of the individual genes composing the ensemble.

The homeotic loci of *Drosophila* are believed to effect their developmental control by the regulation of groups of downstream or 'realisator' genes (García-Bellido, 1977; Gould *et al.*, 1990; McGinnis and Krumlauf, 1992). Among the growing number of documented examples are found genes coding for the potential cell adhesion molecule connectin (Gould *et al.*, 1990; Gould and White, 1992); the transcription factors encoded by the *Brista/Distalless* (Vachon *et al.*, 1992) and *spalt* (Wagner-Bernholz *et al.*, 1992) genes; the mammalian TGF-β growth factor homolog *decapentaplegic* (Immerglück *et al.*, 1990; Reuter *et al.*, 1990; Capovilla *et al.*, 1994); the *scabrous* locus known to be involved in eye development (Graba *et al.*, 1992); and a gene of as yet unknown function (Mahaffey *et al.*, 1993). The homeotic genes, along with a growing number of other cloned genes implicated in developmental control, encode the highly conserved 60 amino acid homeodomain motif (for reviews see Gehring *et al.*, 1990; Hayashi and Scott, 1990). In several cases homeodomain proteins have been demonstrated to function as transcription factors, consistent with their DNA binding activities, nuclear localization and developmental control functions. DNA binding studies *in vitro* have shown that many different homeodomain proteins may bind to identical sequences with very similar affinities. These similarities, for proteins with very different effects in the animal, might simply reflect the inherent limitations of *in vitro* studies to fully replicate *in vivo* conditions. Alternatively it might be that target genes *in vivo* are exquisitely sensitive to differences between homeoproteins. Small differences in affinity might be amplified by co-operative interactions of the homeoprotein, with itself or with co-factors, and subtle differences in binding affinities and/or nuclear concentrations of homeodomain proteins could have a profound impact on the developmental outcome observed.

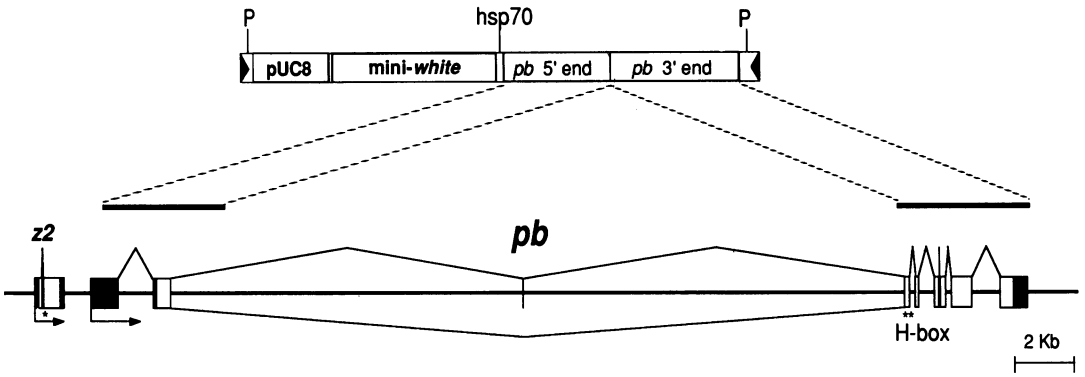

Fig. 1. Structure of the HSPB element. The HSPB element was constructed by inserting two genomic restriction fragments constituting the 5′ and 3′ halves of the *pb* transcription unit downstream of the conditional Hsp70 promoter. The construct contains all coding sequences for *pb* protein except for a 15 bp, alternatively spliced microexon, which was removed together with most of the central 25 kb of the gene. Also shown adjacent to the 5′ end of the *pb* transcription unit is the homeobox-containing *z2* gene, which has no known function (see Pultz *et al.*, 1988).

The homeotic *proboscipedia (pb)* gene of the ANT-C codes for a homeodomain protein conserved in flies, mice and humans (Cribbs *et al.*, 1992a). Normal *pb* expression occurs in a spatially restricted domain; the labial and maxillary segments of embryos and in larval labial imaginal discs (Pultz *et al.*, 1988; Randazzo *et al.*, 1991). Consistent with its spatial expression pattern, pb^+ function is required for the correct development of the adult mouthparts comprising the labial and maxillary palps. In the absence of *pb* function, prothoracic legs replace the adult labial palps (Bridges and Dobzhansky, 1933; Kaufman, 1978; Pultz *et al.*, 1988) while the maxillary palps appear transformed toward antennal identity (Kaufman, 1978). In contrast, a second class of recessive *pb* mutations results in a distinct transformation of the labial palps to antennal aristae (Kaufman, 1978; Pultz *et al.*, 1988). The *pb* gene is unique among the known homeotic loci in two important respects. First, *pb* appears to be adult specific. Despite localized embryonic expression (Pultz *et al.*, 1988), *pb* function appears not to be required at this stage; indeed animals entirely lacking *pb* may survive to adulthood though they soon die from starvation. Secondly, different recessive mutations believed to remove all but part of pb^+ functions may yield distinct developmental transformations: labial palps to prothoracic legs, or to antennal aristae.

Although more than 40 recessive *pb* mutations exist, the only known dominant gain-of-function *pb* mutation provokes thoracic defects rather than a homeotic transformation (Cribbs *et al.*, 1992a,b). Gain-of-function mutations resulting from ectopic expression have often proven powerful tools for studying the roles of homeotic proteins in developmental control, and in the present paper we examine the consequences of ectopic PB protein expression from a *pb* mini-gene fused to the conditional Hsp70 promoter. The phenotypes observed for transgenic lines demonstrate a positive selector function for *pb* in adult development, directing a homeotic transformation of antennae to maxillary palps. Uninduced levels of ectopic PB protein expression suffice to rescue *pb* mutant alleles, while simultaneously inducing the novel dominant segmental transformation. Both rescue and ectopic transformation depend on quantitative aspects of *pb* function. The phenotype of double mutant combinations generated by placing *Hsp70:pb* (antenna transformed to maxillary palp) with dominant mutant alleles of the homeotic gene *Antennapedia* (antennae to mesothoracic legs) depends on the quantity of PB produced: lower levels of PB expression permit the antennae to develop as mesothoracic legs, while higher levels of PB protein may override the effects of *Antennapedia* and replace the ectopic legs with mouthparts. The data presented support a crucial role in segmental differentiation not only for the specific homeotic proteins present but for their respective levels of expression or function.

Results

Construction of the HSPB element

In order to examine the potential of PB protein to alter morphogenetic processes in different tissues, a chimeric mini-gene was constructed (HSPB element: Figure 1) and introduced by P element-mediated germline transformation (Rubin and Spradling, 1982; Spradling and Rubin, 1982; Robertson *et al.*, 1988). This element places *pb* coding sequences downstream of the *Drosophila* Hsp70 promoter (Ingolia *et al.*, 1980; Karch *et al.*, 1981) to permit ubiquitous expression of PB at elevated temperatures (Struhl, 1985). The HSPB element also carries the visible marker gene mini-*white* (*w*) (Pirrotta *et al.*, 1985). Lacking a suitable *pb* cDNA, the HSPB mini-gene was constructed by joining two genomic restriction fragments that contain all *pb* protein-coding sequences except for a 15 bp microexon situated just 5′ of the homeobox. The microexon, which is alternatively spliced and is absent from some classes of *pb* mRNA (Cribbs *et al.*, 1992a), is not mandatory for *pb* function in the adult mouthparts (Randazzo *et al.*, 1991). Apart from *pb* coding sequences, HSPB contains *cis*-regulatory elements located within intron 2 (Randazzo *et al.*, 1991; A.Kapoun and T.C.Kaufman, in preparation). It is not yet clear what combination of regulatory elements generates the basal level of PB expression from HSPB. The Hsp70 promoter remains functional and PB expression may be supplemented by elevated temperatures (see below). Whether *cis*-regulatory sequences of *Hsp70*, *pb* or both modulate aspects of mini-gene transcription, the crucial point for the experiments presented here is that the resulting ectopic expression (as judged by the induced dominant adult phenotypes) for a

