Ectopic Expression of the Drosophila Homeotic Gene *proboscipedia* Under *Antennapedia* P1 Control Causes Dominant Thoracic Defects

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ABSTRACT

A deletion mutation in the Antennapedia Complex of *Drosophila melanogaster*, *Df*(3R)*SCB*^{XL2}, induces both dominant and recessive loss-of-function phenotypes. The deletion is associated with diminished function of *proboscipedia* (*pb*), a homeotic gene required for mouthparts formation. *Df*(3R)*SCB*^{XL2} also has associated dominant thoracic defects related to diminished expression of the homeotic *Antennapedia* (*Antp*) gene copy on the homologous chromosome. This is shown to be a consequence of ectopic *pb* expression in the thorax. Newly juxtaposed *Antp* sequences provide the *pb* gene on the deletion bearing chromosome with a second promoter, *Antp* P1, in addition to its own. Ectopic *pb* protein expression occurs under *Antp* P1 control, by alternate splicing, and results in diminished accumulation of *Antp* protein in the imaginal disc cells where *Antp* P1 is normally expressed. The analysis of this mutant chromosome thus demonstrates that *pb* protein is capable of participating in the negative regulation of a more posteriorly expressed homeotic gene, as well as serving a homeotic "selector" function in the head.

THE fruit fly *Drosophila melanogaster* is composed of a series of related but distinct segments or parasegments. These reiterated pattern elements, the outcome of a series of tightly coupled events in early embryogenesis [see AKAM (1987) and INGHAM (1988) for reviews], define the domains of action of the homeotic genes. The diversification of structures distinguishing one segment from another is believed to reflect homeotic gene regulatory functions directed toward "downstream" or "realisator" genes (GARCIA-BELLIDO 1977; LEWIS 1978; GOULD *et al.* 1990). In Drosophila there are two major clusters of homeotic genes, the Bithorax Complex (BX-C) and the Antennapedia Complex (ANT-C), which together control the segmental identity of most of the fly body [for reviews, see PEIFER, KARCH and BENDER (1987), DUNCAN (1987), MAHAFFEY and KAUFMAN (1988), and KAUFMAN, SEEGER and OLSEN (1990)]. The genes of the BX-C are required for specification of posterior thoracic and abdominal segments, while those of the ANT-C are required in the anterior thorax and the head. In the trunk, homeotic genes are expressed in overlapping domains and may interact among themselves at the transcriptional level to alter one another's pattern of expression (STRUHL 1982; HAFEN, LEVINE and GEHRING 1984; STRUHL and WHITE 1985; CARROLL *et al.* 1986). In contrast in the head it appears that the homeotic genes are expressed in nonoverlapping regions and that these exclusive domains do not result from negative cross regulatory interactions (MAHAFFEY, DIEDERICH and KAUFMAN 1989; KAUFMAN, SEEGER and OLSEN 1990).

The homeotic *proboscipedia* (*pb*) gene of the ANT-C is required to make adult mouthparts. In its absence a transformation of the labial palps to prothoracic legs occurs (BRIDGES and DOBZHANSKY 1933; KAUFMAN 1978; PULTZ *et al.* 1988). A distinctly different adult transformation of the labial palps to antennal aristae is seen in mutants which retain partial *pb* function. Consistent with the adult mutant phenotype, larval expression of *pb* protein is detected in cells of the labial imaginal discs which form the adult labium (RANDAZZO, CRIBBS and KAUFMAN 1991). Embryonic expression of *pb* protein is also observed in the labial and maxillary lobes of the gnathocephalon. These two segments are the likely site of origin of the progenitor cells of the adult head which are affected in *pb*$^-$ animals (PULTZ *et al.* 1988; JÜRGENS *et al.* 1986). However, no embryonic or larval cuticular phenotype has been associated with the *pb*$^-$ condition (PULTZ *et al.* 1988).

The *Antennapedia* (*Antp*) gene of the ANT-C, in contrast, is required for mesothoracic identity both in embryonic/larval and adult development (WAKIMOTO and KAUFMAN 1981; STRUHL 1981; ABBOTT and KAUFMAN 1986; MARTINEZ-ARIAS 1986). The Antp protein is apparently sufficient to specify mesothoracic

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identity; a conclusion drawn from genetic and molecular analyses of dominant gain-of-function *Antp* mutations which transform head tissues to mesothorax (*e.g.*, see DENELL 1973; HAZELRIGG and KAUFMAN 1983; FRISCHER, HAGEN and GARBER 1986; SCHNEUWLY, KUROIWA and GEHRING 1987; SCHNEUWLY, KLEMENZ and GEHRING 1987). *Antp* also contributes to adult development of the prothorax, notably the humeral callus and the mesonotum (ABBOTT and KAUFMAN 1986). Two distinct promoters, P1 and P2, direct *Antp* expression (LAUGHON *et al.* 1986; SCHNEUWLY *et al.* 1986; STROEHER, JORGENSON and GARBER 1986; JORGENSON and GARBER 1987). Whereas P2 is necessary from embryogenesis onwards and is required in the ventral thorax, the P1 promoter is required for adult viability and for proper development of dorsal thoracic structures (WAKIMOTO and KAUFMAN 1981; STRUHL 1981; ABBOTT and KAUFMAN 1986).

Loci homologous to the homeotic genes of the BX-C and the ANT-C are united in a single complex in various species including the flour beetle *Tribolium castaneum*, mice and humans (BEEMAN 1987; BEEMAN *et al.* 1989; DUBOULE and DOLLÉ 1989; GRAHAM, PAPALOPULU and KRUMLAUF 1989; ACAMPORA *et al.* 1989; KAPPEN, SCHUGHART and RUDDLE 1989). The protein products of the homeotic genes are seen to be potent developmental effectors through their known or inferred activities as transcriptional regulators. The extraordinary conservation of the homeotic gene complexes seems likely to reflect a necessity to maintain a fine balance of the homeotic genes' functions and hence their spatial, temporal and quantitative expression for proper development. A knowledge of what interactions may comprise homeotic gene regulation, and how such interactions may be modulated, is thus central to our understanding of developmental control.

This paper deals with the regulation of *Antp* expression by the *pb* locus of the ANT-C. We have analyzed a deficiency mutation in which the *Antp* P1 promoter is placed upstream of the homeobox genes *pb* and *z2*. This chromosomal rearrangement generates overlapping chimeric transcription units in which two homeotic gene promoters, *pb* and *Antp* P1, respectively direct normal *pb* expression in the head as well as ectopic expression in a subset of the *Antp* domain in the thorax. The misdirected expression of *pb* protein is associated with a dominant *Antp* loss-of-function phenotype caused by a loss of *Antp* expression. Thus *pb* protein is apparently capable of modulating *Antp* expression, in addition to serving as a homeotic selector in its normal domain of expression.

MATERIALS AND METHODS

Stocks and culture conditions: Flies were raised on standard cornmeal-molasses-brewer's yeast medium at 25° unless otherwise noted. Mutant strains and nomenclature are as in LINDSLEY and ZIMM (1992). The *Df*(3R)*SCB*^{XL2} chromosome was supplied by C. NÜSSLEIN-VOLHARD and was recovered in her laboratory as a deletion of the *bicoid* (*bcd*) locus of the ANT-C. This deletion also removes the *Antp*, *fushi tarazu* (*ftz*), *Sex combs reduced* (*Scr*), *Deformed* (*Dfd*), *Amalgam* (*AMA*), and *zerknüllt* (*zen*) loci as well as showing a partial and variegating loss-of-function phenotype for the *pb* locus. In addition to the recessive lethal effects of the deletion it is associated with a dominant phenotype most readily seen as a deletion of bristles and cuticle in the region of the humeral callus. This dominant effect closely resembles recessive loss-of-function phenotypes associated with certain alleles of the *Antp* locus (ABBOTT and KAUFMAN 1986).

