Point Mutations Within and Outside the Homeodomain Identify Sequences Required for *proboscipedia* Homeotic Function in Drosophila

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ABSTRACT

The Drosophila homeotic gene *proboscipedia* (**pb**) encodes a homeodomain protein homologous to vertebrate HoxA2/B2 required for adult mouthparts formation. A transgenic Hsp70-**pb** (HSPB) element that rescues **pb** mutations also induces the dominant transformation of antennae to maxillary palps. To identify sequences essential to PB protein function, we screened for EMS-induced HSPB mutations leading to phenotypic reversion of the HSPB transformation. Ten revertants harbor identified point mutations in HSPB coding sequences. The point mutations that remove all detectable phenotypes *in vivo* reside either within the homeodomain or, more unexpectedly, in evolutionarily nonconserved regions outside the homeodomain. Two independent homeodomain mutations that change the highly conserved Arginine-5 in the N-terminal hinge show effects on adult eye development, suggesting a previously unsuspected role for Arg5 in functional specificity. Three additional revertant mutations outside the homeodomain reduce but do not abolish PB* activity, identifying protein elements that contribute quantitatively to **pb** function. This *in vivo* analysis shows that apart from the conserved motifs of **pb**, other elements throughout the protein make important contributions to homeotic function.

The repetitive segments of *Drosophila melanogaster* established in early embryogenesis come to differ from each other due to the action of a small set of homeotic selector genes. These genes attribute unique developmental fates to segment primordia, then maintain these identities throughout development (García-Bellido 1975, 1977; Lewis 1978). The general biological importance of these genes is apparent in their remarkable conservation across the animal kingdom from nematodes to humans (McGinnis and Krumlauf 1992; Kenyon 1994). Dramatic functional demonstrations of their developmental roles have been obtained in ectopic expression assays, where homeotic selector proteins can induce the development of normal structures in novel positions (Schneider et al. 1987; Kuziora and McGinnis 1988; Gibson et al. 1990; Malicki et al. 1990; Mann and Hogness 1990; Gonzalez-Reyes et al. 1992; Heuer and Kaufman 1992; Lamka et al. 1992; Cribbs et al. 1995; Halder et al. 1995).

Eight Drosophila homeotic loci are clustered in the Bithorax and Antennapedia Complexes (Peifer et al. 1987; Kaufman et al. 1990; McGinnis and Krumlauf 1992). These HOM genes encode nuclear proteins possessing the 60-amino acid homeodomain motif that contributes to sequence-specific DNA binding and confer segmental identity through the transcriptional activation or repression of downstream genes (García-Bellido 1977; Gehring et al. 1990; Hayashi and Scott 1996; Andrew and Scott 1992; Botas 1993). The growing number of identified targets comprises genes that encode regulatory molecules such as the Drosophila TGF-β growth factor homologue decapentaplegic (Capovilla et al. 1994), the transcription factor Distal-less (Vaughan et al. 1992; O’Hara et al. 1993), and structural molecules such as connectin (Gould and White 1992; Nose et al. 1992) and centrosomin (Heuer et al. 1995; Li and Kaufman 1996).

Most HOM protein homeodomains bind the same or closely related sequences with similar affinities *in vitro* (Ekker et al. 1991, 1992, 1994), but these similarities poorly reflect the very different developmental programs induced *in vivo*. Target genes might be sensitive to subtle differences in DNA binding by different homeoproteins *in vivo*, for example via cooperative DNA binding as detected for the homeotic Ultrabithorax protein (Beachy et al. 1993). Alternatively, small differences in affinity might be amplified by interactions of the homeoprotein with protein cofactors. One cofactor identified to date is the extradenticle (**exd**) gene, whose highly conserved EXD protein product acts in combination with the homeotic genes to help specify distinct developmental fates (Peifer and Wieschaus 1990; Rauskolb et al. 1993, 1995; Gonzalez-Crespo and Morata 1995). This partnership occurs at least in part through combinatorial DNA binding (Chen et al. 1994; van Dijk and Murré 1994; Johnson et al. 1995).

To examine how HOM proteins achieve their specific functions *in vivo*, one fruitful approach has been to...
study the properties of modified HOM proteins. Studies using chimeric HOM proteins have shown that much of the specificity resides in the homeodomain and a small number of nearby residues, including sequences immediately downstream of the homeodomain and the YPWM motif involved in specific interactions with the cofactor EXD (Gibson et al. 1990; Mann and Hoggness 1990; Chan and Mann 1993; Zeng et al. 1993; Johnson et al. 1995; Mann 1995; Zhao et al. 1996). However, several studies have reported that homeodomain DNA binding activity is neither strictly required nor sufficient for some homeodomain protein functions in vivo (Ananthan et al. 1993). For example, the Drosophila segmentation gene fushi tarazu (ftz) encodes a homeodomain-containing protein that can act as a direct DNA binding transcriptional regulator in vivo (Schier and Gehring 1992). A FTZ polypeptide rendered incapable of DNA binding by removing its homeodomain can still regulate ftz expression in vivo and can still control expression of its target gene wingless through direct interactions with the pair-rule protein Paired (Fitzpatrick et al. 1992; Copeland et al. 1996). Taken together, these results suggest that DNA binding and protein-protein interactions involved in homeoprotein function may involve not only the homeodomain and adjacent regions but also other domains along the protein.