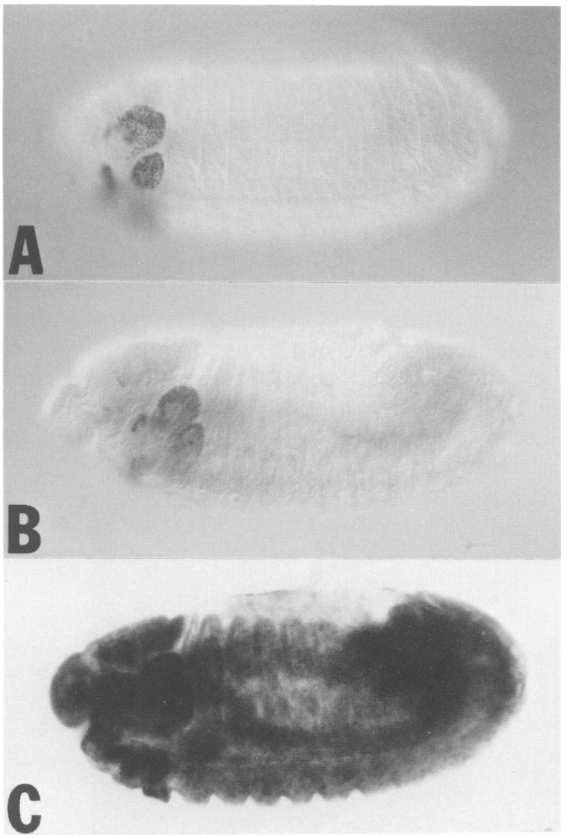

Fig. 2. Embryonic expression of PB protein from the HSPB element. Embryos were subjected to a heat shock of 35°C for 3 h then allowed 0.5 h recovery at 22°C before collection and immunostaining with a polyclonal anti-PB sera. Embryos are shown with anterior to the left and dorsal up. The w^{1118} embryo in (**A**) shows the normal pattern of *pb* staining shortly after the end of germ band contraction and as the gnathocephalic lobes are beginning to migrate anteriorly and to rotate ventrally in the process of head involution (10 h; see Campos-Ortega and Hartenstein, 1985). Prominent nuclear staining is seen in the labial and the maxillary lobes. Panel (**B**) shows an HSPB 2–5 embryo, without heat induction. In (**C**), heat shock-induced nuclear accumulation of PB is observed throughout the embryo, demonstrating ubiquitous expression of the Hsp70 promoter.

fixed temperature and HSPB copy number is remarkably constant.

The HSPB construct directs conditional, ubiquitous pb protein expression

Nine independent HSPB lines were established (see Materials and methods), most of which are associated with dominant mutant phenotypes as described below. To verify that the mini-gene construct functions as a conditional *pb* allele, embryos or larvae carrying the HSPB element were stained with antisera against PB protein (Pultz *et al.*, 1988; Randazzo *et al.*, 1991; see Materials and methods). The normal pattern of PB accumulation in the labial and maxillary lobes of a control (*w*) embryo is shown in Figure 2A while Figure 2B shows an HSPB embryo, both without heat induction. This HSPB embryo appears to be lightly stained outside the normal *pb* domain in the labial and maxillary lobes, compared with *w*. However, this generalized staining outside the normal expression domain was difficult to detect reliably on mixing immunostained embryos carrying zero or one HSPB copy. Ectopic embryonic expression from HSPB is thus markedly reduced compared with wild type accumulation in the head, on a per cell basis, and is not detectably localized. Following heat induction (3 h at 35°C, 0.5 h recovery at 22°C) nuclear accumulation of PB could be detected throughout the embryo (Figure 2C). The Hsp70 promoter thus remains functional, regardless of any additional effects of *pb* regulatory sequences (discussed further below).

Uninduced HSPB expression reveals a selector function for pb in adult development

Adults of several transgenic HSPB lines show a novel domimant homeotic transformation of antennae to maxillary palps (components of the adult mouthparts), without heat induction. This transformation, apparently typical for HSPB, is restricted to the third antennal segment (A3) and the aristae, while A2 is perturbed but not explicitly transformed and A1 is unaffected (compare Figure 3a and c with b and d). The five dominant HSPB lines that transform the antennae toward maxillary identity all affect A3, but the extent to which they transform the aristae varies. The aristal transformation appears to be a reliable marker for HSPB function since effects on the antennae correlate well with the penetrance of defects of the legs and wings, and with rescue of recessive *pb* mutations (described below). Transformation of the aristae can also be augmented by altering the culture temperature from 22 to 29°C. Assuming PB accumulation from HSPB can be supplemented by increasing temperature, these observations all suggest a dose-sensitive response to PB accumulation, or function, in several different segments.

Other dominant phenotypes were observed in addition to the homeotic transformation of the antennae. In general, HSPB-bearing adults exhibited reduced viability and mobility. Discrete dose-sensitive defects were noted for the prothoracic legs (perturbation of the sex combs, and appearance of ectopic sex comb teeth on the second tarsal segment); head capsule (malformed outer vertical bristles on the posterior of the head, reduction of the anterior aspect of the eye); and wings (loss or modification of bristles on the anterior wing margin, and formation of microchaetae at the distal extremity of the longitudinal vein L3). These mutant phenotypes thus indicate that HSPB is expressed and acts in a variety of segments outside the normal *pb* domain, notably in the head capsule, prothorax and mesothorax.

An important aspect of our experimental protocol was the absence of a heat shock. Although the different phenotypes were less penetrant and/or expressive for flies raised at 22 than at 29°C, for a given constant temperature the phenotypes observed were reproducible. Given the dynamic nature of larval/pupal development and the difficulty of precisely staging large populations of larvae, the opportunity to avoid heat induction minimizes fluctuations in the experimental conditions to which the animals are subjected. That the observed defects could be aggravated by rearing the flies at increasing temperatures is likely due to increased 'leakiness', as seen for Hsp70 protein accumulation at varying temperatures within the ambient range (see Ashburner and Bonner, 1979). The adult phenotypes presented here reflect uninduced expression of the HSPB element at 22°C, perceived by the phenotypic effects of HSPB on the fly rather than by direct immunodetection.

The observed effects of HSPB insertions are due to pb$^+$ function

Several HSPB insertion lines show dominant defects including the homeotic transformation of antennae to maxillary palps, which we presumed to reflect functional PB protein product. However, some defects might be related to the chromosomal sites disrupted by HSPB insertion. Also, the mini-gene used contains *pb* regulatory sequences that might titrate a limiting nuclear factor(s). In order to test for the contributions of such complicating factors to HSPB insertion-associated defects, we isolated phenotypic revertants of the HSPB 2-5 line, i.e. flies with colored eyes but normal antennae (mini-w^+; HSPB$^-$). One such EMS-induced revertant line, HSPB^{Rev14}, is viable in homozygous females and shows no discernable defects of the head or thorax, with or without heat induction. All coding sequences in Rev14 were screened for alterations by hydroxylamine-induced cleavage (Montandon *et al.*, 1989). This analysis and subsequent sequencing identified a single point mutation that alters the evolutionarily invariant Arg53 in Helix 3 of the homeodomain to His. This observation led us to conclude that while wild type PB protein is essential for the effects of the HSPB 2-5 line, the site of insertion has little, if any, effect.