Genetic reversion analysis: Males of genotype *Df*(3R)*SCB*^{XL2}/*TM3,Sb* were placed overnight (about 16 hr) in vials containing tissues wetted with 10% sucrose solution/6 mM diepoxybutane (DEB) (OLSEN and GREEN 1982), or with ethyl methanesulfonate (EMS) using standard conditions (BACHER and LEWIS 1968). Males were then removed to fresh vials, allowed several hours to recover, then mated with virgin *pb*^{f13}*p*^{p} females. The weak *pb*^{13} allele in combination with *Df*(3R)*SCB*^{XL2} only rarely gives a phenotype which is visible under a dissecting microscope. F_1 progeny carrying the deficiency chromosome (*i.e.*, *Sb*$^{+}$) were screened for reversion of the humeral defects, and for labial defects indicating the introduction of a *pb* lesion on the *Df*(3R)*SCB*^{XL2} chromosome. The humeral defects associated with this chromosome were thought to be caused by a negative regulation of the *Antp* locus resident on the homologous chromosome. Thus "reversion" of the dominant phenotype should be associated with the inactivation of the entity on the deletion chromosome causing the shut down. Candidates expressing either phenotype were recrossed to *pb*^{13} *p*^{p} to confirm the introduction of a new *pb* lesion and/or a reversion and then balanced over *TM3,Sb*. The DEB-induced alleles were first tested for complementation of lethal *labial* mutations, the next most proximal member of the ANT-C, and stronger alleles of *pb*. The DEB induced alleles were also mapped by genomic Southern blots. The mutation frequency observed with DEB for *pb* was about 0.02% and EMS was similarly efficient; hence the expected frequency of chromosomes doubly hit at *pb* and any other second site should be on the order of 4×10^{-8}.

Gene dosage tests: To vary the dosage of *Antp*$^{+}$, an additional copy of the ANT-C was introduced via the *Y* chromosome using *Dp*(Y;3)*ANT-C*$^{+}$ (a *Y* chromosome-linked translocation of the entire ANT-C, duplicated for polytene region 83DE-84D; obtained by R. DENELL, Kansas State University). Males carrying the *Dp*(Y;3)*ANT-C*$^{+}$ and balancer third chromosomes were crossed to *Df*(3R)*SCB*^{XL2}/*TM3,Sb* females. The male progeny of this cross carrying the duplication and the deletion (*X*/*Dp*(Y;3)*ANT-C*$^{+}$;*Df*(3R)*SCB*^{XL2}/*TM3*) were collected after scoring (see below) and crossed to *Antp*^{w10}/*TM3,Sb* females. Males of the genotype *Dp*(Y;3)*ANT-C*$^{+}$; *Df*(3R)*SCB*^{XL2}/*ANT-C*$^{+}$ (with two copies of *Antp*$^{+}$) were compared to sibling females of genotype *Df*(3R)*SCB*^{XL2}/*ANT-C*$^{+}$ carrying a single copy of the ANT-C, or to *Dp*(Y;3)*Antp*$^{+}$; *Df*(3R)*SCB*^{XL2}/*Antp*^{w10} males with two intact copies of all ANT-C genes except *Antp*$^{+}$, which in this genotype is present in one copy on the *Y*-linked duplication.

Genomic Southern analyses: Genomic DNA was prepared as described by SCOTT *et al.* (1983). For genomic Southern blots, 5 μg of digested DNA were fractionated by size using electrophoresis through 0.8% agarose gels, partially depurinated in the gels by treatment with 0.1 M HCl, denatured with alkali/high salt, neutralized and transferred to Nytran (Schleicher and Schuell) filter membranes. DNA

probes were prepared by oligonucleotide priming of purified restriction fragments (FEINBERG and VOGELSTEIN 1983) or by nick translation of recombinant λ phage. Hybridizations were in high salt (MANIATIS, FRITSCH and SAMBROOK 1982).

Cloning of the fusion point of the deficiency: The interpretation of the chromosome structure obtained by genomic Southern mapping was confirmed by cloning the ~15-kb fusion *Bam*HI restriction fragment (seen in Figure 3, probes A and B). Genomic DNA from *Df*(3R)*SCB*^{XL2}/*TM3,Sb* adults was digested with *Bam*HI, ligated into the bacteriophage λ vector EMBL3, and the fusion fragment was identified by probing duplicate filters with sequences from the first exon of *Antp* (4.0-kb *Eco*RI fragment located at +200 kb; SCOTT *et al.* 1983) and the 3.9-kb *Eco*RI fragment which contains most of *z2* exon 2 (PULTZ *et al.* 1988). Restriction mapping places the proximal breakpoint within a 0.5-kb interval about 1 kb 5′ of the *z2* cap site (RUSHLOW *et al.* 1987). The breakpoint in the P1 region was positioned with a corresponding precision.

Northern analyses: RNA for Northern and S1 nuclease protection analyses were prepared by the methods of SPRADLING and MAHOWALD (1979). Oligo-dT cellulose chromatography, glyoxal gel electrophoresis and blotting of RNA to Nytran membranes were done as described by MANIATIS, FRITSCH and SAMBROOK (1982) and HAGENBÜCHLE *et al.* (1984).

S1 protection analyses: Nuclease S1 protection analyses were done as described in CRIBBS *et al.* (1992). The specific probes used here are indicated in the corresponding figure legends.

P1/z2: To test for "hybrid" mRNAs fused between the first exon (E1) of *Antp* P1 and the second exon (E2) of *z2*, a plasmid was constructed which contained *Antp* P1 sequences from the *Xba*I site at position 710 to the *Bam*HI site at position 2137 in E1 (coordinates as in LAUGHON *et al.* 1986) ligated to the 5′ end of a 200-bp *Bgl*II/*Eco*RI fragment from exon 2 of *z2* (spans amino acids 124–188, coordinates as in RUSHLOW *et al.* 1987) cloned in the polylinker of pGEM2 (ProMega). The 260-bp *Xho*I/*Eco*RI probe extends from the *Xho*I site in *Antp* P1 sequences (position 2071; LAUGHON *et al.* 1986) to the *Eco*RI site in *z2* (above). This chimeric fragment was end-labeled with [α-^{32}P]dATP plus [α-^{32}P]dGTP using the Klenow fragment of DNA polymerase I, and the strands were separated on a preparative 5% polyacrylamide/0.1% bis-acrylamide gel (as in MANIATIS, FRITSCH and SAMBROOK 1982). To minimize the background protection due to contaminating noncoding strand, the purified strands were self-annealed overnight at 65° in 1 × SSC, then repurified as before to separate double-stranded sequences from contaminating noncoding strand. The probe (20,000 Cerenkov cpm) was hybridized overnight at 44° (50% formamide, 0.8 M NaCl, 20 mM HEPES, pH 6.4) to 20 μg RNA, then digested with S1 nuclease (200 units/ml, Boehringer Mannheim), as in HAGENBÜCHLE *et al.* (1984) and FAVALORO, TREISMAN and KAMEN (1980). Protection products were separated by size using electrophoresis on 6% polyacrylamide sequencing gels, then detected by autoradiography.

P1/pb: To test alternate splicing from the *Df*(3R)*SCB*^{XL2} chromosome between *Antp* P1 E1 and *pb* E2, a plasmid was constructed (in the vector pGEM2) with *Antp* P1 sequences as above fused to a 1.55-kb *Bam*HI/*Eco*RI fragment extending from the middle of *pb* E2 into the second intron. The coding strand probe joins 65 nucleotides of Antp P1 sequence, beginning from the *Xho*I site at position 2071 (LAUGHON *et al.* 1986) to 735 nucleotides of *pb* sequence. An 800-bp *Xho*I fragment from the above chimera was isolated and 3′ end-labeled with [α-^{32}P]dATP plus [α-^{32}P]dGTP, and the strands separated by polyacrylamide gel electrophoresis (4% acrylamide, 0.08% bis-acrylamide, with Tris-borate buffer; as in MANIATIS, FRITSCH and SAMBROOK 1982). Each hybridization contained 80,000 Cerenkov cpm of single-stranded probe. Hybridizations and digestions with nuclease S1 were as described above.

Antibody staining: Embryos were prepared and stained as described in MAHAFFEY and KAUFMAN (1987) and PULTZ *et al.* (1988). The rabbit polyclonal antibody used in the experiments shown here was raised against a lacZ/*pb* exon9 fusion protein (GUO *et al.* 1984; CRIBBS *et al.* 1992), then purified by affinity chromatography on a column carrying trpE/E9 protein (as described by DRIEVER and NÜSSLEIN-VOLHARD 1988). Antibody staining of imaginal discs was as described in DIEDERICH, PATTATUCCI and KAUFMAN (1991).

