A functional analysis of 5′, intronic and promoter regions of the homeotic gene proboscipedia in Drosophila melanogaster

Ann M. Kapoun* and Thomas C. Kaufman

Howard Hughes Medical Institute, Department of Biology, Indiana University, Bloomington, Indiana 47405 USA

*Present address: Division of Hematology-Oncology, MS#57, Childrens Hospital Los Angeles, 4650 Sunset Boulevard, Los Angeles, CA 90027-6016, USA

SUMMARY

In Drosophila, the homeotic gene proboscipedia (pb) is required for the formation of the adult mouthparts. To determine the functional significance of putative pb regulatory DNA, we have performed an in vivo analysis of sequences upstream of and within pb using a series of minigenes. Additionally, we have initiated a dissection of pb’s promoter and enhancer elements using lacZ reporter gene constructs. Our results establish that a conserved region located in the second intron is essential for proper formation of the adult mouthparts. A 0.5 kb fragment from this region was shown to direct lacZ expression in a pb pattern in both embryos and third instar labial discs when combined with a 600 bp pb basal promoter sequence. A 32 bp element contained within the 0.5 kb region functions as a labial disc enhancer for pb. Surprisingly, the conserved second intron pb enhancers do not function properly with a heterologous hsp70 promoter, suggesting that promoter-specific interactions occur at the pb locus. We also found redundant and cryptic enhancers in the large introns of pb that are not required for pb function. Finally, we demonstrate that the pb transcription unit does not require sequences upstream of –98 bp to provide pb function in the labial discs. Rather, pb’s upstream DNA appears to contain negative regulatory DNA required for silencing PB accumulation in inappropriate domains of third instar imaginal discs. Thus, we have defined many of pb’s cis-controlling sequences to an experimentally manageable size, thereby making this an attractive system for the discovery of trans-acting proteins and, consequently, for elucidating the mechanisms of homeotic gene regulation.

Key words: homeotic, proboscipedia, minigene, enhancer, promoter-specificity

INTRODUCTION

Segmental identities in the body plan of Drosophila melanogaster are specified by the products of the homeotic genes. These selector genes are expressed in specific domains along the body axis (Cohen, 1993; Kaufman et al., 1990). When homeotic gene products are absent or inappropriately expressed, certain body segments are morphologically transformed into other segments. Therefore, the regulatory machinery governing the transcriptional activation of homeotic genes must be precisely controlled.

Studies to date have shown that the cis-acting regulatory regions controlling the expression patterns of homeotic genes are often spread out over relatively large genomic intervals. For example, genetic analyses of Sex combs reduced (Scr) and Ultrabithorax (Ubx) have revealed that sequences necessary for proper function span regions of DNA over 70 kb (Lewis, 1978; Pattatucci and Kaufman, 1991). Additionally, molecular studies have shown that multiple enhancer sequences conferring certain aspects of the expression patterns of these genes can be separated by 20-35 kb of intervening DNA (Gindhart et al., 1995; Muller and Bienz, 1991). The pattern of homeotic gene expression is thought to be maintained by the actions of multiple silencers that serve to restrict the expression of each gene to its wild-type domain (Busturia and Bienz, 1993; Muller and Bienz, 1991). These silencer elements appear to function at significant distances from their cognate promoters; for example, a Ubx silencer is positioned over 40 kb from the Ubx promoter (Muller and Bienz, 1991). Therefore, the complex regulatory interactions of multiple and separable cis-controlling regions appear to be important for defining the expression patterns of homeotic genes.

Genetic analysis of the homeotic gene proboscipedia (pb) revealed that sequences required for pb function are located within an 84 kb region that includes an approximately 35 kb transcription unit (Pultz et al., 1988). The homeotic phenotype of pb (labial palp-to-leg transformation) has been rescued with a 16 kb minigene constructed from genomic sequences that lie within this genetically defined boundary (Randazzo et al., 1991). The transgene generates a wild-type pattern of PB accumulation in the ectodermal cells of the embryonic labial and maxillary lobes, and in the labial discs of third instar larvae (Randazzo et al., 1991). However, some aspects of PB accumulation in the central nervous system (CNS) are not conferred by this minigene. Because the transgene rescues pb null animals, apparently pb function in the CNS is not essential. Since the cis-controlling regions of pb appear to be less complex (at least physically less extensive) than those of other homeotic genes, analysis of the
controlling mechanisms of pb may provide a manageable system through which homeotic gene regulation can be understood.

Initial investigations of pb regulatory DNA revealed that a conserved region of intron two is capable of conferring a pb-like expression pattern when positioned in a pb-lacZ reporter gene containing 7.3 kb of upstream pb DNA (Randazzo et al., 1991). This result suggests that pb enhancers are present in the second intron fragment, but it does not address whether these sequences are necessary for determining the identity of labial palps. In the study described here, a functional dissection of DNA sequences in the pb locus was performed. To test the significance of the conserved intron two DNA, and to determine how much upstream sequence is required for function, we analyzed a series of pb minigenes containing deletions in these regions. Additionally, we set out to determine which sequences of pb are required to function as the promoter in vivo. Finally, using a series of hsp70 promoter- or pb promoter-lacZ fusion genes, we tested for the presence of pb enhancers over an approximately 30 kb region of the pb locus.

**MATERIALS AND METHODS**

**pb minigene construction**

Minigenes of pb (Fig. 1A) were created by subcloning genomic fragments derived from λ phage isolated from the ANT-C chromosomal walk (Pultz et al., 1988; Scott et al., 1983). The SPL minigene contains 7.3 kb of upstream sequence, all of pb's exons, and 3' DNA extending just downstream of the second polyadenylation site (Cribbs et al., 1992). Most of the large intronic region (25 kb) of pb is absent in this minigene. SPL includes all the sequences present in the minigene that Randazzo et al. (1991) used to confirm the cloned pb gene. Additionally, the 15 bp alternatively spliced microexon that was not included in the original minigene (Randazzo et al., 1991) is included in SPL. All of the other minigenes have deletions in some of the pb DNA sequences contained in SPL. The two internal deletion minigene lines, ΔXho and ΔPvu, remove sequences in the second intron conserved region that was identified by Randazzo et al. (1991). This region spans approximately 700 bases and contains numerous blocks of sequence that are identical to the corresponding regions of D. pseudoobscura. Minigene ΔXho deletes the 3'-most 55% (396/721 bases), and minigene ΔPvu removes the 5'-most 83% (597/721 bases) of this conserved region. In addition, two transgenes with 5' deletions were created: ΔSal and ΔKpn delete sequences upstream of −1.6 kb and −98 bp, respectively. Details concerning the construction of the minigenes are available upon request.