In the present work we wished to study homeotic structure-function relationships for the Drosophila proboscipedia (pb) gene. This locus codes for a protein strongly conserved in mice and humans (for example, the three homeodomains are 95% identical (Cribbs et al. 1992)). pb function is required for the correct development of the labial and maxillary palps composing the adult mouthparts. The pb gene is unique among the known homeotic loci in two respects. First, it appears not to be required for embryonic development despite its prominent localized early expression (Pultz et al. 1988). Indeed, pb animals eclose as adults with labial palps and maxillary palps transformed toward prothoracic leg and antennal identities, respectively (Kaufman 1978). Second, pb function is dose sensitive. Partial loss-of-function mutations lead to a distinct transformation of the labial palps to antennal aristae instead of prothoracic legs (Kaufman 1978; Pultz et al. 1988). Inversely, ectopic PB expression in transgenic Hsp70:pb (HSPB) flies directs a fully penetrant and dose-sensitive dominant transformation of antennae toward maxillary palps. The level of pb function present in a segment is central to determining its identity since different levels of PB function or protein expression can give rise to distinct developmental outcomes (Cribbs et al. 1995).

To better define PB protein structural elements important to its in vivo function, we have performed a novel screen to isolate point mutations that abolish the dominant HSPB antennal transformation. The genetic and molecular analysis of these mutants reinforce the notion that the homeodomain plays a preponderant role in homeotic function. However, we show that point mutations in nonconserved regions can also abolish in vivo PB homeotic function as completely as a mutation within the homeodomain. Our analysis has also identified protein elements situated outside the homeodomain in the C-terminal and N-terminal portions of the protein that contribute quantitatively to pb function with qualitative developmental consequences in vivo.

**MATERIALS AND METHODS**

**Fly culture and phenotypic analysis:** Stocks were cultured on standard cornmeal/agar medium at 22°C unless otherwise indicated. Phenotypes were initially examined under a stereomicroscope; detailed analyses were performed by scanning electron microscopy or by light microscopy (Zeiss Axiopt) after mounting in Hoyer's medium.

The transgenic strain used for the screen, HSPB:2-5, has been described previously (Cribbs et al. 1995). The pb allele is a partial loss-of-function mutation truncated for its C-terminal region (Cribbs et al. 1992).

**Phenotypic reversion analysis:** Male HSPB:2-5 flies were mutagenized with 25 mM EMS overnight (Lewis and Bacher 1968) then mated to w¹¹¹(b) (henceforth, w) virgin females. After 4 days the males were removed to ensure that only postmeiotic 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 males, and HSPB-bearing progeny with normal antennae were kept as revertants. Due to multiple mutagenetic events on the X chromosome of interest, only female progeny with colored eyes were initially obtained in most cases. Continued outcrosses with w males yielded viable and fertile males with colored eyes (hence X chromosomes lacking accessory lethal mutations), permitting pure revertant lines to be established. To examine the phenotypic effects of mutant PB (PB*) overexpression, heat shocks were administered to embryos or larvae by placing the culture tubes in a 35°C water bath for 3 hr. This treatment was repeated up to four times.

**Testing rescue of pb mutations by HSPB revertant lines:** To test the rescuing capacity of the revertant elements for the pb⁻/pb⁻ mutant, females carrying wild-type or mutated HSPB elements were crossed with w; Ki pb⁺/pb⁺/TM6B, Hu Tb males. Male progeny carrying both the wild-type or mutated HSPB element and the pb⁻ chromosome were recrossed with w; Ki pb⁺/pb⁺/TM6B, Hu Tb females. All female progeny carry the HSPB element and of these one-fourth are homozygous for pb⁺ (w HSPB⁺/w; Ki pb⁺/pb⁺/Ki pb⁺/pb⁺). We then examined the pb⁺-induced homeotic transformation in the mouthparts of these females. To test for possible dosage effects on the rescue, we also crossed w HSPB⁺; Ki pb⁺/TM6B, Hu Tb males with w HSPB⁺/w; Ki pb⁺/TM6B, Hu Tb virgin females, both obtained in the F₁ of the previous cross. This allowed us to examine pb⁻ males hemizygous for the HSPB⁺ element (compared with heterozygous females, above) and pb⁻ females homozygous for HSPB⁺.

**Immunolocalizations:** Antibodies and staining reactions were essentially as described (Randaizzo et al. 1991). Heat shocks were administered to nonstaged embryos on 35 mm collection plates in an incubator (3 hr/35°C). The temperature of the incubator was found to be crucial for the HSPB element (containing a mini-gene with seven introns, and not a pre-
were systematically carried out on the same gels for DNA located within genomic protein-coding sequences.

Spliced cDNA, a variation of 0.5° changing the pattern of PB expression detected from the Hsp70 promoter.