However, if the regulatory sequences present within the *pb* mini-gene act as 'sinks' titrating limiting nuclear factors, increasing the copy number of these sequences might aggravate aspects of the mutant phenotype. Hence, a chromosome was constructed carrying the mutated P

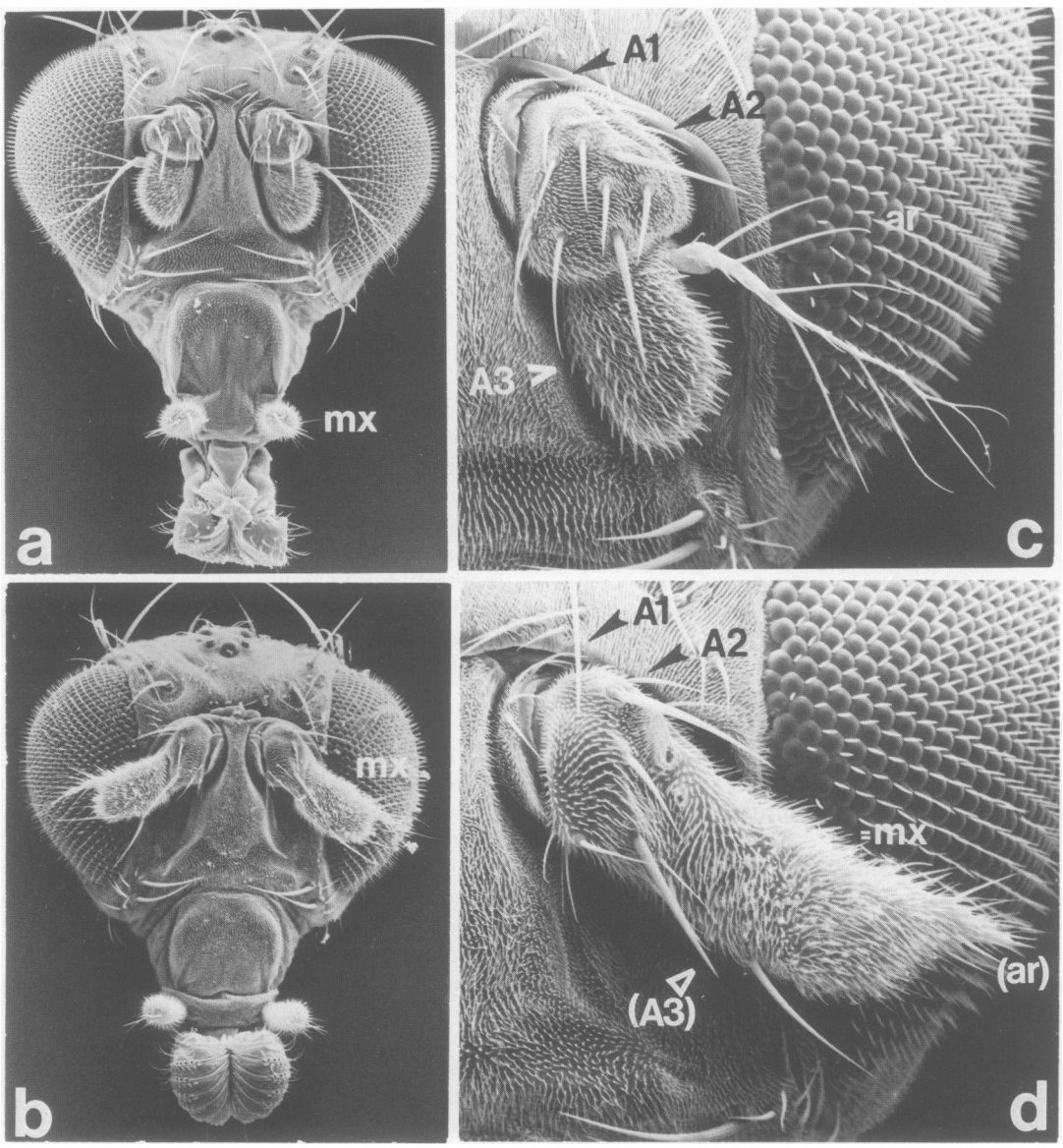

Fig. 3. The dominant phenotype of the HSPB element. (**a**) Wild-type head (Oregon R) showing the mouthparts and the antennae: mx, maxillary palps; lb, labial palps. (**b**) Head of a *w* HSPB2 fly, raised at 29°C, showing a strong homeotic transformation of the antennae toward maxillary palps (mx). The labial and maxillary palps are normal. (**c**) Higher magnification of the wild type head in (a). Antennal segments A1, A2, A3 and the aristae (ar) are indicated. (**d**) Higher magnification of the head from (b). A1 appears completely normal. A2 is somewhat misshapen and shows empty bristle sockets, but no evidence is seen for a homeotic transformation. A3 is transformed to an ectopic maxillary palp (mx); its normal position is indicated by an arrow and (A3). A slight remnant of the aristae is indicated as (ar). Formation of edge hairs is also visible on the ectopic maxillary palp.

element $HSPB^{Rev14}$ (® = reverted element) plus a second revertant copy transposed elsewhere on the X chromosome (not shown). No defects beyond those for a single intact HSPB copy were detected in flies carrying five full-size HSPB elements of which only one was functional (®®/®®; 4d/+). Thus, intact *pb* protein-coding sequences within the HSPB element are required for the diverse dose-sensitive effects of the 2-5 line. The common developmental defects induced by this and other HSPB lines are probably entirely attributable to pb^+ function from the transgenic element.

Homeotic transformation by ectopic pb expression depends on protein level

In *Drosophila*, the level of gene product accumulated is generally proportional to the number of functional gene copies (Simon *et al.*, 1991, and references therein). Varying the copy number, or gene dose, thus allows the testing of the influence of different levels of gene product. Such gene dosage experiments were performed where the number of HSPB copies was varied. Two homozygous viable HSPB insertion lines have been isolated that produce a moderate transformation with one copy and a strong transformation with two copies, illustrated by the X-chromosome linked insertion HSPB 2-5 (Figure 4A and C). Males and females with a single copy of this element give the same transformation, showing that the *Hsp70:pb* mini-gene (contrary to the mini-*white* gene in HSPB) is not susceptible to dosage compensation. Similarly strong transformations result from combinations of chromosomes carrying different HSPB insertions (for example 2-5/+;4d/+), showing that the effect is independent of chromosome pairing. The observed responsiveness of the antennal-to-maxillary transformation (and other HSPB-associated phenotypes) to copy number indicates that these developmental effects are very sensitive to the level of PB protein. Further, as the effects of increasing temperature parallel the graded phenotypic effects of increasing gene dose, the temperature-related effects probably likewise reflect PB levels.

Since uninduced levels of PB expression caused a fully penetrant transformation of antennae towards maxillary palps, we wished to examine the effects of high PB levels. The effect on adult development of various heat induction regimens was examined (inductions for 1–3 h, at 35–37°C). Such conditions were sufficient to direct ubiquitous over-expression of PB (Figure 2C). However, such over-expression was surprisingly erratic as detected by immunostaining and did not provoke marked changes in adult cuticular phenotypes. Pre-mRNA splicing has been shown to be sensitive to elevated temperatures (Yost and Lindquist, 1986), and the presence of seven introns within the *pb* mini-gene may thus limit the effects of heat induction. Indeed, the fully penetrant antennal phenotype induced by two copies of HSPB 2-5 is stronger than any adult phenotype we have observed after heat induction.