**Construction of enhancer test vectors**

P-element vectors HZR (Gindhart et al., 1995) and pbZR were used in this analysis (Fig. 1B). The pbZR vector was created by replacing the hsp70 promoter of HZR with a 0.6 kb pb sequence. One of the two PstI restriction enzyme sites contained in HZR was removed; this was accomplished by performing a PstI partial digestion, making the fragment ends blunt with T4 DNA polymerase (New England BioLabs, NEB), and ligation with T4 DNA ligase (NEB). A subclone that contained the unique PstI site in the hsp70 leader sequences was selected. This subclone was digested with PstI, blunt-ended with T4 DNA polymerase, and then digested with KpnI to remove most of the hsp70 sequences. A 0.6 kb KpnI-NcoI pb fragment that includes from −98 bp to +503 bp of the pb transcription start site was cloned into the resulting vector.

The pb genomic fragments diagrammed in Fig. 1B were cloned into HZR and/or pbZR; these fragments are subclones of the phage clone λ644 except for the 13.0 kb EcoRI fragment, which is isolated from λ859 (Scott et al., 1983). In all constructs, the pb fragments are in their normal orientation with respect to the hsp70 or pb promoter, except in one case (13.0+HZR) in which the orientation is reversed. Plasmid r1r2+pbZR contains two copies of the following oligonucleotide (named r1r2) that was inserted into the Nol site of pbZR:

5'GCGGGCGGABATTATGTGCTGGCCGAAAGATCCTATCTTATTTGTAACATTTTCGAGGCTACACGGCCGTTTCTAGACAATAAACGCTAGTAAACATTGTTAAAAGCTCCGCCGG

This oligonucleotide was designed from a block of sequence identity located within the highly conserved region of pb's second intron (beginning at +3 kb of the pb start site), and contains two motifs, 32 bp (r1) and 7 bp (r2), which are repeated in the second intron (Randazzo et al., 1991). r1r2 was created with an internal EcoRI site (underlined in above sequence) and Nol cohesive ends to facilitate subcloning into pbZR. Clones were sequenced (Sequenase kit, US Biochemical Corp.) to determine orientation. r1r2+pbZR contains two copies (in the same orientation) of r1r2 in their normal orientation with respect to the pb promoter; three bases (GCC) of the internal Nol site were inadvertently deleted in the plasmid construction process.

The en+pbZR construct has a 1 kb fragment from the D. melanogaster engrailed (en) gene (provided by J. Kassis) inserted into pbZR (Kassis, 1990). This first intron fragment contains en regulatory sequences required for the generation of en-like stripes in early embryogenesis.

**Fly stocks**

Minigene transgenic stocks were established in Kh pbr p/TM3,Sb and Kh pbh p/TM6B, Hu Tb backgrounds (Lindsay and Zimm, 1992; FlyBase 1994). The gamma ray induced pb5 allele is a protein null (Cribbs et al., 1992; Kaufman, 1978). Homozygous pb5 flies were scored both by the presence of the Sb+ and the Ki/Ki phenotypes. Third instar pbh/pbh larvae were identified by the Tb+ phenotype. Transgenic rescue experiments were performed with one copy of the minigene unless otherwise stated. In the embryonic analysis, the TM3 chromosome in the above genotypes contained a P-element insertion (HZR+6.8Xb) that directs the expression of β-galactosidase in the posterior three quarters of the embryo and in the hindgut and anal plates (Gindhart et al., 1995). Homozygous pb5 embryos were identified by the absence of β-galactosidase accumulation in this pattern.

**P-element transformation**

Germ-line transformation (Robertson et al., 1988) was performed using a 0.5 mg/ml solution of each minigene. Transformants were selected by the rescue of the white+ phenotype of the recipient flies. Multiple independent transformant lines of each construct were examined: SPL=2; ΔXho=5; ΔPvu=5; ΔSal=9; ΔKpn=6; UASpb=4; 5.3+HZR=5; 10.6+HZR=6; 13.0+HZR=3; 2.1+HZR=8; 2.1+pbZR=8; 1.9+2.1+pbZR=4; 0.5+pbZR=5; 0.8+pbZR=6; r1r2+pbZR=4; en+pbZR=7.

**Immunological staining**

Embryos and imaginal discs were fixed and stained as described by Mahaffey and Kaufman (1987) and Pattatucci and Kaufman (1991), respectively.

In the minigene analysis, anti-PB rabbit polyclonal antisera were used (Cribbs et al., 1992). Antiserum used for imaginal tissues and embryos were directed against the C-terminal region of PB (anti-E9) and the N-terminal region of PB (anti-E2), respectively. FAB’ goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies were used for third instar larvae staining (Protos Immuno Research). For the embryonic analysis, double staining reactions with polyclonal rabbit anti-PB antisera and monoclonal mouse anti-β-galactosidase (Boehringer Mannheim) were used in conjunction with HRP-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies (BioRad). The staining reactions were allowed to proceed 30 minutes for embryos and 10 minutes for discs.

In the enhancer analysis, most embryos were stained using mono-
clonal mouse anti-β-galactosidase from Boehringer Mannheim except for the animals shown in Fig. 6, which were stained using monoclonal mouse anti-β-galactosidase obtained from M. Mazzula. Polyclonal rabbit anti-β-galactosidase antisera (supplied by M. Gorman) were used for the en+pbKN embryos, and for all imaginal tissues from the transformant lines containing the enhancer test vectors. HRP-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies from BioRad were used in the staining reactions.

Embryos were staged as described by Campos-Ortega and Hartenstein (1985). Analysis and photography of stained embryos and imaginal tissues were performed using a Zeiss Axiophot with Nomarski optics. All embryos and discs were photographed at 200× magnification unless otherwise indicated.

Scanning electron microscopy

Scanning electron microscopy was performed as described in Merrill et al. (1987). Minigene transgenic flies in Ki pb5 p° / Ki pb5 p° or Ki pb5 p° /TM3, Sb backgrounds were stored in 70% ethanol until they were prepared for microscopy.