Characterization of the HSPB lesions: To prepare genomic DNA, fly mixes from each HSPB revertant line were homogenized into 250 μl of HB medium [0.2 M sucrose, 0.1 M Tris (pH 9.2), 50 mM EDTA, 0.5% SDS] then incubated at 68° for 10 min. Two hundred fifty microfilters of TE [10 mM Tris HCl (pH 7.5)/0.1 mM EDTA] containing 0.5% SDS and 100 μg/ml protease K were added and incubated at 37° for 1 hr, then at 50° for 30 min. After adding 1.25 M potassium acetate followed by incubation at 4° for 10 min and centrifugation, DNA was extracted three times with phenol, phenol/chloroform, then precipitated from a 0.3 DNA solution with 2 volumes of ethanol. DNA precipitates were washed with 70% ethanol then dissolved in 40 μl of TE.

Typically 1 μl was sufficient for a 50 μl PCR reaction. Single base pair changes were detected by testing for hydroxylamine sensitivity as described by MONTANDON et al. (1989). Using this approach, it was possible to localize most of the HSPB revertants. Sequences of the located mutations were determined directly following asymmetric amplification of appropriate PCR product (McCabe 1990) using the oligonucleotides listed in Table 1.

To ensure the accuracy of this approach, we sequenced the entire PB coding sequence of the characterized mutants. Double-stranded sequence was obtained for all putative mutations presented here. Double-stranded sequence was likewise obtained for the homedomain of all lines. In four cases (HSPBrev1, HSPBrev3, HSPBrev7 and HSPBrev9) the transgenic allele was cloned in lambda EMBL3. For the others, the PCR amplification products contained a mix of the endogenous and transgenic sequences. To ensure that the mutation resides on the transgene and is not a simple polymorphism of the endogenous copy, parallel sequence runs were systematically carried out on the same gels for DNA from w1118, the parental HSPB:2-5 line and the HSPB plasmid injected to yield the transgenic lines. No polymorphisms were located within genomic protein-coding sequences.

Screen for HSPB phenotypic revertants: We performed a structure-function analysis of the homeotic gene proboscipedia (pb) to identify as precisely as possible structural elements contributing to pb° protein function. Several previous studies have addressed the question of structure/function relationships for homeotic proteins, by the construction of transgenic lines expressing internally deleted and/or chimeric homeotic proteins. Function is then assessed following heat-induced ectopic expression. Our strategy differed from those previously used in three respects. First, to minimize variability we employed the single representative transgenic line HSPB:2-5 line, which induces a fully penetrant, dose-sensitive dominant transformation (Figure 2Ab). Second, we chose as mutagen the alkylating agent EMS that provokes primarily point mutations (G to A, and C to T), thus introducing minimal changes at random in vivo. Third, the new presumptive point mutations were selected, rather than constructed, as phenotypic revertant mutations that annul the developmental effects of HSPB°. Newly isolated mutant stocks were characterized genetically for functional properties and molecularly to identify the new lesion.

The strategy of the reversion screen is described in Figure 1B. Males carrying the HSPB:2-5 element (see Figure 1A) were mutagenized with EMS and crossed with virgin w females. Female progeny with orange eyes (P[min-white°]) were screened in search of revertant females with wild-type antennae. Such revertant lines

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**Table 1**

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>nt position*</th>
</tr>
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<tbody>
<tr>
<td>LS10</td>
<td>5' GTCACCGAGCTGAGGAATATCTTAATACGC 3'</td>
<td>Exon 2: 21–49</td>
</tr>
<tr>
<td>LS11</td>
<td>5' CAAATTCAGATGCTGATGC 3'</td>
<td>Exon 2: 717–697</td>
</tr>
<tr>
<td>pbr3</td>
<td>5' ATTCGGACGGCTGATGGCTGA 3'</td>
<td>Exon 2: 359–331</td>
</tr>
<tr>
<td>pbr4</td>
<td>5' ATTCGGACGGCTGATGGCTGA 3'</td>
<td>Exon 2: 401–422</td>
</tr>
<tr>
<td>LS3</td>
<td>5' CATTCCACTCGCAAGTCTTCATAACGC 3'</td>
<td>Exon 2: 492–470</td>
</tr>
<tr>
<td>LS11</td>
<td>5' GGAATTCGCGTGCGGTGTCG 3'</td>
<td>Introm 3'</td>
</tr>
<tr>
<td>LS1</td>
<td>5' GCAACACGCTGTCGGAACGTC 3'</td>
<td>Introm 4'</td>
</tr>
<tr>
<td>I4</td>
<td>5' GCACAGCGACGTGCGGCGGACGTC 3'</td>
<td>Introm 4'</td>
</tr>
<tr>
<td>pb3</td>
<td>5' TTGTTTGTGTCTGAGCAGGCGACGTC 3'</td>
<td>Exon 6: 860–837</td>
</tr>
<tr>
<td>pb1</td>
<td>5' GAACCGCGATCGGATGATATATACGC 3'</td>
<td>Exon 6: 799–821</td>
</tr>
<tr>
<td>pb6</td>
<td>5' GGAATTCGAGGAGAATAGCCTG 3'</td>
<td>Exon 6: 1639–1620</td>
</tr>
<tr>
<td>LS26</td>
<td>5' CGGCCTTCAAATCGGATGATATATACGC 3'</td>
<td>Exon 7: 1019–1042</td>
</tr>
<tr>
<td>ES5</td>
<td>5' ATGGGCGACGACGAGGCG 3'</td>
<td>Exon 8: 1488–1507</td>
</tr>
<tr>
<td>LS5</td>
<td>5' GCAATGGTTAATCGGCGCGCG 3'</td>
<td>Exon 8: 2190–2089</td>
</tr>
<tr>
<td>pbr6</td>
<td>5' GGAATTCGAGAATGACGTC 3'</td>
<td>Exon 8: 1639–1620</td>
</tr>
<tr>
<td>pbe8</td>
<td>5' GCATCGGACGTCAGGGAGGCG 3'</td>
<td>Exon 8: 1734–1755</td>
</tr>
<tr>
<td>LS6</td>
<td>5' GGAATTCGAGGAGAATAGCCTG 3'</td>
<td>Exon 9: 37–62</td>
</tr>
<tr>
<td>pb2</td>
<td>5' ATTCGGCGGAGAATAGCCTG 3'</td>
<td>Exon 9: 466–445</td>
</tr>
<tr>
<td>E9</td>
<td>5' TTCCGAGGAGAATAGCCTG 3'</td>
<td>Exon 9: 359–338</td>
</tr>
<tr>
<td>LS29</td>
<td>5' GGAATTCGAGGAGAATAGCCTG 3'</td>
<td>Exon 9: 235–217</td>
</tr>
</tbody>
</table>