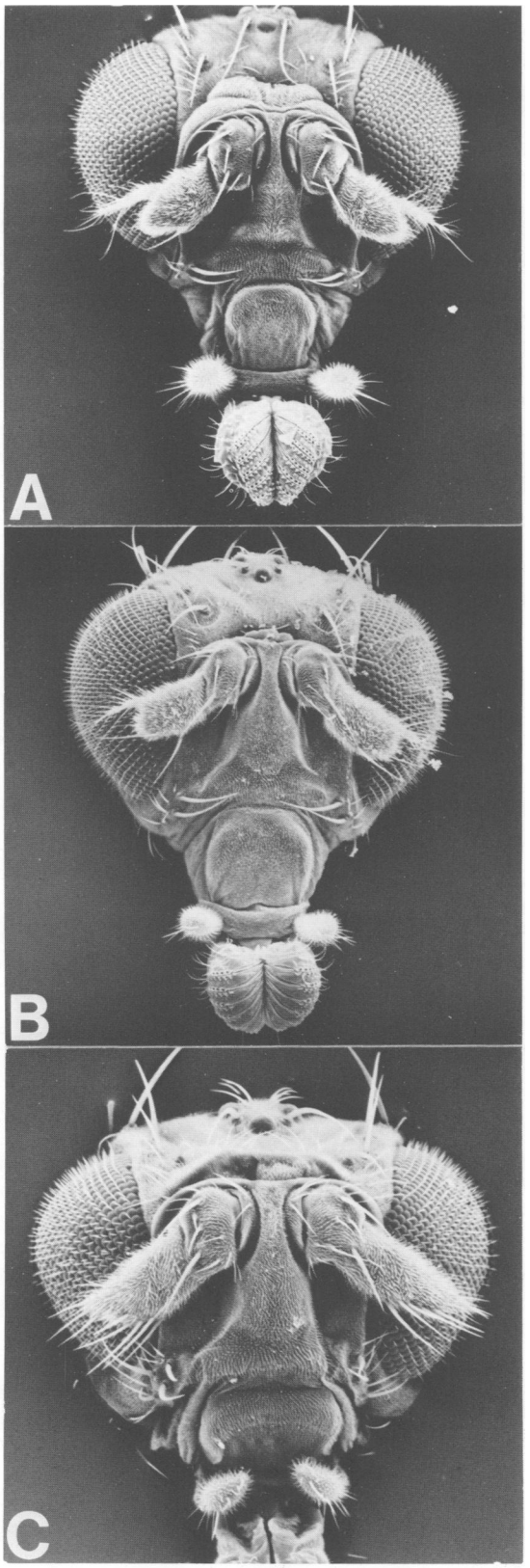

Fig. 4. Effect of HSPB dose on antennal development (**A**) *w/w* HSPB 2-5 heterozygous female, raised at 22°C. A partial transformation of the antennal segment A3 and aristae toward maxillary palp is observed. Note particularly that much of the aristal tissue is still present. (**B**) *w/w* HSPB 2 heterozygous female, raised at 29°C. The antennal transformation is more complete than in (**A**) but less severe than in (**C**) below. (**C**) *w* HSPB 2-5/HSPB 2-5 homozygous female, raised at 22°C. Aristal tissue is no longer observed, and numerous edge hairs typical of maxillary palps are observed along the rim of the ectopic palp. These phenotypes show that the extent of transformation depends on dose, and that HSPB 2 is intermediate between one and two copies of HSPB 2-5 in its effects.

Elevated levels of PB due to increasing copy number are likely to be lethal, as for other homeotic proteins. Despite several attempts, we have never obtained a fly containing three dominant elements. While either 2-5/2-5 or 2-5/+; 4d/+ are viable and fertile (though weak), the combination 2-5/2-5; 4d/+ has never been obtained, suggesting that ectopic expression at this level is lethal. In contrast, as noted above, heat shocks applied periodically throughout development have little effect on the adult antennal phenotype and are not fully lethal. Perhaps the PB^+ functions required for the adult transformation must be applied at a sufficiently elevated level and across an extended period of time, a requirement that transient inductions do not fulfill.

HSPB rescues loss-of-function pb mutations and reveals quantitative aspects of determination in the mouthparts

Recessive *pb* mutations may transform the labial palps to very different structures: antennal aristae or prothoracic legs. The accumulated *pb* alleles may be ordered as an allelic series from wild type (Figure 5a), weak antennal, antennal (Figure 5b), mixed antennal/T1 leg or T1 leg (the null condition as defined by deletions or protein null alleles of *pb*; see Figure 5c). The antennal transformation encompasses a smaller region of the labium than the null condition and permits the formation of some of the pseudotracheal rows necessary for feeding. Antennal alleles thus direct the formation of a labium that is more nearly wild type than for the null condition. Since the positive function of *pb* is to make labial and maxillary palps, these observations suggest that the antennal phenotype results from a partial loss of the same *pb* functions as with leg-forming alleles. Alternate developmental outcomes would reflect different 'quantities' of *pb* function (Cribbs *et al.*, 1992a).

The preceding interpretation might be tested by adding limiting amounts of transgenically derived wild type PB to the products of mutant endogenous *pb* alleles. We therefore asked whether the HSPB element could contribute sufficient pb^+ function to modify the phenotypic effects of antennal or leg-producing *pb* mutations. The alleles tested were pb^4, a putative hypomorph causing an antennal transformation; and the protein null mutation pb^5 (Pultz *et al.*, 1988). The labium-to-antenna phenotype of pb^4 bearing animals was often fully rescued by a single copy of HSPB 2-5, whereas the pb^5 null mutation that produces legs was incompletely rescued by the same element (compare Figure 5b with e, and 5c with f, respectively). Strikingly, the incomplete rescue of pb^5 often results in a weak transformation of labium to antenna rather than to prothoracic leg. The leg tissue due to the pb^- condition can thus be transformed to a mix of labial plus aristal tissue (indicated by arrow in Figure 5f, and labelled 'ar' in g) by simple addition of pb^+ protein from the same HSPB element that can rescue an antennal allele. Thus the transformation of labium to antennal arista results from a partial loss of the same functions as for leg alleles, not from inhibitory effects of the mutated PB protein. The two qualitatively different transformations of the labium are thus best interpreted as landmarks within an allelic series rather than the loss of distinct functions of the PB protein.

Partially rescued HSPB 2-5;pb^5 flies were also obtained with a different phenotype, where small patches of labial tissue were transformed to distal leg tissue (for example the claw 'cl', in Figure 5h). Some adults possessed leg and antennal tissue on the adjacent labial palps. It is possible to obtain structures of three different identities from the labial imaginal discs of a single animal, under identical conditions of genotype and temperature: prothoracic or antennal, plus wild type (labial) surrounding the mutant tissue. These data suggest that the quantity of pb^+ function provided by the HSPB 2-5 element in this tissue falls very near a threshold allowing for different determination in an identical context. The discrepancy between the degree of rescue of pb^4 and pb^5 emphasizes that the amount of PB sufficient to rescue a hypomorphic allele does not entirely replace the lost functions of a null allele. The results indicate an important quantitative aspect of *pb* function in distinguishing among three available developmental outcomes for the labial palps, the normal site of *pb* function.

Quantitative interactions of HSPB with dominant Antennapedia mutations in determining tissue identity

The *pb* and *Antennapedia* (*Antp*) genes are normally expressed in non-overlapping sets of imaginal disc cells. Nevertheless, these two homeodomain proteins may act on the same ectopic target tissue, the antennae, but with divergent and readily distinguishable results. Expression of PB from the HSPB element results in the transformation of antennae to maxillary palps, while ectopic expression of protein from $Antp^{Dom}$ alleles (e.g. Frischer *et al.*, 1986; Schneuwly *et al.*, 1987a) or from an Hsp70-cDNA transgene (Schneuwly *et al.*, 1987; Gibson and Gehring, 1988) results in transformation of the antennae to mesothoracic legs. Further, alteration of head tissue is a dose-dependent process for both proteins: two HSPB copies yield a more extensive transformation than one alone, as described above, while pairs of $Antp^{Dom}$ alleles may yield transformations of head tissue to thorax markedly more complete than for one allele alone (in males bearing a Y-chromosome linked duplication of $Antp^+$ to rescue recessive lethality; data not shown). We examined the consequences of ANTP and PB co-accumulation in responsive antennal cells by placing together one or more HSPB elements with $Antp^{Dom}$ alleles. Potential outcomes of this novel combination of homeotic proteins in the antennal anlagen include a novel appendage (new combinatorial identity; Lewis, 1978), mixed leg/maxillary identity (no definitive developmental decision), or a choice between leg and maxillary structures (epistatic interactions between *Antp* and *pb*).