RESULTS

Mouthpart phenotypes and PB accumulation in the labial discs in minigene transformant animals

A series of pb minigene deletion constructs were made in order to determine the functional significance of pb’s upstream and second intron conserved DNA (Fig. 1A and Materials and Methods). To test the ability of the five minigenes to supply pb function, we examined the adult mouthparts and the anlagen of these structures, the labial discs, from transformant animals.

Fig. 1. (A) The molecular organization of the five pb minigenes used in this study. Coding and noncoding exons are represented by the black and white boxes, respectively. The direction of transcription (arrow) is from distal to proximal, with respect to the centromere. A detailed description of the pb transcription unit is reported in Cribbs et al. (1992). The mRNAs of pb can be comprised of either eight or nine exons, depending on a 15 bp alternatively spliced microexon (third exon, indicated by a vertical line). The translation start site is located at the 5′ end of exon two, and the homeobox is encoded by exons four and five. An unrelated gene, z2, is adjacent to the pb transcription unit; z2 can be deleted from the genome without any discernable consequences (Pultz et al., 1988). The shaded boxes represent DNA that is conserved between D. melanogaster and D. pseudoobscura. One conserved region (0.06 kb) is located between z2 and the pb start site, and the other (0.7 kb) is located in intron two. The symbol ‘Δ’ indicates that a deletion was made, and the number that follows is the size of the deletion (all units are in kilobases). The horizontal line that is interrupted between exons two and four shows that most of the intronic DNA is absent (approximately 22 kb) from the transgenes. (B) Below the molecular map of pb are the genomic fragments that were cloned into HZR (hsp70 promoter-lacZ fusion gene) and/or pbZR (pb promoter-lacZ reporter construct).

The P-element reporter constructs are named as follows: ‘size of the cloned pb fragment’ (in kilobases) + ‘vector name’, for example, 5.3+HZR. Restriction enzymes: H, HindIII; R, EcoRI; B, BamHI; S, SaII; N, Ncol; P, PvuI; X, XhoI.
in a \( pb \) null background (Figs 2, 3). In \( pb \) null animals, the labial palps are transformed into first thoracic legs, and the maxillary palps are reduced in size (Fig. 2A). Labial discs in these animals do not accumulate PB and morphologically resemble leg discs (Fig. 3A). In contrast, the mouthparts of \( pb \) null animals containing one copy of the SPL minigene were
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indistinguishable from that of wild-type animals (Fig. 2B). Labial discs from transformant SPL animals exhibited wild-type levels of PB accumulation (compare Fig. 3C to B). These results demonstrate that the SPL minigene completely rescues the pb homeotic phenotype.

Transformant animals containing the two 5' deletion minigenes, ∆Sal and ∆Kpn, had significant levels of pb expression in the labial discs (Fig. 3D,E). Although PB accumulation levels were somewhat variable from animal to animal, many labial discs were indistinguishable from wild-type discs stained with pb antisera (compare Fig. 3D,E to B). In addition, a majority of these minigene lines (4/6, ∆Kpn and 7/9, ∆Sal) almost completely rescued the pb null phenotype (Fig. 2C,D). Pseudotracheal rows were present in the labial palps, and maxillary palps appeared to be normal in size and morphology (compare Fig. 2C,D to B). However, small patches of aristal tissue were often seen on the lateral aspect of the labial palps (Fig. 2C,D). Consistent with the somewhat variable pb expression in the labial discs, a minority of the adult animals in each line displayed more severe labial palp-to-aristae transformations. This aristae transformation is characteristic of pb hypomorphic alleles (Kaufman, 1978). In addition, 2 of 6 ∆Kpn and 2 of 9 ∆Sal transformant lines showed this type of transformation in a majority of the animals, and some transformants exhibited a partial labial palp-to-leg phenotype. Because most of the lines exhibited better rescue, the transformations seen in these lines are likely to be the result of position effects (down-regulation) caused by sequences flanking the insertion sites (Levis et al., 1985). These results suggest that the spatial information needed to specify pb expression in the labial discs is not contained in pb's upstream DNA, and that these sequences are not required for at least partial rescue of pb− animals.

Fig. 2. Scanning electron micrographs (SEMs) of adult mouthparts. (A) Mouthparts of an animal carrying a pb null mutation (pb5/pb5). The labial palps are transformed to prothoracic legs (arrow points to a claw) and the maxillary palps (Mx) are reduced in size. (B) The mouthparts of a P[w+, SPL]; pb5/pb5 animal are shown. This animal is indistinguishable from a wild-type individual (Hodgkin and Bryant, 1978). Note the normal pseudotracheal rows on the labial palps (arrow). The mouthparts of (C) P[w+, ∆Sal]; pb5/pb5 and (D) P[w+, ∆Kpn]; pb5/pb5 animals are almost completely rescued. Note the presence of small patches of aristal tissue on the lateral aspects of the labial palps in these transformants (arrow). (E,F) P[w+, ∆Xho]; pb5/pb5 animals display a range of mouthpart transformations. The animal in E shows a near-wild-type phenotype: the mouthparts are almost completely rescued, but often small patches of aristal tissues are present (arrow) and there are abnormal pseudotracheal rows (arrowhead). The mouthparts in F depict the most severe transformation observed in ∆Xho transformants; the labial palps are transformed into aristae (arrow). (G,H) The range of phenotypes exhibited by P[w+, ∆Pvu]; pb5/pb5 animals. The mouthparts in G show a labial palp-to-aristae transformation. The animal in H displays a labial palp-to-leg transformation; note the claw present on the transformed labial palps (arrow).