* Figure 3, B–D (Cribbs et al. 1992).

**NOTES**

RESULTS

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spliced cDNA, a variation of 0.5° changing the pattern of PB expression detected from the Hsp70 promoter.

**Figure 1B.** Males carrying the HSPB:2-5 element (see Figure 1A) were mutagenized with EMS and crossed with virgin w females. Female progeny with orange eyes (P[min-white°]) were screened in search of revertant females with wild-type antennae. Such revertant lines
should carry either a nonfunctional HSPB element, or a second-site suppressor of HSPB+. Since the dominant antennal phenotype is obtained without heat induction, we anticipated that most of the mutations would reside within protein coding sequences of the HSPB element and not in the heat shock response pathway itself. However, this screen could in principle also yield other classes of recoverable mutations, notably dominant second-site modifiers. From ~50,000 mutagenized chromosomes screened, we obtained 21 revertant candidates (Figure 1C). For all 21 revertant lines, the revertant phenotype was fully penetrant and has not in any case been separated from the HSPB mini-w' marker. This indicates that the newly induced mutations are all located very near to or within the HSPB element.

**Phenotypic characterization of the different revertant lines:** Adult phenotypes were examined for each established revertant line with one or two copies of the HSPBrev' element or after a heat induction. We also tested the capability of the HSPB revertant lines to rescue a partial pb loss of function mutation (pb4 allele). We previously showed that this pb mutation, which leads to a reliable partial transformation of the labial palps to antennal aristae (Figure 2Ag), can be fully rescued by a single wild-type HSPB:2-5 transgene copy (Figure 2Ah). Assuming that the observed dominant homeotic transformation in the antennae reflects PB+ function, reversion of PB function in the antennae should be paralleled by a loss of rescuing activity in the mouthparts.

The collection of revertant lines can be divided into three classes (Table 2). The first class contains apparent pb "null" mutations that abolish all HSPB effects detected in the antennae, mouthparts, wings, legs, and eyes (Cribbs et al. 1995). This class comprises 14 lines, called HSPB-. Such flies possess wild-type antennae in all tested conditions with no additional dominant phenotypes (Figure 2Ae). Similarly, none of these lines detectably rescues the pb4 mutation with one or two transgene copies (Figure 2Ak). We should note that these revertant flies, which still contain the P insertion in the original site, are much more active and agile than the starting HSPB:2-5 line. This observation suggests that the reversion event has also abolished detrimental effects of PB in the nervous system.

A second class of HSPB revertant contained two lines (HSPBrev'17 and HSPBrev'5) that behaved in a fashion qualitatively different from the others (Table 2). These lines showed a complete reversion of the dominant
ant to max transformation in a single copy, whereas one of the lines showed a slight but novel defect in two copies (appearance of a single, fine bristle on antennal segment 3; Figure 2Aa). Following heat induction both lines showed detectable reductions of the adult eye. This reduction was weak for HSPBrev1 (not shown), whereas the eyes of HSPBrev3 flies were often markedly reduced (Figure 2Bn). These mutations remove pb homeotic activity in the mouthparts (Figure 2Al) and antennae but show a novel eye loss phenotype.

Revertant lines of the third class act like partial loss of function mutations. This class comprises three lines, called HSPB++/− (Table 2), that exhibit wild-type antennae with one HSPB++/− copy (Figure 2Ac), which is in the conditions used to select the revertant. However, these lines show a restored transformation of the antennae toward maxillary palps with two HSPB++/− copies similar to the transformation induced by one copy of the original HSPB element (compare Figure 2, Ad to Ab). Furthermore, two copies of these HSPB++/− elements suffice to detectably rescue a pb phenotype (Figure 2Aj) whereas a single copy shows partial rescue (Figure 2Ai). The dominant phenotypes and the partial rescues provided by the three lines are comparable. These mutations appear to affect quantitative aspects of PB function, modifying the number of HSPB copies required to give a threshold homeotic transformation.