RESULTS

The *Df*(3R)*SCB*^{XL2} chromosome is associated with both dominant and recessive defects: The mutant chromosome *Df*(3R)*SCB*^{XL2} (kindly provided by C. NÜSSLEIN-VOLHARD) carries a deletion removing most of the ANT-C. This chromosome fails to complement mutations in loci from *Antp* through *zerknüllt* (*zen*) (see Figure 3). It only partially complements null *pb* mutations, and confers a recessive variegating loss-of-function phenotype (PULTZ *et al.* 1988). The proximal breakpoint of the *Df*(3R)*SCB*^{XL2} deficiency was previously shown to fall near the 5′ end of the *pb* transcription unit. The variegating loss-of-function phenotype likely reflects defective transcriptional regulation of *pb*, due to removal of *cis*-regulatory elements and/or to position effects exerted by the newly juxtaposed sequences (PULTZ *et al.* 1988).

The dominant developmental defects associated with the *Df*(3R)*SCB*^{XL2} chromosome are the focus of this work. This chromosome is associated with defects in a number of structures dependent on *Antp* function (ABBOTT and KAUFMAN 1986). Defects of the humeral callus are observed with nearly full penetrance, notably the frequent malformation or absence of one or both of the large bristles normally present there (compare Figure 1, b to a). Additionally, the wings of animals heterozygous for the deficiency are often held abnormally, away from the body and drooping. This condition of the wings, as well as the humeral phenotype, can be further aggravated by the presence of the *TM6B* balancer chromosome carrying the *Humeral* (*Hu*) mutation of the *Antp* gene. The dominant phenotype of *Hu* (supernumerary bristles on the humeral callus) is enhanced when *Hu* is combined with loss-of-function *Antp* alleles. However the phenotype of *TM6B, Hu/Df*(3R)*SCB*^{XL2} flies is more extreme than that of *TM6B,Hu/Antp*$^{-}$. Other dominant effects associated with the *Df*(3R)*SCB*^{XL2} chromosome are pupal lethality, a failure of pupae to evert their anterior spiracles and adult leg defects [usually fusions of proximal structures (in ~1–10% of adults varying with genetic background)].

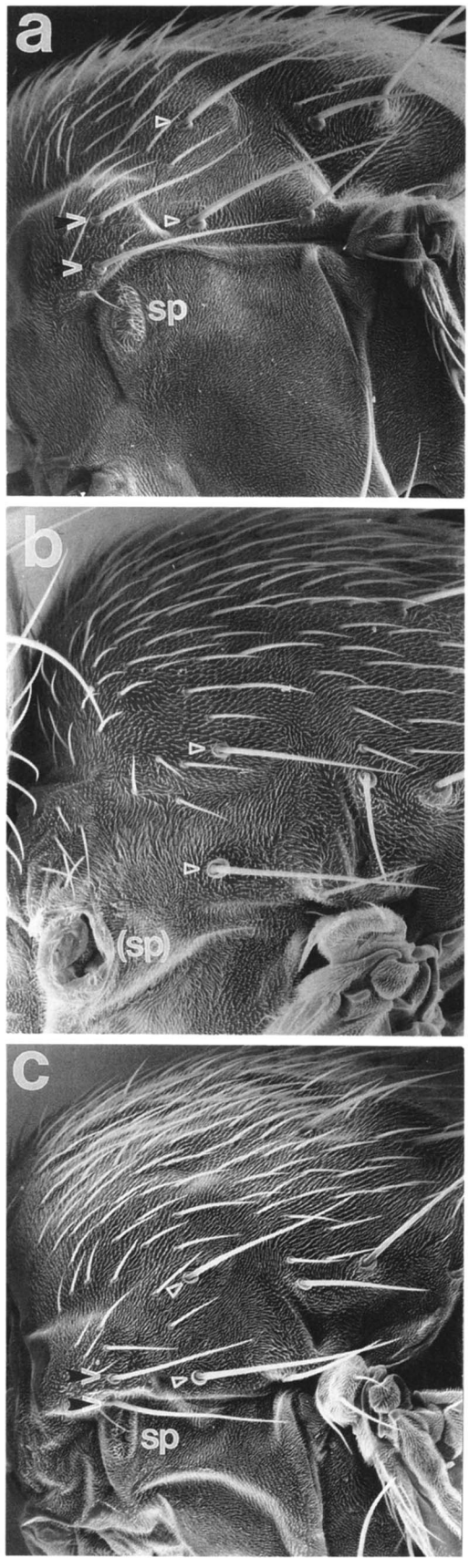

FIGURE 1.—Dominant phenotype associated with the *Df*(3R)*SCB*^{XL2} chromosome. (a) Shown is a portion of the dorsal thorax of a *pb*^{13} *p*^{p} fly, with open arrows indicating the two macrochaetae of the humeral callus (left), and closed arrows the anterior notopleural (right, bottom) and presutural (right, top) bristles. The spiracle (sp) is also indicated. This thoracic bristle pattern is the same as that seen in normal individuals. (b) Dorsal thorax of a *Df*(3R)*SCB*^{XL2}/*pb*^{13} *p*^{p} individual. The expressivity of the dominant defects seen for this fly is extreme, and approximates the *Antp* P1 null condition. Note that both of the macrochaetae of the humeral callus are absent (no arrows) and that the spiracular opening (sp) is malformed. (c) Dorsal thorax of revertant *Df*(3R)*SCB*^{XL2}-*Dr1*/*pb*^{13} *p*^{p}. *Dr1* has superimposed a deletion which removes *z2*, *pb* and *lab* from the original deficiency chromosome, and eliminates all aspects of the dominant defects, restoring the humeral callus (arrows, left) and the spiracle (sp) to wild type.

We emphasize that the formation of all of the aforementioned adult or pupal structures depends on functions of the *Antp* gene, and particularly on the P1 promoter (ABBOTT and KAUFMAN 1986). Thus the array of defects associated with *Df*(3R)*SCB*^{XL2} resembled a dominant, partial loss-of-function phenotype. Gene dosage experiments were done to address the nature of the effect exerted by the deficiency chromosome on *Antp* expression. Animals of the genotype *Df*(3R)*SCB*^{XL2}/+ possess a single functional copy of *Antp* and exhibit the dominant phenotypes described above. These dominant defects were largely ameliorated by the presence of a second copy of the entire ANT-C, but not if the entire ANT-C except for *Antp* was present in two copies. Therefore the dominant defects depend on the dosage of *Antp*$^{+}$ but not on the other loci in the ANT-C. This result suggests that the *Df*(3R)*SCB*^{XL2} chromosome is antimorphic with respect to *Antp* function, a result which was unexpected since *Df*(3R)*SCB*^{XL2} deletes all *Antp* protein coding sequences on this chromosome (see below).

pb protein is ectopically expressed in the thorax of embryos carrying the *Df*(3R)*SCB*^{XL2} chromosome: The unusual variegating *pb* loss-of-function phenotype and the dominant thoracic defects associated with *Df*(3R)*SCB*^{XL2} led us to examine *pb* expression in embryos carrying this chromosome, using antisera directed against pb protein. Ectopic accumulation of pb protein was observed in the thorax of embryos carrying the *Df*(3R)*SCB*^{XL2} chromosome, in a pattern that strongly resembles the normal *Antp* expression pattern (Figure 2). This ectopic expression is localized to the ventral nerve cord in posterior prothorax (pT1) through posterior metathorax (pT3) (Figure 2, D and A). Normal Antp protein expression in the thorax is first observed at the beginning of germ band retraction (~7 hr 30 min; CARROLL *et al.* 1986; WIRZ, FESSLER and GEHRING 1986) (stages as in CAMPOS-ORTEGA and HARTENSTEIN 1985) and precedes ectopic expression of pb protein by as much as 56 hr (data not shown). Still, the striking similarity of the *Antp* and *pb* patterns at approximately the end of ventral nerve cord contraction (stage 16; ~15 hr) indicates that *pb* expression has come at least partially