Fig. 3. Expression of minigenes in the labial discs of third instar larvae. Labial discs of third instar larvae were stained with antisera that recognize PB. (A) The labial discs of pb5/pb5 animals do not accumulate PB. (B) Protein levels in wild-type labial discs. (C) Transformant labial discs of a P[w+, SPL; pb5/pb5] animal is shown; the levels of PB in these discs are similar to that of a wild-type animal. (D) P[w+, ∆Sal, pb5/pb5] and (E) P[w+, ∆Kpn, pb5/pb5] transformant larvae express significant levels of pb in the labial discs. (F) PB accumulates in the proximal portion of the labial discs in a P[w+, ∆Xho, pb5/pb5] transgenic animal. (G) The labial discs of a P[w+, ∆Pvu, pb5/pb5] transformant have extremely low levels of pb expression.
PB accumulation in the labial discs of ΔXho (deletes 55% of pb’s second intron conserved region) transformant animals was localized to the proximal portion of the discs in a pb null background (Fig. 3F). When the staining reaction was allowed to proceed two times longer than the standard interval (see Materials and Methods), PB was detected in more distal portions of the labial discs (not shown). However, the most distal region of the discs in these animals lacked detectable pb expression. A range of mouthpart phenotypes was observed in ΔXho transgenic adults. Labial palps of weakly transformed animals had aristal tissues present on the lateral aspects of the labial palps and sometimes aberrant pseudotracheal rows (Fig. 2E). In animals that had a stronger homeotic phenotype, labial palps were transformed more extensively into aristae (the hypomorphic phenotype) (Fig. 2F). Leg tissues were not observed on the labial palps of ΔXho transformant adults, and maxillary palps appeared to be wild type in size. In contrast, more severe phenotypes were observed in ΔPvu (deletes 83% of the second intron conserved region) transformants in a pb null background. Very low levels or often no detectable PB accumulation was seen in the labial discs of these animals (Fig. 3G). In addition, the labial discs sometimes morphologically resembled leg discs. A range of homeotic phenotypes was observed in pb null adults bearing the ΔPvu transgene. The range extended from a labial palp-to-aristae transformation (Fig. 2G) to a more severe mouthpart transformation (Fig. 2H), as seen in animals carrying pb null mutations. The animals of the latter class often had characteristic leg structures present on the transformed labial palps (compare Fig. 2H to A). Therefore, the sequences deleted in the ΔXho and ΔPvu
minigenes are essential for pb function, and those absent in ΔPvu are relatively more important.

**Ectopic imaginal disc expression of the pb minigenes**

To determine if the minigenes are properly regulated in regions outside of pb’s expression domain, third instar eye-antennal and leg discs from transformant larvae were stained with PB antisera. PB does not accumulate in the eye-antennal discs of wild-type animals (Fig. 4A). Similarly, transformant animals harboring minigenes SPL, ΔXho, or ΔPvu did not accumulate PB in the imaginal eye-antennal discs (not shown). The adult antennal structures in these transformants resembled wild-type animals (Fig 4B), with the exception of a slightly thickened aristae that was observed in two SPL transformant lines. The latter phenotype is likely due to position effects from the genomic DNA flanking the insertions, since two other pb minigenes did not produce this effect (Randazzo et al., 1991). In contrast, a majority of the ΔSal (7/11) and ΔKpn (7/8) minigene transformant lines expressed pb ectopically in the eye-antennal discs (Fig. 4C,E). In these animals, PB accumulated in the antennal nodule that gives rise to the adult arista, and sometimes in the surrounding tissues that give rise to segments one, two and three. Corresponding adult antennal defects were observed in these animals, including a thickening of the arista and often morphologically aberrant third antennal segments (Fig. 4D,F).

The leg discs of wild-type animals do not accumulate PB (Fig. 5A-C). Similarly, pb expression was not detected in leg discs of transformant animals harboring the ΔSPL, ΔXho or ΔPvu minigenes (not shown). Significant PB accumulation in the leg discs was not observed in a majority of the ΔSal transformant lines (Fig. 5D-F). In two ΔSal transgenic lines, however, a few very weakly staining cells were sometimes seen in the distal (central) portion of the discs. Also, one of five ΔSal transformant lines had ectopic pb expression in leg discs that approached the levels observed in ΔKpn transfomants (see below). In contrast to the ΔSal lines, significant levels of ectopic PB accumulation were observed in the leg discs of a majority (4/5) of the ΔKpn transgenic lines (Fig. 5G-I). The most intense accumulation was seen in the

![Fig. 5. Third instar larval leg discs stained with antisera that recognize PB are shown: (A,D,G) prothoracic leg discs (T1); (B,E,H) mesothoracic leg discs (T2); (C,F,I) metathoracic leg discs (T3). (A-C) Wild-type leg discs do not accumulate PB. (D-F) pb expression is not detected in leg discs from a P[w', ΔSal, pb3(pb')] transformant animal. (G-I) A P[w', ΔKpn, pb3(pb')] larva has ectopic PB accumulation in T1-T3. Note the high concentration of protein accumulation in the central regions of these discs (arrow in G). In all imaginal tissues stained with PB antisera, a nonspecific ‘background staining’ was observed in small cells located on the surface of the discs (D-F). These cells are very different in size and in shape from the columnar epithelial cells that accumulate PB in the distal and proximal regions of the leg discs in ΔKpn transformants (G-I).](image)
The ability of the minigenes to express transformant P\textsubscript{w} DNA. The size and morphology of the adult legs in all of the influence of negative position effects from flanking genomic ing that the P-element insertion in this line is under the labial palp-to-leg phenotype in a that did not accumulate PB in the leg discs exhibited a partial central regions of the discs. The single ΔKpn transformant line that did not accumulate PB in the leg discs exhibited a partial labial palp-to-leg phenotype in a pb null background, suggesting that the P-element insertion in this line is under the influence of negative position effects from flanking genomic DNA. The size and morphology of the adult legs in all of the transformant P\{w\+, ΔKpn\}; pb\textsuperscript{5}/pb\textsuperscript{5} lines appeared normal. Since many of the ΔKpn minigene deletion lines are homogygous lethal, the effects of two copies of the transgene could not be unequivocally determined.

The ectopic expression produced from the 5′ deletion minigenes may not be specific to the leg and the antennal imaginal discs. In transformant lines that exhibited strong ectopic accumulation in these discs, PB was detected in other imaginal tissues such as the haltere and the wing discs (not shown). The results presented above suggest that sequences upstream of pb’s transcription start site, which were deleted in the ΔKpn and ΔSal minigenes, contain negative regulatory elements for pb.