Two additional HSPB revertant lines associated with lethal mutations or chromosomal rearrangements have not been established as homozygous stocks. Both lines
exhibit complete reversion of the dominant antennal phenotype, and no rescue of a pb mutation, in the heterozygous condition (see Table 2).

**Molecular characterization of mutations associated with HSPBRev elements:** To begin the molecular analysis, all 21 revertant lines were examined for PB protein accumulation in embryos after heat induction. We employed a polyclonal antiserum directed against the central portion of the protein including part of the homeodomain (Cribbs et al. 1992). Revertant lines showing levels of ubiquitous PB protein accumulation similar to HSPB:2-5 after heat induction (17 of the 21 lines) were retained for molecular analysis. In these cases, the Hsp70 promoter remains functional and expresses at least the homeodomain-containing N-terminal half of the PB protein.

We next sought to locate the new lesion and determine the specific sequence change, presuming that the close genetic linkage reflects mutation of PB coding sequences. Southern blot analysis revealed no detectable deletions or rearrangements in these 17 revertant lines (data not shown). Coding sequences of the 8.6 kb pb mini-gene were then examined to detect mismatched cytosine residues based upon their sensitivity to modification by hydroxylamine (Montandon et al. 1989). This method detects all base changes except A to T and T to A transversions, and we therefore anticipated being able to detect the majority of EMS-induced mutations [most often G to A, or C to T transitions (Sulston and Hodgkin 1988)]. This procedure allowed us to rapidly localize seven mutations.

Guided by these data (not shown), we sequenced the appropriate regions of mutant DNA. For the N-terminal part of the protein including the homeodomain (exons 2–7), unambiguous sequence data were obtained for all 17 revertants. Specific point sequence changes were identified for 10 of the 17 genetically characterized HSPB revertants. Among the 17 revertants, eight carry single identified point mutations, either missense or nonsense, one carries two adjacent point mutations, while one other harbors a frame shift in the homeobox.

These results are summarized in Table 3 and represented schematically in Figure 3. Identified HSPB revertant mutations were situated in the homeodomain or in the C-terminal region. In contrast, the mutations of the HSPB+/− class were outside the homeodomain across the protein. The remaining class led us to identify an amino acid in the homeodomain N-terminal hinge likely to be important for functional specificity.

**Class 1: pb homeotic function requires sequences both within and outside the homeodomain:** Only two complete loss-of-function mutations reside in the homeodomain, among the 17 homeodomains for which we possessed unambiguous sequence data. The third lesion located in the homeodomain is a frameshift mutation generating a truncated PB− protein whose loss of function cannot be attributed to the homeodomain per se (Table 3). The lone previously described revertant, HSPBRev14, acts as a complete loss-of-function and disrupts an evolutionarily invariant position of the homeodomain [arg53 to His; (see Cribbs et al. 1995; Figure 3A). Arginine-53 has been shown to contact phosphates of the DNA backbone in both Engrailed and MATa2 homeodomains (Kissing et al. 1990; Wolberger et al. 1991). Consistent with these observations, DNA binding of the PBRev14 homeodomain to a consensus target sequence in vitro is markedly reduced (Benassayag et al., 1997). This mutation thus behaves as a strong loss-of-function both in vivo and in vitro. The second mutation HSPBRev23 affects cysteine-27 in the non-helical central portion of the homeodomain (Figure 3A) and behaves as a complete loss-of-function in vivo. Taken together, these two alleles thus confirm that a point mutation in the homeodomain can abolish PB function.

Two complete loss of function mutations identified sites outside the homeodomain required for PB function. Both were located C-terminal to the homeodomain, changing Ser450 to Arg and Arg602 to Ala (Table 3 and Figure 3B), within a region containing runs of prolines or glutamines (Opa sequences). Thus a PB protein with an intact homeodomain, but carrying a

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**TABLE 2**

**Different classes of revertant phenotype**

<table>
<thead>
<tr>
<th>Classes</th>
<th>Name</th>
<th>No. of mutants</th>
<th>Phenotype in one copy</th>
<th>Phenotype in two copies</th>
<th>Rescue of pb mature mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HSPB</td>
<td>14</td>
<td>Wild type</td>
<td>Wild type</td>
<td>−</td>
</tr>
<tr>
<td>2</td>
<td>HSPBRev5</td>
<td>1</td>
<td>Wild type</td>
<td>Antennas with a fine bristle on the A3 segment; eye reduction</td>
<td>−</td>
</tr>
<tr>
<td>2</td>
<td>HSPBRev17</td>
<td>1</td>
<td>Wild type</td>
<td>Wild type antennae; mild eye alteration</td>
<td>−</td>
</tr>
<tr>
<td>3</td>
<td>HSPB+/−</td>
<td>3</td>
<td>Wild type</td>
<td>Transformation of antennae toward maxillary palps</td>
<td>+/-</td>
</tr>
<tr>
<td>Nonclassified</td>
<td>HSPBRev7k13</td>
<td>2</td>
<td>Wild type</td>
<td>Lethal</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not determined; +/−, partial rescue; −, a non-rescue.
single amino acid change well downstream of the homeodomain in the C terminal region (193 and 345 residues downstream, respectively), is no longer able to specify segmental identity.