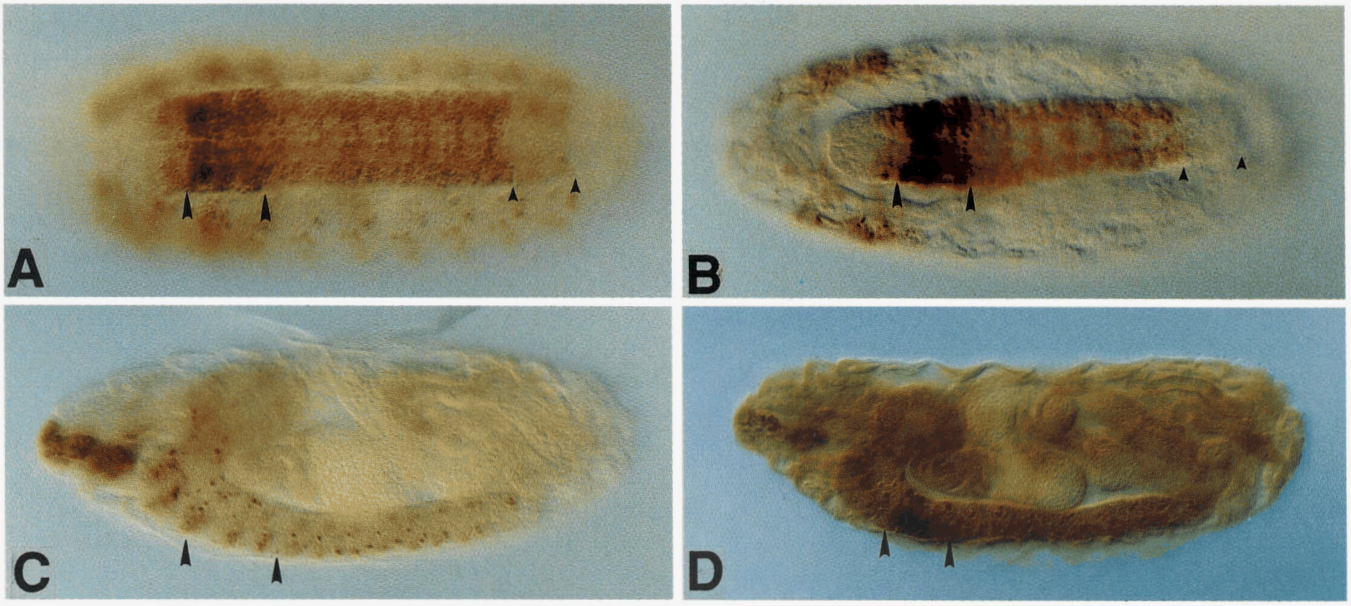

FIGURE 2.—Misexpression of pb protein in embryos carrying the *Df*(3R)*SCB*^{XL2} chromosome occurs in a pattern which closely approximates the normal *Antp* pattern. (A) Oregon R (wild type) stained with anti-*Antp* monoclonal; (B) *Df*(3R)*SCB*^{XL2}/*TM3,Sb* stained with anti-*Antp* monoclonal; (C) Oregon R (wild type) stained with anti-*pb* polyclonal; (D) *Df*(3R)*SCB*^{XL2}/*TM3,Sb* stained with anti-*pb* polyclonal. Panels A and B display embryos from the ventral side in order to more clearly show the accumulation of Antp protein in the ventral nerve chord (vnc). In panels C and D the animals are displayed laterally to show the anterior extent of *pb* expression and the more "patchy" accumulation of pb protein in the vnc. The apparent fading of Antp protein accumulation in the vnc of the animal in B relative to A is accounted for by the flatter preparation and thus uniform focal plane for the entire structure in A and the more advanced age of the animal in B. All embryos shown are about stage 16, or ~15 hr of development (CAMPOS-ORTEGA and HARTENSTEIN 1985), during the period of vnc contraction. The large arrows show the approximate position of parasegments 5 and 6 in the vnc, the position of predominant Antp protein accumulation in this structure. The small arrows show the position of the caudal neuromeres in which Antp protein is not detected. Note that pb protein is found in a pattern resembling Antp accumulation in deletion bearing animals (compare A and C with D) and that Antp protein accumulation in ps5,6 is not apparently affected by *pb* ectopic expression (compare A and B).

under the control of *Antp* regulatory elements.

Ectopic *pb* expression is causative for the dominant defects: Since the ectopic *pb* expression observed in *Df*(3R)*SCB*^{XL2}-bearing embryos resembles *Antp* expression (CARROLL *et al.* 1986; WIRZ, FESSLER and GEHRING 1986) we thought it likely that misexpression of *pb* could account for the dominant thoracic defects associated with the mutant chromosome. This possibility was tested by two types of genetic screens. First, we screened for mutagenized *Df*(3R)*SCB*^{XL2} chromosomes on which *pb* gene function had been lessened or abolished, as identified by a *pb* loss-of-function phenotype in the head. We then asked whether the dominant thoracic defects were simultaneously eliminated. Alternatively, we screened directly for reversion of the dominant defects of the humerus and then asked whether revertants were simultaneously *pb*$^{-}$. Similar results were obtained by both approaches, as the three *pb* mutants isolated were revertant for dominant thoracic defects, and the two revertants selected directly were *pb*$^{-}$.

Four DEB-induced *pb* mutations and one mutation induced by EMS were obtained from mutagenesis of the *Df*(3R)*SCB*^{XL2} chromosome; each mutation is simultaneously revertant for all aspects of the dominant pupal and adult defects. The first revertant isolated, *Dr1*, removes all remaining ANT-C sequences and is no longer associated with any dominant defects (Figure 1c). This demonstrates that the genetic cause of the defects resides within or very near the partially deleted ANT-C of the *SCB*^{XL2} chromosome. The two most informative revertant lines obtained were *Dr2* and *Er1*, induced with DEB and EMS, respectively. *Dr2* is phenotypically *pb*-null and has reverted to wild type as is the case for *Dr1*. However, it shows no discernible deletion in the *pb*/*z2* region and still produces detectable pb protein in the thorax of embryos (data not shown). The *Er1* lesion is associated with a hypomorphic allele of *pb* which also largely ameliorates the dominant effects. It should be noted however, that the "reversion" associated with this chromosome is less complete than that for the *pb*-null alleles, again supporting the hypothesis that *pb* is causative for the dominant defects. The number of simultaneously *pb*$^{-}$ - revertant lines isolated (most pertinently the *Dr2* and *Er1* lines, which are apparent point mutations; see also MATERIALS AND METHODS) makes it unlikely that a gene other than *pb* [for example the homeobox gene *z2*, which is not removed in the *Df*(3R)*SCB*^{XL2} chromosome] can be implicated as a cause of the dominant defects. We therefore conclude that ectopic expression of *pb* directed by the

Df(3R)*SCB*^{XL2} chromosome is causative for the observed thoracic defects. That is, in some fashion, pb protein expressed in the thoracic imaginal tissue interferes with the normal function of the *Antp* gene on the homologous chromosome. Interestingly, there are no detectable phenotypic consequences on larval morphology despite the clearly discernable ectopic accumulation of pb protein in the embryo (Figure 2).

Structure of the ANT-C on the *Df*(3R)*SCB*^{XL2} chromosome: In order to understand how ectopic *pb* expression causes the dominant *Antp* defects, the structure of this interval of the ANT-C was characterized. Genomic Southern mapping of the *Df*(3R)*SCB*^{XL2} chromosome was performed to localize the endpoints of the deficiency. The structure of the *Df*(3R)*SCB*^{XL2} chromosome is shown in Figure 3. The proximal breakpoint, localized previously to the 4.0-kb *Eco*RI fragment containing the *z2* cap site and homeobox (PULTZ *et al.* 1988; RUSHLOW *et al.* 1987) was confirmed. The distal break point resides about 8 kb proximal (3′) to the first P1 promoter-specific exon of *Antp* mRNA (Figure 3). The deficiency thus removes ~240 kb from the ANT-C, of which ~92 kb are from the *Antp* P1 transcription unit. The *pb* RNA-coding sequences and ~3 kb of 5′-flanking sequence remain intact in the deficiency chromosome (the structure of the *pb* gene is described elsewhere; CRIBBS *et al.* 1992). The region thus retains all *pb* and *z2* coding sequences plus the lone *Antp* P1 exon.

All three genes are normally transcribed from centromere-distal to proximal. The P1 promoter on the *Df*(3R)*SCB*^{XL2} chromosome has the potential to express the P1 first exon with its splice donor, and produce a transcript in which this exon can be joined with the downstream splice acceptor sites of the second exons of *z2* and/or *pb*. Though the predicted z2 protein is initiated in the first exon of this gene, the first three nucleotides of the second *z2* exon are an ATG triplet in frame with the *z2* homeodomain (RUSHLOW *et al.* 1987). Hence a P1/*z2* fusion mRNA could plausibly direct the synthesis of a shortened *z2* homeodomain protein under *Antp* control. However, the genetic reversion analysis described above indicates that such a protein, if it exists, is not the cause of the dominant phenotype associated with the deletion bearing chromosome. The predicted pb protein is initiated in the second *pb* exon (CRIBBS *et al.* 1992) and a fusion *Antp* P1/*pb* mRNA is expected to encode a normal pb protein that is subject to regulation by the remaining P1 promoter elements.