**Expression of the minigenes in pb null embryos**

The ability of the minigenes to express pb during embryogenesis was examined. Minigene transformant lines were crossed into a pb\textsuperscript{5}/TM3 background, and selection of homozygous pb null animals was based on the absence of lacZ expression derived from a reporter gene located in the TM3 chromosome (see Materials and Methods) (data not shown). Transgenic expression of SPL in embryos was similar to wild-type pb expression (Pultz et al., 1988), with the exception of the CNS accumulation pattern (see below). The expression generated by SPL was also similar to that of the original pb minigene (Randazzo et al., 1991), except that only SPL conferred proper embryonic mesodermal expression and weak PB accumulation in the ventral nerve cord. The reason for these differences is likely due to the sensitivity of the different polyclonal antibodies used, since transgenic expression is relatively weak in these domains. Expression generated from the two 5′ deletion minigenes was analogous to SPL transgenic expression; however, the accumulation levels varied from animal to animal, and late in embryogenesis (after stage 13) the protein levels were reduced. Transgene accumulation produced from ΔXho was variable in level, but the correct spatial pattern was generated, and many of the animals exhibited expression levels similar to transgenic SPL individuals. Transgenic ΔPvu animals had a normal pb expression pattern in early stages, but the protein levels produced from this construct were lower than what was observed in the other minigene lines; later in embryogenesis (stage 14-16), protein accumulation was very difficult to detect in these animals. These results are consistent with the idea that the 5′ region of pb does not contain spatial information needed to direct pb expression, and that the second intron conserved region is important for expression of pb. Interestingly, ectopic accumulation of PB was not detected in the minigene transformant embryos, suggesting that different methods of gene regulation exist at the two developmental stages.

**Minigene expression in the CNS**

In wild-type animals, PB accumulates in cells of the larval and embryonic CNS (Pultz et al., 1988; Randazzo et al., 1991). None of the pb minigenes confer the proper CNS expression pattern (not shown). Transgenic pb expression in the larval CNS was detected only in a few cells in the brain hemispheres and in the ventral ganglion. In embryos, transgenic pb expression in the ventral nerve cord was drastically reduced from wild-type levels, and PB was not detected in most of the cells in the supraesophageal and the subesophageal ganglia. Thus, the sequences required for proper CNS expression are not fully contained in the pb minigenes.

**Enhancers located in the large introns of pb**

Because essential regulatory information is located in the central regions of these domains. Expression generated from the two 5′ deletion minigenes was analogous to SPL transgenic expression; however, the accumulation levels varied from animal to animal, and late in embryogenesis (after stage 13) the protein levels were reduced. Transgene accumulation produced from ΔXho was variable in level, but the correct spatial pattern was generated, and many of the animals exhibited expression levels similar to transgenic SPL individuals. Transgenic ΔPvu animals had a normal pb expression pattern in early stages, but the protein levels produced from this construct were lower than what was observed in the other minigene lines; later in embryogenesis (stage 14-16), protein accumulation was very difficult to detect in these animals. These results are consistent with the idea that the 5′ region of pb does not contain spatial information needed to direct pb expression, and that the second intron conserved region is important for expression of pb. Interestingly, ectopic accumulation of PB was not detected in the minigene transformant embryos, suggesting that different methods of gene regulation exist at the two developmental stages.

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Regulatory sequences of proboscipedia

We tested the presence of enhancer sequences in the second intron of pb. Additionally, we set out to determine if CNS enhancers are located in the large introns of pb, since these regions are absent from the pb minigenes that did not confer proper CNS expression. Three regions of pb (5.3 kb, 10.6 kb, and 13.0 kb) were cloned upstream of the hsp70-lacZ reporter gene HZR (Fig. 1B). The 5'-most of these fragments (5.3 kb HindIII region) contains 0.7 kb of intron one, all of exon two, and 4 kb of the 5' end of intron two including the highly conserved 0.7 kb stretch of DNA (Randazzo et al., 1991). The 10.6 kb HindIII and 13.0 kb EcoRI fragments together include most of pb’s large intronic region. Transgenic embryos containing these vectors and a control vector, pbG1-intron, (Randazzo et al., 1991) were stained with anti-β-galactosidase antisera.

The pbG1-intron construct is a pb-lacZ reporter gene that contains 7.3 kb of pb upstream DNA and a 1.6 kb fragment that includes the intron two conserved region; this construct confers a pb-like pattern of expression (Fig. 6A). The 5.3+HZR transgene did not direct a reliable β-galactosidase accumulation pattern. A majority of the 5.3+HZR transgenic lines showed no detectable lacZ expression (Fig. 6B). However, in two of the five transgenic lines examined, a weak patchy expression pattern was seen in the maxillary and labial lobes (not shown). This result suggests that the 5.3 kb HindIII fragment does not act as a functional pb enhancer when in combination with the hsp70 promoter. Next, the 10.6 kb HindIII intronic region was found to confer lacZ expression in a late embryonic pb pattern (Fig. 6C). Beginning at approximately stage 13, weak β-galactosidase accumulation in the maxillary lobes was observed in transgenic animals containing the 10.6+HZR transgene. By stage 14, significant levels of lacZ expression were seen in the maxillary lobes and low levels were detected in the labial lobes in these animals (Fig. 6C). Finally, expression from the 13.0+HZR construct was detected in the pharyngeal region and in a few muscle cells in the anterior region of the embryo, but only very late (stage 16) in embryogenesis (Fig. 6D). This pattern is unrelated to the endogenous domain of pb expression, implying that the 13.0 kb EcoRI fragment does not contain pb enhancer sequences that can direct expression through the hsp70 promoter.

A 600 bp region functions as the pb promoter in combination with a 2.1 kb pb enhancer

To define sequences required for pb promoter function, we engineered a pb-lacZ fusion gene (pbZR) that contains a 0.6 kb pb DNA sequence (−98 bp to +503 bp of the transcription start site) fused upstream of a lacZ reporter gene. The pb sequences provide the transcription start site to pbZR; the lacZ gene supplies the translation initiation sequences. Transgenic lines containing pbZR did not exhibit β-galactosidase staining in pb’s embryonic or imaginal expression domains (compare Fig. 7A to B, E to F). However, when a 2.1 kb fragment (Fig. 1B) from the intron two conserved region of pb was inserted upstream of the promoter in pbZR (2.1+pbZR), the reporter gene was able to confer a pb pattern in embryos (compare Fig. 7A to C) and in labial discs (compare Fig. 7E to G). These results demonstrate that a 0.6 kb pb fragment containing only 98 bases of upstream DNA can function as a promoter in com-

Fig. 7. The second intron conserved region acts in combination with a 600 bp pb promoter to generate a pb expression pattern in embryos and third instar labial discs. (A-D) Horizontal views of stage 13 transgenic embryos stained with an antibody that recognizes β-galactosidase. Anterior halves of the animals are directed up. (A) The wild-type pattern of PB accumulation represented in a pbG1-intron transgenic embryo. (B) A transformant embryo harboring the pbZR transgene does not accumulate β-galactosidase. In contrast, (C) 2.1+pbZR and (D) 1.9+2.1+pbZR transformants exhibit a pb-like lacZ expression pattern. (E-G) Third instar labial discs from transformant animals stained with anti-β-galactosidase antisera. (E) A wild-type pattern of lacZ expression is displayed in pbG1-intron transformant discs. (F) β-galactosidase accumulation is not detected in pbZR transformant labial discs, whereas (G) 2.1+pbZR transformants exhibit significant lacZ expression.
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**A 500 bp region containing a discrete 32 bp labial disc enhancer element confers a pb expression pattern**