Ten different $Antp^{Dom}$ alleles were tested in combination with the HSPB lines 2, 2–5 and 4d (see Materials and methods; Figure 6). The homeotic transformation of antennae to T2 legs conferred by various $Antp^{Dom}$ alleles could often be redirected by HSPB from ectopic leg to maxillary palp: that is, PB was epistatic to ANTP. All but one tested allele could be overridden at least occasionally by HSPB 2 or 4d, at a temperature within the interval 22–29°C. The weakest *Antp* allele phenotypically, $Antp^{CB}$, was overridden by all conditions tested; the strongest of the 10, $Antp^{73b}$, was never successfully redirected by

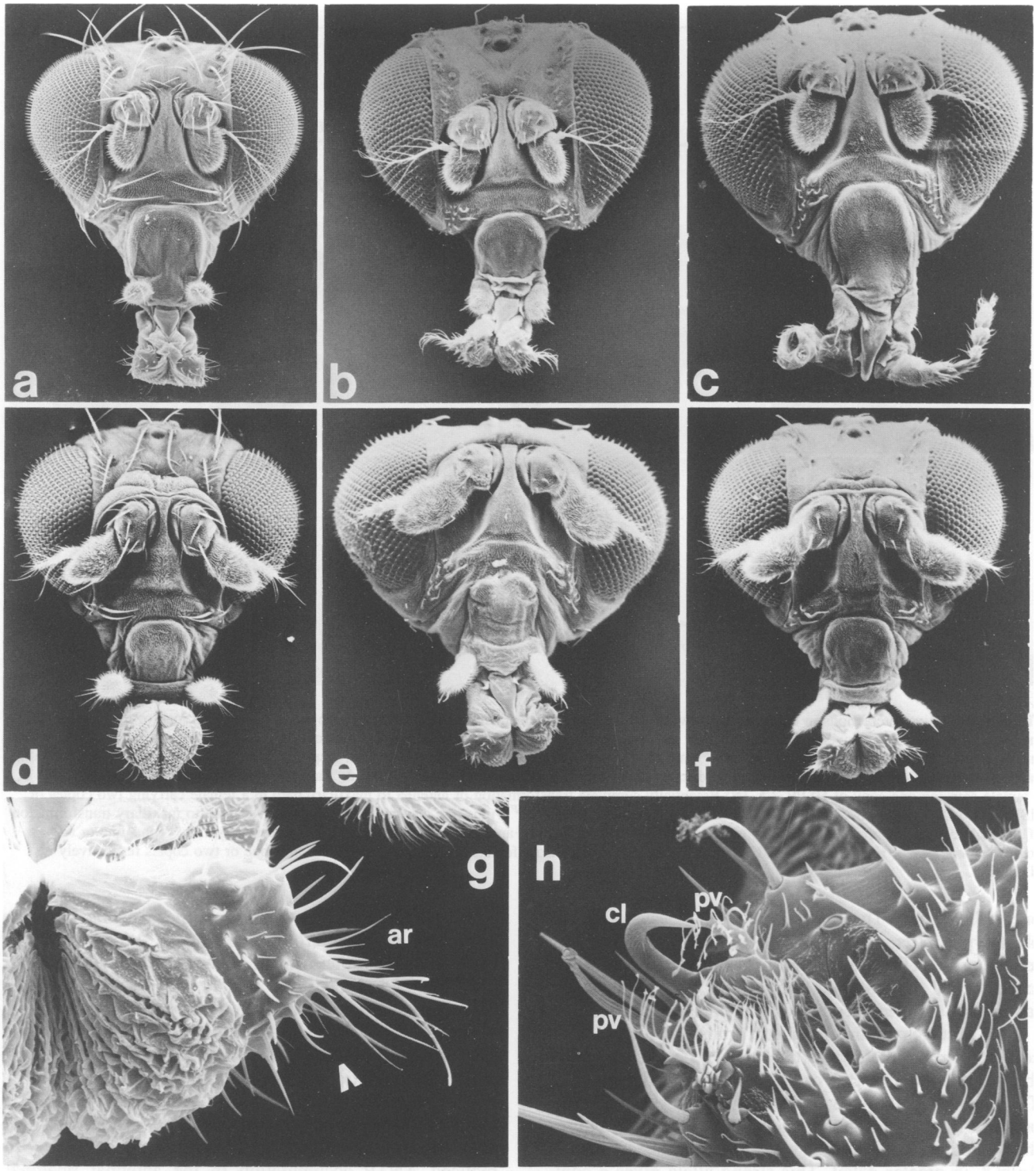

Fig. 5. Effects of HSPB elements on *pb* mutant alleles. (**a**–**c**) These flies lack the HSPB element and are wild type Oregon R (a), *w; Ki* pb^4p^p (b) and *w; Ki* pb^5 p^p (c). (**d**–**f**) These flies carrying the HSPB element are (d) *w* HSPB2-5 [derived from Line 2 by mobilizing the element with the P(ry$^+$, Δ2-3) element], (e) *w* HSPB 2-5; *Ki* pb^4p^p and (f) *w* HSPB 2-5; *Ki* pb^5p^p. The aristal transformation in (b) is rescued by the function of the transgene (e). The transformation of the mouthparts to prothoracic legs due to the pb^5 mutation is largely rescued, and is replaced a tuft of distal aristal tissue (indicated by the arrow in f). (**g**) Higher magnification of (f), showing a small tuft of aristal tissue (ar) in mouthparts which are otherwise restored to wild type. (**h**) The mouthparts of a different adult of genotype *w* HSPB 2-5; *Ki* pb^5p^p. In this animal, one labial palp was as shown in (f) and (g) while the other possessed a small patch of distal leg tissue as evidenced by the presence of a claw (cl) and pulvilli (pv; the asymmetrically branched structures which resemble flag trees found in severe, windy climates). Note that in (d–f), the antennae are transformed toward maxillary palps in equal measure regardless of the *pb* allele present, showing that the transformation of the antennae is independent of endogenous gene function.