Overlapping transcription of *Antp*, *z2* and *pb* from the *Df*(3R)*SCB*^{XL2} chromosome: To examine the possibility that transcripts initiated from the *Antp* P1 promoter yield chimeric *Antp* P1/*z2* or *Antp* P1/*pb* mRNAs by alternate splicing, poly(A$^+$) RNA was prepared from wild type (Oregon-R) and *Df*(3R)*SCB*^{XL2}/TM3 embryos (0–24 hr postoviposition) for Northern blot analysis. The results, as shown in Figure 3, probe C, indicate that one or perhaps two novel transcripts containing P1 first exon sequences are present in the RNA from mutant animals. One of these, marked P1/*z2*, hybridizes to *Antp* P1 exon 1-specific as well as to *z2* (z2 exons 1 and 2)-specific probes (data not shown), and is of the size predicted for a fusion P1/*z2* mRNA (~1,900 nucleotides, including ~200 nucleotides in the polyA tail; LAUGHON *et al.* 1986; RUSHLOW *et al.* 1987). These blots also allowed the detection of a second novel band (P1/*pb*?; Figure 3, probe C) containing *Antp* P1 first exon sequences which suggested the presence of a hybrid P1/*pb* mRNA. This possibility was tested in the experiments described below.

In order to test more rigorously for the existence of hybrid mRNAs containing sequences from the *Antp* P1 first exon fused to sequences from the second exons of *z2* or *pb*, S1 nuclease protection experiments were performed. Chimeric end-labeled probes were prepared that contained sequences from P1 and *z2* or P1 and *pb*, joined in the predicted 5′ to 3′ configuration. Exonic *Antp* P1 E1 and either *z2* E2 or *pb* E2 sequences were fused at convenient restriction sites rather than at predicted splice junctions. The assay used here relies on base-stacking forces to maximize the formation of a nearly continuous duplex between the radiolabeled DNA strand and the looped-out chimeric cognate mRNA, rendering the probe resistant at least in part to S1 nuclease digestion. In the absence of a "hybrid" mRNA, only protection corresponding to *Antp* P1-derived transcripts should be seen; the detection of a "hybrid," mutant-specific protection product may be considered proof that *Antp* P1 and either *z2* or *pb* sequences reside in the same mRNA molecule.

Sequences of the P1 first exon were fused to the *pb* second exon (where the *pb* open reading frame initiates; CRIBBS *et al.* 1992). The 800-nucleotide probe used begins within the transcribed P1 sequences and extends past the end of *pb* E2 (Figure 4). The predicted S1 protection products are a 65-nucleotide fragment (derived from the normal *Antp* P1 and/or P1/*z2* transcripts) and a mutant-specific product of 240 nucleotides ending at the 3′ terminus of the *pb* second exon. These expectations were fully met (Figure 4). A similar experiment was performed using a chimeric P1/*z2* probe (MATERIALS AND METHODS) and the predicted protection products were again obtained (data not shown). Thus, transcripts directed by the *Antp* P1 promoter and initiated at the first *Antp* exon on the deletion bearing chromosome can extend through both the *z2* and *pb* genes. Subsequently, by alternate splicing, elements of the three normally distinct genes are joined to generate at least two novel

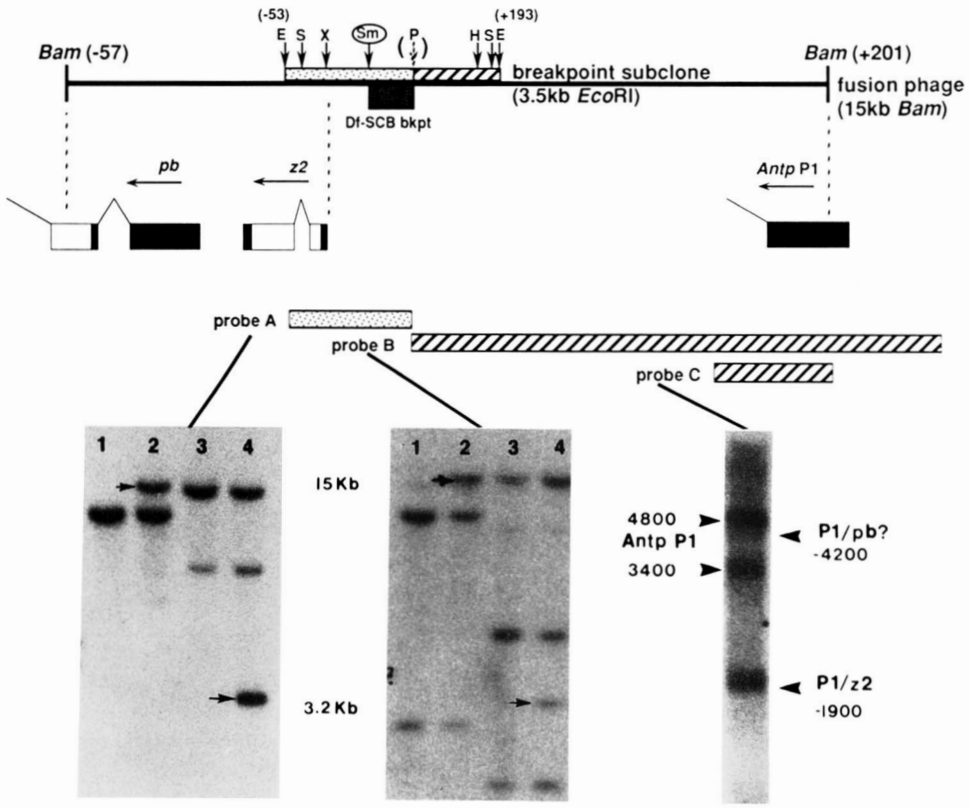

FIGURE 3.—Structure of the ANT-C in the *Df*(3R)*SCB*^{XL2} chromosome. The *Df*(3R)*SCB*^{XL2} chromosome fails to complement mutations in genes from *zen*, *bcd*, *Dfd*, *Scr*, *ftz* and *Antp*, but partially complements *pb* alleles (PULTZ *et al.* 1988) and complements lethal alleles of *l*(3)*84Ba* (the next locus distal to *Antp*). The breakpoints of the deficiency were located by genomic Southern blot analysis shown at the bottom of the figure (probes A and B). Genomic blot data for the breakpoint localization were confirmed by cloning the 15-kb *Bam*HI fusion fragment in bacteriophage λ (MATERIALS AND METHODS) and then performing restriction analysis on the subcloned 3.5-kb *Eco*RI restriction fragment indicated (*pb*/*z2* sequences are stippled, *Antp* P1 sequences are cross-hatched). The position of the breakpoint was delimited to within 0.6 kb by the presence within the 3.5-kb *Eco*RI fragment of a *Sma*I restriction site from *pb*/*z2* (circled), coupled with the absence of a *Pst*I site normally located 0.6 kb upstream of the *Sma*I site. The correspondence between the transcription map of the fusion region and the cloned genomic DNA is indicated by vertical dotted lines. For the genomic blots shown at the bottom, lanes 1 and 3 of each set contain 5 μg of DNA from *Df*(3R)*DC-5 ri p*^{p}/*TM3,Sb* flies (*DC-5* is a deletion which removes all complementation units from *twr* proximal of *labial* through minimally *l*(3)*84B1* and thus the entire ANT-C; D. L. CRIBBS, unpublished observation). Bands seen in lanes 1 and 3 therefore derive exclusively from the *TM3, Sb* chromosome. Lanes 2 and 4 contain 5 μg of DNA from flies of genotype *Df*(3R)*SCB*^{XL2}/*TM3,Sb*. Bands detected in lanes 2 and 4 which are not present in lanes 1 and 3 derive from the *Df*(3R)*SCB*^{XL2} chromosome. *Df*(3R)*SCB*^{XL2}-specific bands detected by both *Antp* P1 and *pb*/*z2* region probes reflect the presence of fusion fragments that span the deficiency breakpoints. DNA was digested with *Bam*HI (lanes 1 and 2 of each set), or with *Sal*I (lanes 3 and 4). (Probe A) The proximal breakpoint was described previously (PULTZ *et al.* 1988) and is confirmed here (the probe used was a 2.0-kb *Eco*RI/*Pst*I fragment spanning coordinates −51 to −53, indicated by the lightly stippled box; see PULTZ *et al.* (1988) for description of coordinates). (Probe B) The distal breakpoint was located by probing with λ phage A577 (cross hatched box), carrying sequences from the *Antp* P1region which span the P1 promoter and first exon of *Antp* (SCOTT *et al.* 1983; LAUGHON *et al.* 1986). The location of the breakpoints was further confirmed, as described above, by cloning and mapping of the 15-kb fusion *Bam*HI fragment (lanes A2 and B2). The exon structure surrounding the deletion breakpoints is shown in the middle of the figure, with *pb*, *z2* and the lone *Antp* P1 exon all transcribed in the same direction. The positions of the protein coding sequences are indicated by open boxes while the nontranslated exonic intervals are black. Note that first exons of *Antp* P1 and *pb* are entirely noncoding and that the open reading frame of *pb* initiates in exon 2. (Probe C) For Northern blot analysis of mutant derived RNA, poly(A+) RNA was prepared from wild type (Oregon R) or *Df*(3R)*SCB*^{XL2}/*TM3, Sb* embryos (0–24 hr). Shown is a gel track containing 10 μg of mutant poly(A^{+}) RNA that was transferred to a Nitran membrane and probed with a 1.8-kb *Bam*HI/*Eco*RI fragment containing most of *Antp* P1 and E1 (probe C; coordinates 2137 to 3959, LAUGHON *et al.* 1986). Similar hybridizations were performed using *z2* and *pb*-specific probes. As indicated in (probe C) (arrows, left), two bands correspond to normal *Antp* P1 transcripts of about 4,800 and 3,400 nucleotides. These are products of the *TM3,Sb* chromosome. A novel RNA class of about 1,900 nt was detected with both *Antp* P1 and *z2*-specific sequences (labelled P1/*z2*). This band is presumed to be a product of the deletion bearing chromosome and to result from alternate splicing. Additionally a second novel band of about 4,200 nucleotides was sometimes detected (labeled P1/*pb*?).