Two subregions of the 2.1 kb second intron regulatory region (0.8 kb and 0.5 kb) (Fig. 1B) were tested for enhancer activity in embryos and imaginal tissues. Transforms carrying 0.5+pbZR displayed β-galactosidase accumulation in an embryonic and imaginal pattern similar to that observed in the 2.1+pbZR lines (Fig. 8C and not shown). The only observed difference in the pb-like expression patterns was the absence of staining in the triangular belt of ventral mesodermal cells just anterior to the maxillary segment in 0.5+pbZR transformant embryos. Weak ectopic dorsal ridge β-galactosidase accumulation was also observed in most 0.5+pbZR transformant embryos. In contrast, the 2.1+pbZR transgene did not generate a reproducible pattern of this ectopic expression, suggesting that sequences absent from the 0.5 kb fragment are needed to prevent expression in the dorsal ridge.

Transformants containing the 0.8 kb fragment linked to pbZR showed no β-galactosidase expression in early embryogenesis (not shown). Beginning at approximately stage 14, however, very low levels of lacZ expression were detected in the labial lobes, and this weak staining persisted until the end of embryogenesis. Protein accumulation in the maxillary lobes was not detected in these animals. Third instar labial discs of 0.8+pbZR transformants showed levels of β-galactosidase accumulation comparable to that of 0.5+pbZR transformant discs (Fig. 8C,D). In both cases, however, some variability in protein levels was observed from animal to animal (compare Fig. 8F to C,D). The variability suggests that these fragments are lacking a full complement of the endogenous pb labial disc enhancer elements.

The observation that the 0.8 kb and 0.5 kb regions confer labial disc expression establishes that labial disc enhancer sequences are contained in each fragment. To begin defining these labial disc regulatory elements, we compared the sequences of the two fragments. Interestingly, a 32 bp motif (ANCTGTATTTNGNTCATTTGTNAATNT; Randazzoz et al., 1991) is contained in both the 0.5 kb and 0.8 kb regions. To test if labial disc enhancers are contained within this sequence, we cloned a synthetic oligonucleotide (r1r2) containing the 32 bp motif into pbZR. The oligonucleotide was designed from one of the endogenous second intron sequence motifs, and contains 17 bp of flanking DNA that is unique to the 0.5 kb region (13 bp and 4 bp on the 5' and 3' ends, respectively). Transformants carrying P-elements that contain two copies of r1r2+ pbZR did not exhibit a reproducible pb-like expression pattern in embryos. However, one out of four lines examined showed extremely weak late protein accumulation (stages 14-16) in the labial lobes. In some lines, lacZ...
expression was observed in various regions of the embryo, but the pattern varied from line to line (not shown). In contrast, third instar labial discs from these transgenic animals had levels of β-galactosidase comparable to that observed in trans- formant 0.5+pbZR and 0.8+pbZR larvae (Fig. 8E). These results demonstrate that a 49 bp region of pb’s second intron contains labial disc enhancer sequences. Moreover, since the 32 bp motif is common to both the 0.5 kb and the 0.8 kb fragments, it is likely to be sufficient for generating this enhancer activity.

**pb enhancers do not function properly with a heterologous promoter**

The 5.3 kb fragment does not confer a reliable pb-like expression pattern when linked to an hsp70 promoter-lacZ fusion gene, but a subsequence of this region (2.1 kb, Fig. 1B) confers a pb pattern when in combination with a pb promoter-lacZ fusion gene (pbZR). These results suggest that the second intron regulatory DNA requires sequences present in the pb promoter to function. To begin understanding the nature of this pb promoter-regulatory DNA interaction, we cloned the 2.1 kb subfragment upstream of an hsp70 promoter fused to the lacZ gene. Expression of β-galactosidase produced from this transgene (2.1+HZR) was compared to that of the pb promoter driven reporter gene (2.1+pbZR). Transforms harboring 2.1+HZR exhibited lacZ expression in cells located in pb’s domain of expression; however, the accumulation was patchy in both embryos and labial discs (Fig. 9B,D). There were regions of the gnathal lobes and the labial discs where staining was not detected (Fig. 9B,D). Patchy staining was not observed in a majority (6/8) of the 2.1+pbZR trans- formant animals (Fig. 9A,C). Ectopic accumulation of PB in the gastric caeca was detected in all of the 2.1+HZR transgenic lines, and many of the lines displayed expression patterns in other inappropriate locations depending on the differing genomic positions of the P-element insertions (not shown). In contrast, only a few transformant lines (2/8) harboring 2.1+pbZR showed ectopic embryonic expression, suggesting that the 2.1+HZR transgene is more sensitive to position effects from flanking genomic DNA than the 2.1+pbZR construct.

Next, we compared the function of a pb minigene that utilizes the pb promoter (∆Kpn) to a similar minigene that operates with an hsp70 promoter (UASpb). The hsp70 promoter in this minigene is analogous to the one contained in HZR; both constructs lack the heat-shock element consensus sequences in their promoters. The only other difference between these minigenes is the microexon that is absent in the hsp70-minigene, but present in the pb-minigene. Since the microexon is dispensable for the homeotic function of pb, its absence in UASpb is not likely to affect the function of this minigene. Both minigenes were crossed into a pb null background in order to assay the effects of transgenic expression on the identity of the adult mouthparts. The transgene that utilizes the pb promoter almost completely rescues the pb homeotic phenotype (compare Fig. 10C to A,B). However, these animals often have small patches of aristal tissues present on the lateral aspects of the labial palps (Fig. 10C), probably due to the absence of an upstream general pb enhancer. In contrast, the hsp70-pb minigene transformant animals showed only weak or partial rescue; they displayed some of the characteris tics of the pb null homeotic labial-to-leg transformation. Partial labial palps formed in these animals as evidenced by the presence of some pseudotracheal rows, but distinctive prothoracic leg tissues were present on the labial palps (Fig. 10D,E). Thus, the 2.1 kb pb intronic fragment does not function properly with the hsp70 promoter to specify the identity of the adult mouthpart structures.