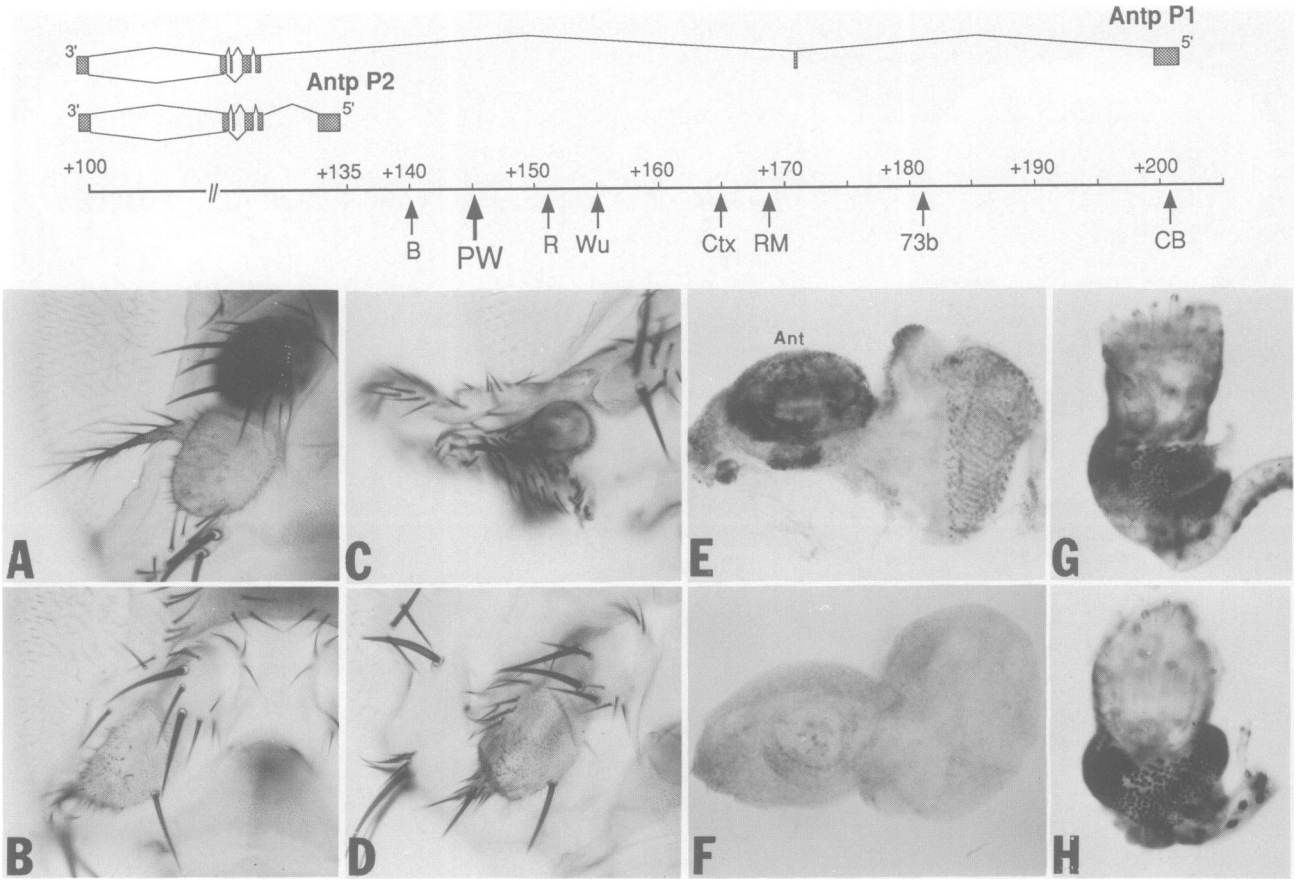

Fig. 6. Quantitative interactions in the antennae between HSPB and dominant *Antp* alleles. Shown at the top is a structural map of the *Antp* gene, indicating the positions of inversion breakpoints for the dominant alleles tested relative to the positions of the P1 and P2 promoters. The breakpoints are not known for two other tested alleles, $Antp^{W}$ and $Antp^{DC}$ (isolated by A.Wohlwill and D.Cribbs, respectively). All flies shown are females from parallel crosses raised at 22°C, and all imaginal discs derived from female late third instar larvae. (**A**) Partial antenna to maxillary transformation of a female carrying one copy of HSPB 2-5. (**B**) Essentially complete transformation of antenna to maxillary palp in a female carrying two copies of HSPB 2-5. (**C**) Antenna to T2 leg transformation of a female carrying $Antp^{PW}$ plus one copy of HSPB 2–5. (**D**) Antenna to maxillary transformation of a female carrying $Antp^{PW}$ plus two copies of HSPB 2-5. (**E** and **F**) Eye–antennal discs from female larvae carrying $Antp^{PW}$ plus one or two copies respectively of HSPB 2-5. (**G** and **H**) Dorsal prothoracic discs from female larvae carrying $Antp^{PW}$ plus one or two copies respectively of HSPB 2-5.

one HSPB copy. These observations suggested that the phenotypic strength of the *Antp* allele (presumably reflecting ANTP accumulation) was more critical as an experimental variable than the position of the breakpoint (and hence the *Antp* sequences still present).

In several cases the homeotic transformation due to certain $Antp^{Dom}$ alleles was observed to be overridden by the presence of HSPB 2 or 4d at 29°C, but not at 22°C. This suggested that the level of PB was central to the outcome of antennal specification. We therefore examined the interactions of HSPB with one $Antp^{Dom}$ mutation, $Antp^{PW}$, in greater detail. This strong allele gives a completely penetrant transformation of antenna to mesothoracic leg, as well as a variable reduction of the anterior aspect of the eye. Gene dosage experiments were performed in which $Antp^{PW}$ was combined with one or two copies of HSPB 2-5. Whereas one HSPB 2-5 copy had little or no effect on the antennal transformation of $Antp^{PW}$ (Figure 6C), a second copy caused a majority of antennal appendages to resemble maxillary palps (compare Figure 6B and D). HSPB is conditionally epistatic to $Antp^{PW}$ in the antennae as a function of HSPB copy number and, we infer, of PB protein level.

This marked conditional dominance of PB over ANTP could be explained by two different mechanisms: (i) dose-dependent negative cross-regulation of *Antp* by the PB homeodomain protein in the antennal imaginal discs; or (ii) competition between ANTP and PB proteins for binding to common *cis*-regulatory sites, modulating downstream target gene expression. If the relevant target of transcriptional control by PB is *Antp*, whether directly or indirectly, conditions sufficient to replace ectopic mesothoracic legs with maxillary palps should result in diminished ANTP protein accumulation in the imaginal disc cells. However, a phenotypic switch not accompanied by reduced ANTP accumulation in the antennal imaginal discs would strongly favor a mechanism of competition for the regulation of common downstream genes.

We therefore examined ANTP accumulation in imaginal discs. As the critical period for *Antp* function leading to transformation of antennae to mesothoracic legs is the third larval instar (Gibson and Gehring, 1988), we examined discs from female third instar larvae bearing $Antp^{PW}$ plus one or two copies of HSPB 2–5 ($1\times;Antp^{PW}$ or $2\times;Antp^{PW}$ respectively). ANTP accumulation was monitored by immunodetection with a monoclonal anti-ANTP serum (see Materials and methods). The expression pattern of ANTP in the eye–antennal discs of $1\times;Antp^{PW}$ larvae,

shown in Figure 6E, reflects the fully penetrant development of ectopic mesothoracic legs. Accumulation of ANTP was detected in both the eye and the antennal portions of the composite eye-antennal disc. Staining was most intense in the antennal portion (indicated by 'Ant' in Figure 6E) giving rise to the ectopic leg (Figure 6C). Expression in the eye disc was also observed, both within developing ommatidia posterior to the morphogenetic furrow and in cells that form part of the anterior head capsule. The latter expression correlates well with the reduction of the anterior of the adult eye and the occasional presence of a mesothoracic sternopleural bristle below the eye (not shown). Apart from expression in precursors of the eye that occurs without obvious developmental consequence, the pattern of ANTP eye−antennal disc expression from $Antp^{PW}$ detected here corresponds well to the discernible phenotypic effects of this allele on the antennae and head capsule.

Parallel immunostaining of female larvae that would yield adults with ectopic legs ($1\times;Antp^{PW}$) or ectopic maxillary palps ($2\times;Antp^{PW}$) revealed strikingly different ANTP accumulation in the eye−antennal imaginal discs of the head. All aspects of ANTP accumulation in these discs in $1\times;Antp^{PW}$ animals were markedly reduced in $2\times;Antp^{PW}$ larvae (Figure 6E versus F). This result indicates that the phenotypic transition of the adult 'antenna' from ectopic leg to maxillary palp is largely attributable to negative cross-regulation of *Antp* expression in the head, an event requiring a critical level of PB protein. Interestingly, the reduced accumulation of ANTP is head specific, since staining in the dorsal prothoracic discs (normal sites of *Antp* expression, Figure 6G and H), the wing and leg discs and ventral nerve cord (not shown) was not altered.

The dose-dependent negative regulation exercised by PB toward $Antp^{PW}$, while surely crucial to the observed phenotypic threshold, does not appear to provide a complete explanation. All detectable elements of ANTP staining were strongly reduced throughout the eye−antennal discs ($2\times;Antp^{PW}$; Figure 6F). Nevertheless many adult females from these crosses possessed 'antennae' of mixed identity (resembling maxillary palps but carrying groups of thoracic bracted bristles or of overt leg structures). The residual reduced levels of ANTP protein detected in these experiments are sufficient to direct at least some cells toward a thoracic and not maxillary identity. Since ANTP protein expressed from $Antp^{PW}$ cedes its functional place to PB in many (but not necessarily all) cells of the antennae, it is likely that competition between ANTP and PB proteins for the transcriptional regulation of shared downstream targets also plays an important role in the developmental control of at least certain cell types in the head, in addition to cross-regulation of *Antp* by PB.

Discussion

pb selector function

In the present work, *pb* was found to exert a positive, dose-sensitive selector function in adult development. With these results all of the *Drosophila* homeotic selectors of the ANT-C and BX-C have been tested as Hsp70 fusions [i.e. *Antp*, Schneuwly *et al.*, 1987b; Gibson and Gehring, 1988; *Sex combs reduced (Scr)*, Gibson *et al.*, 1990; *Deformed (Dfd)*, Kuziora and McGinnis, 1988; *labial (lab)*, Heuer and Kaufman, 1992; *Ultrabithorax (Ubx)*, Mann and Hogness, 1990; *abdominal-A (abd-A)*, Gonzalez-Reyes *et al.*, 1992; *Abdominal-B (Abd-B)*, Lamka *et al.*, 1992]. For *pb*, expression of the HSPB mini-gene causes a dominant homeotic transformation of adult antennae to mouthparts, and may simultaneously rescue loss-of-function mutations. In keeping with the reciprocal relationship that has been observed between the identities of tissues affected by loss- and gain-of-function homeotic mutations (Lewis, 1978), the HSPB-directed transformation of antennae to maxillary palps bears an inverse relationship to the inferred loss-of-function transformation of the maxillary palps as well as to the hypomorphic transformation of the labium. Taken together with the antenna-to-maxillary transformation obtained with HSPB, *pb* is both necessary for formation of the maxillary palps and autonomously capable of conferring a maxillary identity to the antennae.