**Class 2: The amino terminus of the homeodomain is involved in PB-induced eye loss:** Two additional point mutations located in the homeodomain, HSPBRev5 and HSPBRev17, constitute a second class of phenotypic revertants. For HSPBRev17 mild but detectable eye defects were observed following heat induction. In contrast, elevated expression of HSPBRev5 protein led on occasion to a complete eye loss no longer associated with a homeotic transformation. Both lines carry a single point mutation affecting the same amino acid, changing the highly conserved arginine-5 in the homeodomain to His (HSPBRev5) or to Cys (HSPBRev17; see Table 3 and Figure 3A). These two independent revertants can detectably affect the eyes and carry a missense mutation of residue 5 of the N-terminal hinge region of the homeodomain implicated in functional specificity of homeotic proteins (LIN and McGINNIS 1992; ZENG et al. 1993; GHAN and MANN 1996). Wild-type PB protein expression in the eyes can induce an eye reduction resembling that induced by expression of these revertants proteins. However, the reversion and non-rescue associated with the mutant alleles show that the lesion is not a simple loss of function. Changing Arg5 to a new identity can apparently separate explicit homeotic selector functions of the PB+ protein from the eye defects. These novel properties prompted us to perform a detailed analysis of these mutations presented elsewhere (BENASSAYAG et al. 1997).

**Class 3: Nonconserved regions outside the homeodomain contribute quantitatively to PB function:** One of the most interesting aspects of the pb model is that partial or complete loss-of-function mutations give rise to distinct transformations (CRIBBS et al. 1992). Furthermore, different quantities of pb protein can apparently result in alternate developmental outcomes (CRIBBS et al. 1995). In our analysis, three revertants were found to exhibit a partial reversion of the dominant PB homeotic transformation in the antennae and in the mouthparts. These harbor point mutations outside the homeodomain, in three distinct and nonconserved regions of the protein (Figure 3B). The first, HSPBRev17, carries a double point mutation changing prolines 67 and 68 in the N-terminal portion of the protein to glutamine and threonine (Table 3). The second, HSPBRev17, harbors a point mutation changing Ser281 to Tyr (24 amino acids downstream of the homeodomain). The third mutation, HSPBRev2, creates a stop codon leading to a truncated protein lacking its C-terminal 123 amino acids. These data suggest that some C or N terminal sequences of the PB protein contribute quantitatively to normal pb+ function.

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Phenotype</th>
<th>Rescue of pb+ mutants</th>
<th>Nucleotide position*</th>
<th>Nucleotide change*</th>
<th>Codon change</th>
<th>Amino acid change*</th>
</tr>
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<tbody>
<tr>
<td>HSPB Rev no. 9</td>
<td>Class 1, HSPB+</td>
<td>–</td>
<td>Beginning of exon 5</td>
<td>Deletion of one A (between 352 and 356)</td>
<td>Frameshift within the homeobox</td>
<td>Detectable protein</td>
</tr>
<tr>
<td>HSPB Rev no. 14</td>
<td>Class 1, HSPB−</td>
<td>–</td>
<td>Exon 5: position 376</td>
<td>G→A</td>
<td>CGC→CAC</td>
<td>Arg250→His</td>
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<td>HSPB Rev no. 23</td>
<td>Class 1, HSPB−</td>
<td>–</td>
<td>Exon 4: position 123</td>
<td>T→G</td>
<td>TGC→GCC</td>
<td>Cys224→Gly</td>
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<tr>
<td>HSPB Rev no. 1</td>
<td>Class 1, HSPB−</td>
<td>–</td>
<td>Exon 8: position 1556</td>
<td>A→C</td>
<td>AGT→CGT</td>
<td>Ser450→Arg</td>
</tr>
<tr>
<td>HSPB Rev no. 5</td>
<td>Class 1, HSPB−</td>
<td>–</td>
<td>Exon 8: position 2012</td>
<td>A→C</td>
<td>GAC→GCC</td>
<td>Asp602→ala</td>
</tr>
<tr>
<td>HSPB Rev no. 17</td>
<td>Class 2, complete reversion + mild eye reduction</td>
<td>–</td>
<td>Exon 4: position 117</td>
<td>C→T</td>
<td>CGC→TGC</td>
<td>Arg202→Cys</td>
</tr>
<tr>
<td>HSPB Rev no. 3</td>
<td>Class 2, complete reversion + eyes reduction</td>
<td>–</td>
<td>Exon 4: position 118</td>
<td>G→A</td>
<td>CGC→CAC</td>
<td>Arg202→His</td>
</tr>
<tr>
<td>HSPB Rev no. 2</td>
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<td>+/−</td>
<td>Exon 9: position 158</td>
<td>G→A</td>
<td>CGC→TGC</td>
<td>Gln675→amb</td>
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<td>HSPB Rev no. 19</td>
<td>Class 3, HSPB+/−</td>
<td>+/−</td>
<td>Exon 2: positions 297, and 299</td>
<td>C→T</td>
<td>CCA→CAA</td>
<td>Pro67→Gln</td>
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<tr>
<td>HSPB Rev no. 20</td>
<td>Class 3, HSPB+/−</td>
<td>+/−</td>
<td>Exon 6: position 754</td>
<td>C→A</td>
<td>TCC→TAC</td>
<td>Ser281→Tyr</td>
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</tbody>
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*Refers to nucleotides and amino acid position within the pb sequence [Figure 3, A–D (CRIBBS et al. 1992)].
+/-, partial rescue; −, a non-rescue.