![Fig. 9. Second intron pb enhancers do not act properly when linked to an hsp70 promoter; an en promoter-specific enhancer functions with the pb promoter. Transformant animals are stained with anti-β-galactosidase antisera. Embryos are oriented with their anterior ends to the left. Compare the lacZ expression in the maxillary (Mx) and labial (Lb) lobes in a (A) 2.1+pbZR transformant embryo to that of a (B) 2.1+HZR animal; there are regions where β-galactosidase does not accumulate in the 2.1+HZR embryo (arrow). (C) A third instar labial disc from a 2.1+pbZR transgenic animal shows significant lacZ expression. In contrast, (D) a 2.1+HZR transformant labial disc exhibits weak patchy β-galactosidase accumulation. (E) An en enhancer confers on en-like stripe expression pattern when linked to the pb promoter, as shown in an en+pbZR transformant embryo.](image)
Can the promoter sequences of *pb* interact with enhancers from other genes that display similar promoter-specific interactions? We cloned an *en* enhancer region upstream of the *pb* promoter in pbZR. This first intron fragment generates an *en*-like stripe expression pattern in embryos when positioned upstream of an *en* promoter, but not when 5′ to an hsp70 promoter (Kassis, 1990). Transformant embryos harboring the *en* DNA linked to the *pb* promoter-*lacZ* reporter gene express β-galactosidase in *en*-like stripes in embryos (Fig. 9E), implying that *pb* promoter sequences can interact properly with promoter-specific enhancers from other genes.

**DISCUSSION**

**Function of the conserved intron two sequences in *pb* regulation**

We demonstrate that a conserved region within *pb*’s second intron (Fig. 11) functions to specify the identity of the adult mouthparts. Previous sequence analysis uncovered a highly conserved 0.7 kb region in the second intron of *pb* (Randazzo et al., 1991). This sequence contains numerous DNA stretches that are similar to the corresponding regions of *D. pseudoobscura*, including blocks of sequence identity ranging in size from 18 to 59 bases. To test if this intronic conserved region is important to the function of *pb*, two minigenes (ΔXho and ΔPvu) with deletions in these sequences were analyzed. Our results demonstrate that this region is essential for *pb* function. Perturbations in this region result in the formation of aberrant adult mouthparts that are characteristic of *pb* hypomorphic or amorphic mutations. Additionally, the abilities of the two minigenes to rescue the *pb* null phenotype were discernibly different from each other. The mouthparts of P{w+, ΔXho}; *pb*/*pb* animals ranged from wild-type to hypomorphic phenotypes. In contrast, the mouthparts of P{w+, ΔPvu}; *pb*/*pb* animals ranged from hypomorphic to amorphic phe-
Fig. 11. Summary of the cis-regulatory regions in the pb locus. The pb locus is depicted as in Fig. 1. The solid lines underneath the pb molecular map indicate the locations of cis-acting regulatory sequences. The dotted lines indicate the intron two sequences present in the ΔXho and ΔPvu minigenes. The sequences of the repeated elements are as follows: \( r_1 = \text{ANCCTTATTTNNGTTCATGGTNNAAATNTT} \); \( r_2 = \text{TCATTTG} \); \( r_3 = \text{TTTTCCTGTCC} \); \( r_4 = \text{GTTGC} \); \( r_5 = \text{TTGCAA} \) (Randazzo et al., 1991). All units are in kilobases unless otherwise indicated.

...notypes. These results indicate that the 5' most 597 bases of the intron two conserved region (deleted in ΔPvu) are essential for pb function, but may not be completely sufficient, since partial rescue of the homeotic phenotype is occasionally achieved when this sequence is deleted. In addition, this region is necessary both for significant PB accumulation levels in the labial discs and for normal levels of embryonic pb expression.

**Function of pb's upstream DNA**

Sequences upstream of −98 bp from the transcription start site are not required for activation of pb in its wild-type expression domain. Transforms containing 5' deletion minigenes (ΔKpn and ΔSal) in a pb null background accumulate PB in the labial and maxillary lobes of the embryo, in cells of embryonic mesoderm and in the labial discs of third instar larvae. The pb gene is the only homeotic gene tested to date that is capable of almost completely rescuing an adult homeotic larva.

While ectopic expression of pb in the leg discs has no discernible effects (at the levels conferred by the minigenes), inappropriate PB accumulation in the eye-antennal discs causes an abnormal (thickened) adult aristae. This gain-of-function phenotype has been interpreted to be a transformation toward maxillary identity (Cribbs et al., 1995).

A region important for thoracic repression is located between −1.6 kb and −0.098 kb of the pb transcription start site (Fig. 11) because ΔKpn transgenic animals, but not a majority of ΔSal transforms, direct significant PB accumulation in the leg discs (see also Kapoun and Kaufman, 1995). It is likely that there are several negative regulatory regions located in the upstream DNA of pb, some of which may be redundant. For example, sequences upstream of −1.6 kb could repress pb in the thorax in the absence of the −1.6 kb to −0.098 kb region, or sequences located downstream of −1.6 kb may act in combination with elements further upstream. Multiple regions capable of gene repression have been observed at homeotic loci such as Ubx and Abdominal-B (Abd-B), and they appear to act together to restrict these gene’s expression patterns to their wild-type domains (Busturia and Bienz, 1993; Muller and Bienz, 1991). Further investigations will define the sequences and mechanisms required for the negative regulation of pb.

**Discrete promoter and enhancer regions defined at the pb locus**

By investigating the expression patterns generated from various pb DNA-lacZ fusion genes, we have identified several discrete cis-acting sequences that are important for the regulation of pb (Fig. 11). The basal pb promoter was defined as a 600 bp region that includes DNA sequences from −98 bp to...
+503 bp of the transcription start site. A 2.1 kb second intron conserved region contains regulatory DNA that can generate a pb expression pattern when combined with the basal pb promoter. Furthermore, a 0.5 kb subregion of this intronic sequence retains the same spatial information as the 2.1 kb fragment, since the pb-like expression pattern produced from the two fragments are analogous. The 0.5 kb fragment, however, does not contain all of the pb enhancer elements present in the 2.1 kb sequence; a 0.8 kb stretch of DNA that lies just downstream of the 0.5 kb region (in the endogenous pb locus) confers labial disc and weak embryonic labial lobe expression.