The segment specificity of the *pb* homeotic selector function as detected in *Drosophila* adults is evolutionarily conserved, since gain- and loss-of-function mutations of *maxillopedia (mxp)*, the presumptive *pb* homolog in the flour beetle *Tribolium castaneum*, affect the same segments in a similar fashion (Beeman *et al.*, 1989). However, loss-of-function *mxp* mutations lead to an embryonic/larval transformation (*ibid*), whereas the first evidence of the pb^- condition in *Drosophila* is the morphological transformation of the labial imaginal discs to a leg-like form (Randazzo *et al.*, 1991). If *pb* exercises an adult-specific selector function in the fly, this absence of embryonic function may be the exception rather than the rule.

HSPB reveals combinatorial interactions of pb with Sex combs reduced

Although expression of the homeotic genes is normally confined to a limited domain, this restriction is not the sole source of functional specificity. Indeed, the homeotic proteins themselves show striking specificity of action when expressed outside their normal domains. Ubiquitous ANTP protein expression from an *Hsp70:Antp* element following heat shocks during larval imaginal disc development leads to a developmental transformation restricted to head tissues, especially those of the antennae; other homeotic proteins when ubiquitously expressed show a similar restraint. The adult phenotypes induced by HSPB suggest that PB likewise possesses a specificity intrinsic to the protein itself and apart from the different HSPB-induced phenotypes cited above, other epidermal tissues appear largely unaffected by ectopic PB protein.

The correct formation of the labial palps requires the combined functions of pb and of another homeotic gene of the ANT-C *Sex combs reduced* (*Scr*). Both *pb* and *Scr* are expressed in the labial imaginal discs (Randazzo *et al.*, 1991; Mahaffey and Kaufman, 1987). In the pb^- condition the labium is transformed to prothoracic legs, reflecting the autonomous capacity of *Scr* to establish prothoracic identity (Pattatucci and Kaufman, 1991). Somatic clones of Scr^- cells in turn result in transformation of the labium to maxillary palps (Lewis *et al.*, 1980; Wakimoto, 1981; Struhl, 1982). In light of the complementary natures of the pb^- and Scr^- transformations in the labium, the labial imaginal discs appear to require the combined actions of both *pb* and *Scr* to yield an adult labium. In *Tribolium*,

a gain-of-function *maxillopedia* mutation can transform prothoracic legs to apparent mouth palps (Beeman *et al.*, 1989). The prothoracic legs in the fruit fly, whose formation depends on Scr^+ function, are modified (malformed sex combs) but not detectably transformed toward palps by addition of HSPB-derived PB to the leg imaginal discs. While the combination of PB and SCR proteins was not sufficient to generate a labial palp in place of a leg, at least at the levels and durations of expression attained in our experiments, it remains an open and interesting question whether regulated expression of PB in the prothoracic segment could induce mouthpart formation there in combination with SCR.

In contrast to the combined actions of *pb* and *Scr* in the mouthparts, *pb* and *Antp* exhibit a dose-sensitive epistatic relationship in the antennae involving negative regulation of *Antp* by PB. These interactions were observed at the phenotypic level of segmental identity. It will now be of interest to establish models permitting us to examine how identity is attributed at the cellular level.

Homeotic cross-regulation

Multiple parameters undoubtedly contribute to the complex and specific natures of homeotic transformations. The observed dose-sensitive cross-regulation exercised by PB towards *Antp* indicates a role for the quantity of different homeotic proteins present that is perhaps best rationalized as a means to change the terms of a competition, as previously suggested for interactions between *Ubx* and *Adb-B* in embryonic development (Lamka *et al.*, 1992). Another likely factor is the tissue or cell-specific distribution of co-factors, and modifiers such as protein kinases and phosphatases. The observation that *pb* function provided by the HSPB element can extinguish *Antp* function in the head in a dose-dependent manner, without marked reduction of normal *Antp* expression in the thorax, suggests an important contribution from such accessory factors. It is worth noting that the striking dose sensitive down-regulation of $Antp^{PW}$ by PB observed in head tissue runs contrary to the conclusions of Gonzalez-Reyers *et al.* (1990), who suggested that cross-regulatory interactions might be irrelevant to developmental control in the fly. While this divergence of results may be stage or gene-specific, the dose sensitivity of the present data clearly signals a limitation for interpreting heat shock experiments.

Levels of homeotic function and developmental identity

The importance of protein concentration for segmental identity has long been recognized through the existence of haplo-insufficient homeotic mutations, e.g. for *Scr* and *Ubx*. Individuals that are Scr^-/Scr^+ or UBX^-/Ubx^+ show evidence of a partial transformation toward the identity associated with the absence of that gene's function (T1 legs towards T2 legs, or halteres towards wings, respectively). In the case of *Scr*, the haplo-insufficient state results in reduction of the sex combs found on the prothoracic legs of males, with the sex comb teeth removed progressively from body-proximal towards distal. Each tooth of the sex comb is a clonally derived structure, and the reduction can equally be regarded as a change in cell identity as a re-direction of segment identity. The phenotype of Scr^-/Scr^+ male flies suggests a segmental transformation, but is not yet one.

Crucial thresholds of *pb* protein concentration or function appear to exist in mouthparts separating wild type (labial), hypomorphic (antennal) and null (prothoracic) segmental identities. This property of the endogenous gene was also detected for HSPB in several different tissues including the eye–antennal discs of the developing head. These results are in accord with accumulating results of *in vitro* studies examining the DNA binding specificity of various homeodomain proteins. Target DNA sequences have generally been found to be very similar and to be bound with similar affinities (reviewed in Scott *et al.*, 1989; Hayashi and Scott, 1990; Gehring *et al.*, 1990; see also Ekker *et al.*, 1991, 1992). Variable numbers of binding sites in concert with overlapping affinities may yield very finely tuned response thresholds, as documented for the transcriptional regulation of *hunchback* by *bicoid* early in embryogenesis (Driever and Nüsslein-Volhard, 1989; Struhl *et al.*, 1989) or of *even skipped* by several homeodomain proteins (Small and Levine, 1991). The involvement of phenotypic thresholds in the process of segmental identification, thresholds that depend at least in part on protein concentration, appears to support the interpretations given to *in vitro* DNA binding properties of homeodomain proteins *vis-à-vis* their *in vivo* behavior at various stages of the developmental process. Our results point to the existence of quantitative thresholds for homeotic selector function effected at a suprastructural rather than a cellular level. pb^+ function acts in a finely tuned fashion to re-direct the morphogenesis of non-equivalent cells in a tissue normally subject to *pb* regulation, namely the mouthparts, or in a novel environment such as the antennae. Interestingly, one way to integrate a fine tuned response might involve intercellular communication, consistent with the observation of Struhl (1981) that clones of $Antp^-$ cells displayed non-autonomous transformation of leg to antennae.

Thresholds and evolution

Thresholds distinguishing one segmental identity from another might exist generally for homeotic proteins, even though the level required could differ considerably from one cell type to another within the confines of a segment. For *pb* this critical level of function falls well below the normal level of expression (pb^-/pb^+ is normal), and allows a transition from labial through antennal to prothoracic identity. Potential evolutionary benefits of such an arrangement include the capacity to modify morphology rather easily by altering the threshold level through incremental, non-radical genetic changes. Modulations in absolute and relative expression levels, in the number or quality of target binding sites or in the susceptibility of the homeoprotein to to post-translational modifications, all might lead to marked changes. The necessity for such finely tuned regulation, of both the absolute and relative levels of homeotic proteins, may thus be an important factor contributing to the remarkable evolutionary conservation of the HOM/HOX complexes.