"hybrid" transcripts, the P1/*z2* and P1/*pb* species (Figure 5). Additionally, the *Df*(3R)*SCB*^{XL2} chromosome must be capable of directing the synthesis of normal *pb* mRNA and protein under normal *pb* control. This is inferred from the *pb* mutant phenotype conferred by this deletion (*i.e.*, a partial loss-of-function). Moreover, *pb* protein is detected in *Df*(3R)*SCB*^{XL2}/*pb*^{null} embryos, demonstrating that the remaining approximately 3 kb of *pb* 5′ flanking sequences are sufficient to direct spatially correct *pb* expression though in

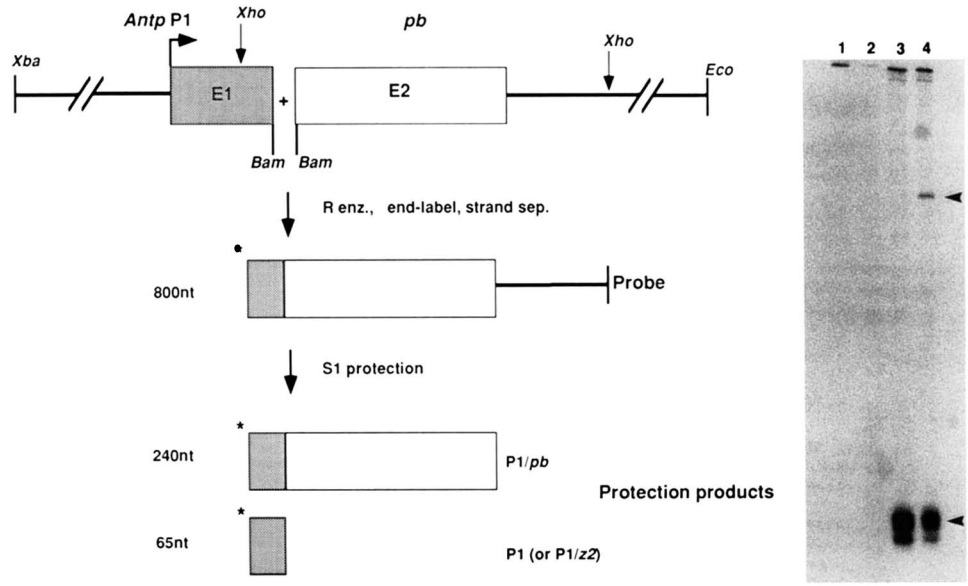

FIGURE 4.—Demonstration of alternate splicing from the *Df*(3R)*SCB*^{XL2} chromosome between the first exon (E1) of *Antp* P1 and the second exon (E2) of *pb*. The probe, prepared as described in MATERIALS AND METHODS, contains 65 nucleotides (nt) of *Antp* P1 sequence and 735 nt of *pb* sequence, and fuses P1 E1 to *pb* E2 as indicated. Each hybridization contained 80,000 Cerenkov cpm of single-stranded probe. Hybridizations and digestions with nuclease S1 were as described in MATERIALS AND METHODS, and only the results obtained with the coding strand probe are shown. Lanes shown are: (1) Probe only, approximately 20 Cerenkov cpm; (2) probe hybridized to 20 μg of *Escherichia coli* tRNA; (3) probe hybridized to 20 μg of wild-type (Oregon R) embryonic (0–24 hr) poly(A$^+$) RNA; and (4) probe hybridized to 20 μg embryonic (0–24 hr) poly(A$^+$) RNA from a *Df*(3R)*SCB*^{XL2}/*TM3,Sb* stock. The predicted classes of protection products are shown to the left, and the corresponding protection products indicated (arrows) to the right of the autoradiograph. The upper band (240 nt) is specific to the mutant RNA track and corresponds to *Antp* P1/*pb* E2 fusion mRNA.

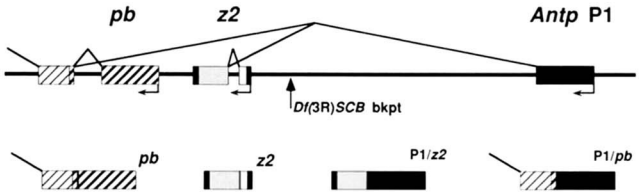

FIGURE 5.—Summary of expression from the *pb* region of the ANT-C on the *Df*(3R)*SCB*^{XL2} chromosome. The structure shown on top indicates the novel juxtaposition of the *Antp*, *z2* and *pb* transcription start sites on the deletion chromosome. Dark motifs within boxes indicate the position of transcribed but non coding portions of the three loci (*Antp* exon 1 is black). Light motifs within boxes show the positions of the two remaining open reading frames in *z2* (stippled fill) and *pb* (diagonal line fill). Above the indicated exons are shown the normal and postulated alternate splicing events; while below, the four possible mature RNA products are indicated. Note that the two novel products shown at the right result in the splicing of only leader sequence in the case of P1/*pb* and the fusion of *Antp* P1 leader into protein coding sequences in the case of P1/*z2*.

diminished quantities (PULTZ 1988). We have not tested directly for the presence of normal *z2* transcripts. However, assuming that all of the necessary *cis*-acting regulatory elements for this locus are present, the deletion chromosome does have the potential to encode such a product.

Accumulation of Antp protein is reduced in imaginal tissues of *Df*(3R)*SCB*^{XL2}-bearing animals: Expression of pb protein in the embryonic thorax was correlated with a dominant *Antp* loss-of-function phenotype. This suggested that the nuclear pb homeodomain protein itself might interfere with the function or accumulation of Antp protein in the imaginal discs, perhaps at the level of transcriptional control. If pb protein negatively regulates *Antp* transcription, whether directly or indirectly, then Antp protein levels should be reduced compared to wild type in cells expressing *pb* under the control of the *Antp* P1 promoter. To test this prediction, imaginal tissues derived from *Df*(3R)*SCB*^{XL2}-bearing or from normal third instar larvae were stained with monoclonal antisera directed against Antp protein (CONDIE, MUSTARD and BROWER 1991) (Figure 6, A and B) or with polyclonal anti-*pb* sera (Figure 6, C and D). Cells associated with the dorsal prothoracic and wing discs give rise to the humeral callus, anterior spiracles and anterior mesonotum (ABBOTT and KAUFMAN 1986), all of which are perturbed in *Df*(3R)*SCB*^{XL2}/+ bearing adults. Staining experiments show that *pb* protein is present in the dorsal prothoracic discs of deficiency-bearing heterozygotes but is not detectable in wild-type discs (compare Figure 6, C and D). Conversely, the level of Antp protein present in the same discs is markedly reduced in the mutant larvae. It is also possible to detect pb protein in the wing discs in regions where the level of Antp protein is diminished (data not shown). Thus ectopic *pb* expression correlates to reduced accumulation of Antp protein in the imaginal tissues which give rise to the affected adult structures. Further, the diminished accumulation of Antp protein is correlated