 supernumerary reporting
FIGURE 3.—Schematic representation of the PB protein showing the localization of identified mutations. (A) Shown at top is the sequence of the PB homeodomain. Beneath are indicated the locations of revertant homeodomain mutations (arrows) with the normal and replacing amino acids indicated. Complete loss-of-function mutations are indicated by HSPB−. (B) The conserved N, YPWM, homeodomain, C motif and the C-terminal domains of the PB protein are represented by boxes, and the approximate locations of Opa repeat sequences that encode runs of prolines or glutamines, by ovals (CRIBBS et al. 1992). Positions of sequence changes associated with the HSPB revertants are indicated beneath (arrows). Partial revertant mutations (HSPB+) are distinguished by bold type.

DISCUSSION

The present work represents the first characterization of a consequent number of point mutations altering homeotic function. The highly reproducible, dose-sensitive nature of the partial transformation induced by HSPB has permitted the isolation of a diversified collection of mutations, as revertants of a dominant phenotype. Proteins showing complete loss, partial loss or altered functions were generated and recovered in vivo in a homogeneous background.

We found 17 PB-expressing mutations that affect pb function in distinct ways, of which we have identified 10 lesions. The other seven mutations are closely linked to HSPB but are heat inducible and thus do not remove Hsp70 promoter function. It therefore seems probable that they are located within the pb transgene and were not detected due to technical limitations. Two obvious possibilities exist. (1) Several conserved regulatory elements are present within the intron 2 (RANDAZZO et al. 1991; KAPOUN and KAUFMAN 1995), whereas our analysis was limited to exonic coding sequences and thus did not examine those sequences. (2) Further, while clear sequence data were obtained for most protein-coding sequences, difficulties were often encountered in amplifying and sequencing the Opa repeat-rich regions in the C-terminal half of the PB protein.

Point mutations in the homeodomain can abolish PB function: Only four of 17 analyzed revertant alleles contain mutations localized in homeodomain-coding sequences. This result contrasts with the preponderant role attributed to the homeodomain in homeotic function. One homeodomain point mutation, located in helix 3, changes an evolutionarily invariant arginine (Figure 3B), Arg250 (or in the standard homeodomain structure Arg53 → His), that contacts the DNA backbone in co-crystals with Engrailed and Matα2 homeodomains (KISSINGER et al. 1990; WOLBERGER et al. 1991). Our results are consistent with the structural data concerning the homeodomain, since this mutation causes a strong or complete loss of function in vivo (CRIBBS et al. 1995) and reduced DNA binding affinity (BENASSAYAG et al. 1997). The second simple loss-of-function mutation in the homeodomain alters cysteine-224 (Cys27 → Gly) in the unstructured part of the homeodomain separating helices 1 and 2. Though less is known about this region of the homeodomain, the neighboring residue 25 makes sequence nonspecific contacts to phosphates along the DNA backbone similar to those effected by Arg53 (KISSINGER et al. 1990). We speculate that the strong or complete loss of PB+ function due to the Cys27 → Gly change may result from a modified
local conformation that alters DNA-binding affinity indirectly. Combined with structural data for other homeodomains, these observations also support a crucial role for the homeodomain in homeotic function via its interactions with DNA.

A critical role for homeodomain Arg5 (N-terminal hinge) in homeodomain function: Two other homeodomain mutations identified in this screen change the same amino acid, Arginine-202 (or Arg5, in the N-terminus of the standard homeodomain). Each revertant leads to detectable adult eye defects following heat induction, though to differing extents. Several studies have shown that the N-terminal portion of the homeodomain is critical for functional specificity in vivo (Gibson et al. 1990; Lin and McGinnis 1992; Zeng et al. 1993; Ekker et al. 1994; Mann 1995; Chan and Mann 1996; Zhao et al. 1996). Nonetheless, the role of the nearly invariant Arg5 in functional specificity has never been analyzed, precisely because of its very strong evolutionary conservation. It is notable that our screen, based on dominant gain-of-function phenotypes, permitted us to detect these novel properties of the mutant PB protein whereas the same mutation was viewed as a simple loss-of-function allele by the criterion of normal function in rescue experiments. A detailed analysis of these mutations is presented elsewhere (Benassayag et al. 1997).