Materials and methods

Construction of the HSPB element

The vector was derived from pUChsneo (Steller and Pirrotta, 1985) by deleting the pUC8 polylinker (partial *Hin*dIII digest plus *Eco*RI digest,

fill-in with Klenow polymerase and ligation), reinserting a blunted pUC18 polylinker (*Eco*RI plus *Hin*dIII) into the blunted *Hin*dIII site at position 4833 (Steller and Pirrotta, 1985) and deleting the neo^R gene by digestion with *Pst*I followed by ligation. The polylinker contains unique *Pst*I, *Sal*I, *Xba*I, *Bam*HI and *Sma*I sites consecutively beginning at position 3552, fused to exon 1 of the *Hsp70* gene. The 3.8 kb *pb* 5′ fragment extends from an *Nco*I site in the middle of exon 1 (replaced after blunt-ending by an *Xba*I linker) through exon 2 to a *Sal*I site 1.8 kb downstream of exon 2 in the second intron; the 4.6 kb *Xho*I−*Bam*HI *pb* 3′ fragment encompasses exons 4–9, including the homeobox (exons 4 and 5, marked with asterisks) and the first *pb* polyadenylation site. The mini-*white* 6.0 kb *Eco*RI fragment (Pirotta *et al.*, 1985) was used as the visible marker. It was modified by blunting with Klenow polymerase and addition of *Xho*I linkers, then inserted into the *Xho*I site in the *Hsp70*-derived sequences at position 3277 (Steller and Pirrotta, 1985).

Germline transformation

Standard methods were employed, and injections were performed without dechorionating the embryos (Robertson *et al.*, 1988).

Transgenic HSPB lines employed

We have isolated a number of HSPB lines, either by injection or by mobilization of inserted elements. Lines obtained by injection include HSPB 1 (X chromosome; homozygous viable; poor expressivity of the HSPB phenotype; gives variegating eye color, but the effect is diminished at 29°C, suggesting the insertion is in, or near, heterochromatin); HSPB 2 (X insertion; strong antennal to maxillary transformation, penetrance complete; male sterile due to the site of insertion); HSPB 3 (insertion in the TM6B, Hu Tb balancer; phenotype is weak); HSPB 4c (insertion in the TM6B, Hu Tb balancer; transformation of antennal segment 3 is complete, and about half the arista is removed; penetrance complete, expressivity very reproducible; viability mediocre); HSPB 4d (insertion on chromosome 3; transformation very similar to HSPB 4c, but with better viability; recessive pupal lethality). Other lines were generated by mobilization of inserted elements. These include HSPB 2-5 [X linked insertion derived by mobilization of the element of HSPB 2 males, employing the transposase source P(ry+;Δ2-3) (Robertson *et al.*, 1988); intermediate phenotype, weaker than for lines 4c and 4d; females are homozygous viable and fertile], and HSPB 2g (chromosome 2; weak phenotype). Other lines generated by transposition are CB1 and CB2. Both are insertions on the second chromosome with phenotypes slightly weaker than for their parental HSPB 2-5 element. Homozygotes of CB2 are weakly viable, whereas CB1 is homozygous lethal.

Fly culture and phenotypic analyses

Stocks were cultured on standard cornmeal/agar medium, at 22°C unless otherwise indicated. Phenotypes were initially regarded under a stereomicroscope; detailed analyses were performed by scanning electron microscopy, or by light microscopy (Zeiss Axiophot) after mounting in Hoyer's medium.

Analysis of HSPB/$Antp^{Dom}$ double mutant combinations

All stocks carried the eye color mutation *w* permitting the unambiguous identification of flies carrying HSPB (mini-w^+), while the absence of the balancer chromosome-specific dominant markers *Sb* and *Hu Tb* (TM3 and TM6B) allowed identification of the $Antp^{Dom}$-bearing chromosome regardless of phenotype. Ten different $Antp^{Dom}$ alleles were tested (Figure 6), in combination with the HSPB lines 2, 2-5 and 4d. Apart from the *Antp* allele and HSPB line, other potential variables were sex, culture temperature and density. Crosses were raised at 22, 25 and 29°C, or subjected to various heat induction regimes during larval stages. Additionally, as certain $Antp^{Dom}$ alleles show consistently stronger mutant phenotypes in females than in males, comparisons involved female siblings eclosed from the same or from simultaneous crosses. Since $Antp^{Dom}$/HSPB flies were generally weak, we attempted to maintain low culture densities. The appropriate number of females per cross varied with the genotypes involved and was not strictly controlled. Criteria for assigning identity to the antennal appendage were overall shape, the presence or absence of the prominent apical bristle and bracted bristles typical of mesothoracic leg, and the presence of distinctly placed edge hairs typical of maxillary palps. Female larvae carrying $Antp^{PW}$ and either one or two HSPB copies were obtained by crossing HSPB 2-5/Y; $Antp^{PW}$ TM6B, *Hu Tb* males with w^{1118} or with HSPB 2-5/HSPB 2–5 virgin females.

Immunolocalizations

Antibodies and staining reactions were essentially as described in Randazzo *et al.* (1991). Disc stainings followed the protocol of Pattatucci and Kaufman (1991b) except that an amplification step was included for the detection reaction of ANTP protein from the $Antp^{PW}$ allele (Elite amplification system, Vecta).

Phenotypic reversion analysis

Male HSPB 2-5 flies were mutagenized with 25 mM ethyl methanesulfonate (EMS) overnight (Lewis and Bacher, 1968), then mated to w^{1118} virgin females. After 4 days the males were removed to ensure that only post-meiotic events were recovered. To verify the presence of the HSPB element, only females with orange eyes (mini-w^+) were examined for reversion of the antenna-to-maxillary palp transformation. Candidate females were re-crossed with w^{1118} males, and HSPB-bearing progeny with normal antennae were kept as revertants. Due to multiple mutagenic events on the X chromosome of interest only female progeny were initially obtained in most cases. Continued outcrosses with w^{1118} males yielded viable and fertile males with colored eyes (and hence X chromosomes lacking accessory lethal mutations) permitting pure revertant lines to be established.

Characterization of the lesion in an HSPB revertant line

The mini-gene of the HSPB 2-5 revertant line 14 was cloned as a 14 kb *Sal*I fragment in the bacteriophage λ vector EMBL 3. The point mutation was detected by testing for hydroxylamine sensitivity as described by Montandon *et al.* (1989). Five different 5′-^{32}P-end-labelled probes were generated from the original HSPB plasmid by amplification with *Taq* polymerase, and equivalent amplification products from the mutant transgenic element. The following oligonucleotide pairs were employed. Exon 2:
5′-GTTCCCAAGCTGAGGAATATCTAATACGC-3′ (positions 21–49 of Figure 3b, Cribbs *et al.*, 1992a) and 5′-TCAAATTGACATGGTCGATCGC-3′ (717–697 of Figure 3b). Exons 4, 5 and 6: 5′-CATTCACTCCGCAAGTCTCAAACG-3′ (within intron 3, not shown in Cribbs *et al.*, 1992a) and 5′-TGTTGTTGTTGTGACCCCGGGAA-3′ (Figure 3c, 860–837). Exons 6, 7 and beginning of 8: 5′-GAACTGCCATCCGATGATATACC-3′ (Figure 3c, positions 799–821) and 5′-TGGGACTAGGATAATAGCCTG-3′ (Figure 3c, 1639–1620 in exon 8). Exon 8: 5′-ATGCACCCACCAGCAAGGCG-3′ (Figure 3C, 1488–1507) and 5′-TGGTGGTTAATCGCCGCTGGC-3′ (Figure 3c, 2109–2089). Exon 9: 5′-GCACATGCATCACCTGGGAAATGGGG-3′ (Figure 3d, 37–62) and 5′-ATTCCGGCGCAAAGTCGTTGGC-3′ (Figure 3d, 466–445).

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