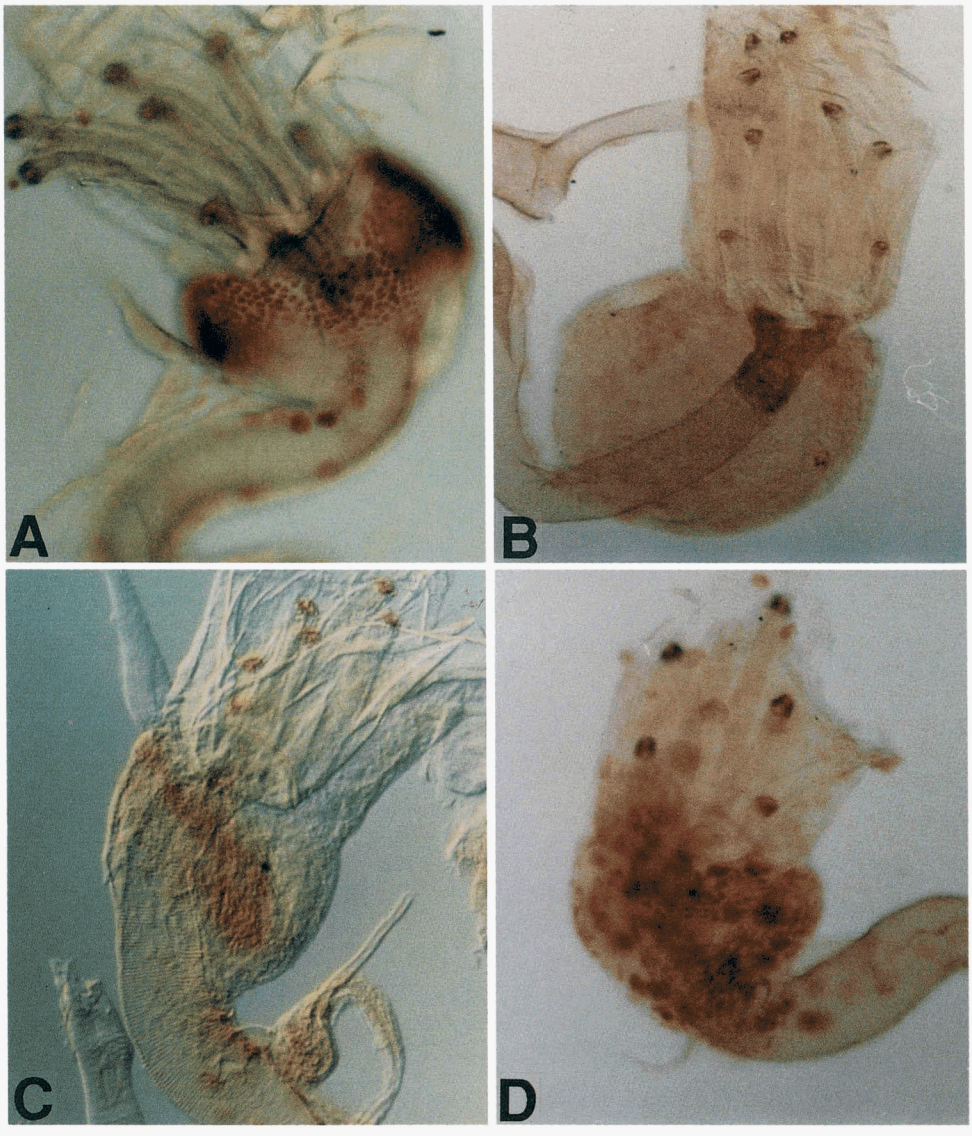

FIGURE 6.—Distribution of Antp and pb proteins in imaginal discs of normal and *Df*(3R)*SCB*^{XL2} larvae. Larvae of the desired genotypes were stained with primary antisera directed against either Antp or pb proteins. Subsequent to treatment with the secondary antibody and enzymatic reactions to reveal the distribution of homeotic protein, the imaginal discs were dissected and mounted for photography. Shown are dorsal prothoracic discs, which give rise to the humeral callus of the adult. The discs are oriented such that the associated spiracles (located anteriorly in the larva) are up and the tracheae (passing posteriorly) are down. (A) Wild type (Oregon R) stained with anti-*Antp* monoclonal; (B) *Df*(3R)*SCB*^{XL2}/+ stained with anti-*Antp* monoclonal; (C) wild type (Oregon R) stained with anti-*pb* polyclonal; (D) *Df*(3R)*SCB*^{XL2}/+ stained with anti-*pb* polyclonal. Note the inverse relationship between accumulation of Antp and pb proteins.

with the ectopic "function" of pb protein, since in larvae with the revertant chromosome *Dr2* (carrying a *pb*^{null} mutation on the *Df*(3R)*SCB*^{XL2} chromosome) Antp protein accumulation appears normal. The mutant phenotype observed in *Df*(3R)*SCB*^{XL2} animals and its absence in the *pb*$^{-}$ revertants thus may be explained by these results. The most economical explanation for this lessened accumulation of Antp protein is that pb protein can directly or indirectly repress transcription from the P1 promoter. Alternatively, pb protein or the product of a gene activated by *pb* may contribute to reduced accumulation of Antp protein by competing with it for binding to target DNA sequences and thereby destabilizing it. This latter possibility seems less likely especially if it is assumed that it is the pb protein which directly competes with *Antp* protein. The abundance of *pb* mRNA, and protein, appears to be considerably less on a per cell basis than for other homeotic loci of the ANT-C (our unpublished observations) and thus would appear to be at a competitive disadvantage to the product of the *Antp* locus. Taken together, these various data show that pb protein is capable of contributing to the negative regulation of *Antp*, and suggest that this may occur at the transcriptional level.

DISCUSSION

***pb* has acquired a second homeotic promoter:** We present here the characterization of a chromosomal rearrangement which has integrated elements of three distinct transcription units into one. The homeotic gene *pb*, with a single promoter and a single domain of expression in the head, has been converted into a gene with two promoters (*pb* and *Antp* P1) which is expressed in two distinct regions of the fly body, the head and thorax. The important consequence for the fly is that a new regulatory regime is imposed on *Antp*. In the new arrangement, ectopic *pb* expression in imaginal tissue causes diminished thoracic expression of remaining copies of *Antp*. Most extant *Antp* domi-

nant gain-of-function mutations result from chromosome rearrangements in which the *Antp* P1 promoter is replaced by other sequences, allowing *Antp* expression in the eye-antennal imaginal disc (*e.g.*, SCHNEUWLY, KURIOWA and GEHRING 1987; FRISCHER, HAGEN and GARBER 1986). Here we have encountered a different situation, where the superimposition of *Antp* P1 control on another gene has resulted indirectly in loss of *Antp* functions. A number of developmentally important Drosophila genes have been described which possess multiple promoters, such as *Antp* (LAUGHON *et al.* 1986; STROEHER, JORGENSEN and GARBER 1986; SCHNEUWLY *et al.* 1986), *caudal* (MACDONALD and STRUHL 1986; MLODZIK and GEHRING 1987), and *hunchback* (TAUTZ *et al.* 1987; BENDER *et al.* 1988). The deletion analyzed in this work is an example of how gene fusion can result in the superimposition of two distinct regulatory paradigms. The acquisition or shuffling of elements necessary for regulation of gene expression is likely an important component of the evolution of regulatory systems (for review, see SCHIBLER and SIERRA 1987), and naturally occurring juxtapositions similar to the one studied here may serve at least in part to explain the origin and existence of multiple independent promoters for a single gene.

Mutant chimeric homeotic genes other than the one observed on the *Df*(3R)*SCB*^{XL2} chromosome have previously been described in Drosophila. The *Ultrabithorax* (*Ubx*) mutation C1 of the BX-C is associated with a deletion which fuses the *abdominal-A* (*abd-A*) and *Ubx* loci. In this case the result is a chimeric *abd-A/Ubx* transcription unit and protein (ROWE and AKAM 1988). Because the spatial expression domain of *abd-A* overlaps that of *Ubx*, and the protein contains the *Ubx* homeobox, some *Ubx* functions are retained even in the absence of its normal control (CASANOVA, SANCHEZ-HERRERO and MORATA 1988; ROWE and AKAM 1988). The encoded chimeric protein gives no evidence of acquired new functions; rather, in this case the intriguing observation is that some functions normally attributed to either *Ubx* or to *abd-A* are retained in the mutant.