Quantitative contributions of sequences outside the homeodomain to PB homeotic function: The three mutations causing a partial loss of PB function are located outside the homeodomain. One is in the N-terminus of the protein, a second 25 residues downstream of the homeodomain, and a third mutation deletes the C-terminal 123 amino acids. The diminished gain-of-function and partial rescues observed could result from lessened protein stability. However, immunostaining experiments did not indicate markedly reduced accumulation in embryos following heat induction (not shown). Further, none of the three point mutations affects apparent PEST-related motifs implicated in protein stability (Rechsteiner 1988). More interestingly, the N-terminal and/or C-terminal regions of PB may affect PB homeodomain functions within the same molecule, or interactions with other proteins necessary for full biological activity in vivo. Given the small reduction in activity of roughly one half detected in vivo, this point will no doubt be difficult to establish at a molecular level.

Implications of PB dose sensitivity: Our genetic screens were performed based on the partial homeotic transformation of antennae to maxillary palps induced by a single HSPB+ element. Two copies of the same element completely transforms antennae to palps. Flies carrying three HSPB+ copies die. This lethality is at the level of the organism, since clones of marked cells carrying three HSPB+ elements can be generated by mitotic recombination (not shown). One partial revertant copy (HSPB+/-) can be considered equivalent to 0.5 wild-type copies (since two HSPB+/- copies are phenotypically similar to one wild-type copy). The difference between levels of PB activity that are phenotypically undetectable and overtly lethal is thus only a factor of about six (0.5 to three copies). Normal regulation of pb activity probably must respect a narrow window of acceptable expression levels. These levels might be important, for example, for combinatorial interactions with other transcription factors. Such considerations may be important for normal pb functions, for example in the labial palps where pb acts in concert with Ser to generate labial rather than prothoracic or maxillary identities (Cribbs et al. 1995). Ultimately, to understand the biological logic of homeotic function it will be necessary to comprehend how these small differences are employed in a coherent way.

Role of the C-terminal region of PB protein: Mutations with different developmental effects alter the C-terminal half of the PB protein. Two alleles, one a nonsense mutation 24 residues downstream of the homeodomain and the other a nonsense mutation that truncates the last 123 amino acids, show similar partial loss of PB+ function. The latter is consistent with previous results showing that loss of PB epitopes in exon 9 (the C-terminal 163 amino acids) can result in partial loss of pb+ function (Cribbs et al. 1992). The separation in the linear protein sequence of these mutations with similar effects might reflect genuine interactions of dispersed elements that are reassembled in the folded protein.

In marked contrast to the partial loss-of-function alleles, two point missense mutations (located ~290 and 350 amino acids downstream of the homeodomain) show complete loss of PB function. Neither mutation resides within an evolutionarily conserved motif, clearly supporting the value of an approach based on function such as the present screen, rather than one guided primarily by structural conservation, in studying homeotic/Hox activity.

Sequences C-terminal to the homeodomain (the C-tail) were found to influence UBX function in vivo, playing an important role in determining UBX specificity without affecting homeodomain DNA binding (Chan and Mann 1993). Similarly, sequences C-terminal of the yeast MATa2 homeodomain are important for interactions with the MATa1 homeodomain and for target gene selection in vivo (Mak and Johnson 1993; Stark and Johnson 1994). The partial revertant 24 residues downstream of the PB homeodomain may be comparable to the loss of the UBX C tail (Chan and Mann 1993). Our results with PB extend the previous study, since simple point mutations of the nonconserved C-terminal region abolish any detectable PB homeotic function. We conclude that sequences downstream of the homeodomain are necessary for normal PB function, as previously deduced for UBX. However,
it is not clear to what extent previous observations for UBX are comparable to PB since the C-tails differ not only in sequence but are also of markedly different sizes (35 amino acids for UBX vs. 640 for PB). If, as for UBX, the C-tail plays a role independent of obvious DNA binding functions, it will be of interest to examine potential protein-protein interactions influencing developmental outcome.

**Evolutionarily conserved protein motifs and requirements for pb function:** The PB protein exhibits striking similarity to its mammalian homologues Hox-A2 and Hox-B2, in the homeodomain as well as several regions outside the homeodomain including the highly conserved Tyr-Pro-Trp-Met (YPWM) motif present in most homeotic/Hox proteins (CRIBBS et al. 1992). Apart from the homeodomain, no identified mutation touches any of the three other strongly conserved sequence elements present in vertebrate Hox-A2/B2 proteins. Given the small size of these elements with respect to the 798 residue PB protein, this point is perhaps not surprising. Still, the high evolutionary conservation of such elements is considered as strong evidence for their important roles in homeoprotein function and a guide for site-directed mutagenesis to test function. Our data lead us to conclude that the apparently nonconserved remainder of the protein also plays a critical role. One way in which this might occur is in the correct folding of the conserved protein elements. Mutations affecting positioning of conserved elements could act as strongly as those affecting the conserved motifs themselves. These mutations might also affect sites governing species-specific rather than general HOM functions. Further studies like the one presented here should help to elucidate the nature of homeotic protein function central to proper development.

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