What is the functional significance of these fragments to the pb gene? If the 0.5 kb fragment contains all the essential pb pattern enhancers, it should rescue the pb homeotic labial palp-to-leg transformation when linked to a minigene. The AΔXho minigene that contains this region, but lacks the downstream 0.8 kb sequence, does not completely rescue the pb homeotic phenotype. However, the SPL minigene that contains the 0.5 kb and 0.8 kb sequences completely rescues the pb null phenotype, suggesting that both regions contain enhancers important for pb function. Sequence comparison between the two fragments revealed the presence of a 32 bp motif, r1r2 (Fig. 11), that is common to both regions (this motif was first reported in Randazzo et al., 1991). No other significant sequence similarities exist between the two regions, implying that this 32 bp motif is an important regulatory element. Our results demonstrate that two copies of a synthetic oligonucleotide containing r1r2 confers expression in labial discs when linked to a pb promoter-lacZ fusion gene. Therefore, r1r2 appears to function as a discrete pb enhancer. Interestingly, a 7 bp element (r2) that is repeated three times in pb’s intron two conserved region exists within the 32 bp sequence: once in each of the two 32 bp motifs and one time in other conserved DNA (Fig. 11). Three other short repeated motifs (r3, r4, r5) are also present in the 0.8 kb fragment (Fig. 11) (Randazzo et al., 1991). The reason the minigene containing the 0.5 kb fragment without the 0.8 kb stretch (ΔXho) is not sufficient to rescue the homeotic pb phenotype may be that it lacks another copy of the 32 bp motif and/or the other repeated elements (contained in the 0.8 kb fragment). The motifs present in the second intron regulatory region of pb probably contain binding sites for trans-acting regulators of pb. Several ATTA core consensus binding sites for Drosophila homeodomain-containing proteins (Biggin and Tijian, 1989; Regulski et al., 1991) are located in the 0.5 kb and 0.8 kb regions. Further investigations will be required to test the significance of these elements to pb function and to search for the trans-acting factors that interact with the repeated motifs.

**pb has redundant and cryptic enhancers**

A 10.6 kb fragment of pb’s large intronic region (Fig. 11) directs the expression of an hsp70-lacZ fusion gene in a pb-like pattern in the embryonic maxillary and labial lobes. Since this region is missing from pb minigenes that rescue the homeotic phenotype, the enhancers contained within the 10.6 kb fragment are redundant and not essential for pb function. Why might redundant enhancers exist? It is possible that these regions guarantee stability to the regulatory mechanisms of genes. Although pb minigenes rescue the homeotic phenotype of pb null animals, there is variability in expression among the rescued animals. A similar observation was made for Ubx minigenes (Castelli-Gair et al., 1992). Enhancers not present in the pb and Ubx minigenes may be needed to ensure consistent transgenic expression. Redundant enhancers may be particularly important for assuring proper homeotic gene expression, since loss of these gene products results in severe consequences to the fly (Kaufman et al., 1990; Duncan, 1987). Interestingly, some regions of the intron that contains pb’s redundant elements appear to be conserved between D. melanogaster and D. pseudoobscura (A. Aplin, personal communication), suggesting that they are important to pb gene regulation.

Cryptic enhancer sequences were found in a second fragment (13 kb) of pb’s large intronic region (Fig. 11). When this sequence is positioned upstream of an hsp70-lacZ reporter gene, β-galactosidase accumulates in a pattern that does not overlap with the pb expression pattern. Cryptic enhancers have also been found to reside in the Scr locus (Gindhart et al., 1995). The mechanism of silencing these regulatory regions in the endogenous loci is presently unknown. Since the distance between the cryptic enhancers and the pb promoter is much greater at the endogenous locus (over 10 kb) as compared to the fusion constructs, spacing may be involved in derepression of the enhancers. It is also possible that the pb promoter and these intronic elements are incompatible and that the resident promoter is incapable of responding to their presence.

**Basal pb promoter essential for function**

We found that the pb enhancers contained in the 2.1 kb second intron fragment do not function properly with a heterologous hsp70 promoter. By comparing the β-galactosidase accumulation generated from reporter genes containing this fragment linked to an hsp70 or a pb promoter, it was found that a patchy staining pattern was conferred by the hsp70 promoter fusion and not by the chimeric pb gene. Moreover, a pb minigene linked to the hsp70 promoter produced a partial labial palp-to-leg transformation, while a pb promoter driven minigene conferred wild-type labial palps.

It is often assumed that all enhancers can function with heterologous promoters (O’Kane and Gerhing, 1987; Wilson et al., 1989). Why, then, do the second intron pb enhancers function improperly in combination with an hsp70 promoter? One possible explanation is that the pb promoter fragment used here contains enhancer elements required for proper pb expression. This hypothesis is unlikely, since β-galactosidase is not detected in transformants harboring the pb promoter-lacZ construct. Another explanation could be that the protein-bound enhancer-promoter interaction is inherently different at the two promoters. It is likely that the particular combination of proteins interacting with a promoter is important; for instance, the hsp70 promoter may lack binding sites for certain factors that the pb enhancer-bound trans-acting proteins require. Consistent with this suggestion is the observation that some basal transcription factors may be promoter-specific (Parvin et al., 1992). Additional mechanisms of promoter-specificity are likely to occur in vivo. It is possible that boundary elements (Eissenberg and Elgin, 1991) or specific repressors keep enhancers of one gene from influencing the promoter of a neighboring gene.

As an initial step towards understanding the promoter-specificity of the pb enhancers, we tested whether the pb basal
promoter can function with a promoter-specific enhancer from *engrailed*. We show that an *en* stripe enhancer can function properly with the basal *pb* promoter. These results suggest that, at least for some enhancers, a general mechanism of promoter specificity may be employed. Interestingly, neither the *en* nor the *pb* promoters contain TATA boxes, but they do contain initiator elements (reviewed in Smale and Baltimore, 1989). Additionally, sequence comparison between the two promoters revealed a 10 bp sequence of which 8 bases are identical (TNNCAGTTCA beginning at −18 bp for *pb* and −63 bp for *en*). It is possible that similar *trans-acting* factors for *pb* and *en* act through the initiator sequence and/or other regions that are common to each promoter. Further experiments will be required to determine the significance of the 10 bp motif and the initiator to the promoter-specific interactions observed at these loci. These studies and others will facilitate a deeper understanding of the regulation of *pb* and may provide insights into the mechanisms of promoter-specificity.

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