Chromosomal rearrangements not associated with deletions provide another sort of plasticity to the genome. This is seen in a spectacular fashion for the dominant *Antp*^{73b} mutation; in these mutants ectopic expression of *Antp* protein in the eye antennal discs results from an inversion mutation that exchanges the *Antp* P1 promoter for the 5′ end of a gene normally expressed in the head (FRISCHER, HAGEN and GARBER 1986; SCHNEUWLY, KURIOWA and GEHRING 1987).

The basis of differential mRNA accumulation/processing: Curiously, though it is clear from the present work that an RNA polymerase can traverse the sequences separating *z2* and *pb*, there is no evidence for the formation of *z2/pb* hybrid mRNA from *Df*(3)*SCB*^{XL2} or from a normal chromosome (PULTZ *et al.* 1988; RUSHLOW *et al.* 1987). Put from a slightly different perspective, it is interesting that transcription initiated at the P1 promoter can extend through both the *z2* and *pb* loci but transcription initiated at the *z2* promoter apparently does not extend over the much shorter interval between *z2* and *pb*. Perhaps this is an indication of the existence of specific transcription factors which distinguish between early zygotic gene promoters such as *z2* and those for later expressed homeotic genes such as *Antp* and *pb*. Consistent with such a possibility, it is of interest to note that in embryos carrying the chromosome *Df*(3R)*LIN*, in which *ftz* and *Sex combs reduced* (*Scr*) 5′ flanking sequences are juxtaposed to the *z2* and *pb* genes, *z2* RNA is detected in stripes as *ftz* is normally (RUSHLOW and LEVINE 1988), while pb protein accumulates in a subset of cells in the foregut where normal *Scr* expression occurs (*pb* expression in stripes and *zen* accumulation in the foregut is not observed) (PULTZ 1988). Another potentially important variable is the time at which the different promoters are expressed. A limited supply of factors required for the excision of large (*e.g.*, *Antp* and *pb*) but not small introns (*ftz*, *zen* and *z2*) soon after the onset of zygotic transcription might suffice to explain the ability to make P1/*pb* mRNAs at 13–16 hr of embryogenesis but not *z2/pb* mRNAs at 2–4 hr. Lastly one can surmise that the transcriptional machinery which normally operates at the *Antp* P1 promoter is qualitatively different from that normally assembled at genes like *z2*. The P1 promoter drives transcription which normaliy extends over 100 kb of DNA and must traverse a segment of the genome containing the internal P2 promoter. The *z2* promoter does not have to accomplish either feat. The nature of any proposed qualitative difference is obviously not addressed by the experiments or results presented and which (if any) of the above speculations is relevant awaits further investigation. However these results have pointed to some interesting differences which do exist among these developmentally significant promoters and have provided avenues for future experimentation.

Ectopic pb protein diminishes normal *Antp* accumulation: The present work shows that pb protein is capable of negative function(s) directed toward other homeotic genes. The dominant thoracic defects due to ectopic *pb* expression reflect the capacity of pb protein to negatively regulate *Antp*. This observation poses an apparent paradox, since if *pb* negatively regulates *Antp* P1 then *pb* under *Antp* P1 control might be negatively autoregulatory. Assuming that the down-regulation of *Antp* occurs through the binding of a protein to relevant target sequences, a possible resolution is that these targets reside, at least in part,

within sequences removed by the deficiency. Thus, pb protein need not participate in negative autoregulation of its newly acquired promoter (that lacks the target sequences), but might restrict its activities to intact *Antp* copies elsewhere.

Posteriorly directed regulation by *pb*: The data presented here indicate that regulation by ectopically expressed pb protein is directed toward a more posteriorly expressed gene, *Antp*. In a study employing the conditional hsp70 promoter to direct ectopic and overexpression of Antp and Ultrabithorax proteins, the notable result was that more posteriorly expressed endogenous genes were apparently refractory to regulation by those normally expressed more anteriorly (GONZÁLES-REYES *et al.* 1990). In double heat shock combinations, hs-*Ubx* was likewise epistatic to hs-*Antp* (more anterior) in attributing segmental identity. In view of that work, it might be considered surprising that *pb* is capable of reducing *Antp* function and accumulation in the thorax. Perhaps the present case is a simple exception to a generally respected rule. However, since the effect of *pb* on *Antp* accumulation in the thorax seems more severe in larval discs than in the embryo (compare Figure 2 and Figure 6) the departure from the above rule of posterior primacy might rather emanate from differences between embryonic and larval developmental control. Indeed such differences have been shown to exist for two other members of the ANT-C, *lab* and *Scr* (CHOUINARD and KAUFMAN 1991; DIEDERICH, PATTATUCCI and KAUFMAN 1991; PATTATUCCI and KAUFMAN 1991).

Negative regulation of *Antennapedia* by *pb* in a normal fly? The analysis of $Df(3)SCB^{XL2}$ presented here shows that *pb* is capable of negative regulation of *Antp* in the thorax. We therefore attempted to assess whether this reflects normal functions of *pb* directed toward *Antp* in the head. This was done first by examining the spatial expression of *Antp* in pb^{null} larvae, or in embryos lacking both *pb* and *Sex combs reduced* (*Scr*), the other homeotic protein known to be expressed normally in the labial discs (MAHAFFEY and KAUFMAN 1987; RANDAZZO, CRIBBS and KAUFMAN 1991). No ectopic *Antp* expression was observed in either case. Hence, if regulation of *Antp* in the head is a normal function of *pb*, it appears to involve a network comprising other as yet unidentified member loci. As an alternative approach, we examined the contribution of *pb* to the regulation of *Antp* gain-of-function alleles, reasoning that sequences recognized by DNA binding regulatory proteins might be removed by the chromosomal rearrangement. However, though one allele ($Antp^{73b}$) may be expressed in the labial discs provided the level of *pb* function is sufficiently reduced, the results obtained were equivocal. As a consequence, we remain unable to distinguish critically whether or not regulation of *Antp* in the labial discs is a normal function of *pb*.

The capacity of *pb* to negatively regulate *Antp* in the imaginal thorax might thus be simply an historical relic, or an artifact of the *pb* homeodomain's functional similarity to other such proteins. Negative regulation of *Antp* by *pb* in coexpressing cells might then provide a powerful evolutionary incentive to establish and maintain nonoverlapping expression domains by means other than and in addition to direct reciprocal repression. Thus it could be that the two have different domains of expression due at least in part to the fortuitous capacity of *pb* to negatively regulate *Antp* and the negative consequences of their coexpression, rather than to the present-day existence of a negative network including *pb* which represses *Antp* in the head.

An intriguing possibility which remains is that the observed capacity of *pb* to regulate *Antp* in the imaginal discs appears to point to a different and alternate relationship in the central nervous system. Spatially restricted expression of homeotic proteins in the central nervous system is a reiterated theme among these genes from diverse species (DUBOULE and DOLLÉ 1989; GRAHAM, PAPALOPULU and KRUMLAUF 1989; COSTA *et al.* 1988; KAUFMAN, SEEGER and OLSEN 1990). Additionally the homeotic genes of Drosophila have been shown to affect the development of the central and peripheral nervous systems (THOMAS and WYMAN 1984; WEINZIERL *et al.* 1987; MANN and HOGNESS 1990; HEUER and KAUFMAN 1992). A small number of cells in the embryonic (Figure 2C; PULTZ *et al.* 1988) and larval (RANDAZZO, CRIBBS and KAUFMAN 1991) nerve cords normally express *pb*. This domain extends far posterior of the labial segment along the anteroposterior axis into a region in which normal *Antp* expression is found. The function(s) of pb protein in these cells remains unknown. However, the reduced motility both of pb^{-} flies and of mini-pb^{+}; transgenic flies (with rescued mouthparts but lacking expression in the nervous system; RANDAZZO, CRIBBS and KAUFMAN 1991) supports an important role. Moreover ectopic expression of *pb* into the normal domain of *Antp* associated with the $Df(3R)SCB^{XL2}$ chromosome does not cause repression of *Antp* expression in the embryonic CNS. Thus coexpression of these two genes at this stage and in this cell type is permitted and indicates that these two loci have a very different relationship in this spatiotemporal domain and that they may act in a concerted rather than antagonistic fashion. The existence of such alternate regulatory logic points out the potential for cell- or tissue-specific cross-regulation of homeotic genes. Such mechanisms might then serve toward the integration of pattern among segments in the construction of a differen-

tiated individual, as well as within the confines of a segment.

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