Control elements of the P2 promoter of the *Antennapedia* gene

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***Antennapedia* (*Antp*), a homeotic gene of *Drosophila* required for proper differentiation of the thorax of the fly, is expressed in complex spatial patterns during development. The gene is >100 kb long and has two independently regulated promoters. To characterize *cis*-acting regulatory elements responsible for the expression pattern, fusions of the *Antp* promoter 2 cap site and upstream sequences to an *Adh-lacZ* gene were introduced into flies. A 10-kb sequence directs β -galactosidase production in a pattern that closely resembles the endogenous P2 pattern. Transcription from the 10-kb fusions is regulated by three genes that regulate *Antp* transcription. Control elements, including a target of action of homeo-domain-containing proteins, were mapped by deleting parts of the 10-kb sequence.**

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The homeotic genes of *Drosophila* are required for the specification of segmental identity during embryonic development and metamorphosis. Mutations in homeotic genes lead to the transformation of one part of the embryo or adult fly into another. Many of the homeotic loci are clustered in two gene complexes: the Bithorax Complex (BX-C) (Lewis 1978; Duncan 1987) and the *Antennapedia* Complex (ANT-C) (Kaufman et al. 1980). BX-C genes are involved in development of the posterior thoracic and abdominal regions of the embryo; the ANT-C homeotic genes are required for the differentiation of the head and thorax.

The spatial domains of function of each homeotic gene in the embryo have been determined by analyzing mutant phenotypes. Within these domains, homeotic mutations can have different effects on different segments, parasegments (PS, the posterior part of one segment and the anterior part of the next most posterior segment; Martinez-Arias and Lawrence 1985), or dorsal and ventral structures (Lewis 1963; Morata and Kerridge 1981; Struhl 1981, 1984; Kerridge and Morata 1982; Hayes et al. 1984; Kaufman and Abbott 1984; Karch et al. 1985; Sato et al. 1985; Abbott and Kaufman 1986). In situ hybridization with probes for each of the known protein-coding genes of the ANT-C and BX-C has revealed very complex patterns of gene expression in embryos. In general, there is good correspondence between the parts of an embryo affected by the loss of gene function and the places in the embryo where gene transcripts accumulate (Akam 1983; Levine et al. 1983; Akam and Martinez-Arias 1985; Harding et al. 1985; Kuroiwa et al. 1985; Martinez-Arias 1986; Chadwick and McGinnis

1987; Mahaffey and Kaufman 1987; Martinez-Arias et al. 1987; Regulski et al. 1987). The complexity of homeotic mutant phenotypes and the elaborate spatial patterns in which the genes are expressed suggest that proper developmental function of homeotic genes depends on precise spatial and temporal patterns of expression.

Homeotic and segmentation genes (including gap, pair-rule, and segment polarity genes) make up a network, or hierarchy, of genes that control differentiation of the embryonic body pattern (for reviews, see Akam 1987; Scott and Carroll 1987; Ingham 1988). Segmentation genes are thought to regulate the expression of other segmentation genes and to control the initial sites of homeotic gene expression. After the patterns of homeotic gene expression have been established, they are maintained by interactions between homeotic genes and by the action of the *Polycomb*-like class of genes that function as *trans*-regulators of ANT-C and BX-C loci. Many genes in the hierarchy encode proteins that contain homeo domains (McGinnis et al. 1984; Scott and Weiner 1984; Regulski et al. 1985). The primary amino acid sequence of homeo domains is similar to that of DNA-binding proteins of bacteria and yeast (Laughon and Scott 1984; Shepherd et al. 1984). Homeo domains are capable of binding to DNA in vitro in a sequence-specific manner (Desplan et al. 1985, 1988; Hoey and Levine 1988). Some of the interactions between genes of the developmental hierarchy may be mediated by DNA-binding activities of the homeo domains in segmentation and homeotic gene products, but this hypothesis has not been tested in vivo yet.

We are studying the regulatory elements of the homeotic gene *Antennapedia* (*Antp*), a member of the ANT-C. The experiments address important questions regarding homeotic gene expression, including: What are

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the regulatory elements that direct patterns of expression as complex as those of homeotic genes? What are the mechanisms by which homeotic genes are controlled by segmentation genes, other homeotic genes, and currently unknown regulators? In particular, what sequences respond to homeo domains, zinc finger proteins, and other known components of the regulatory network?

Embryos homozygous for *Antp* mutations have a homeotic transformation of PS4 to PS3 (posterior first thoracic and anterior second thoracic to posterior labial and anterior first thoracic segments, i.e., pT1 + aT2 to pLb + aT1) and of PS5 into a hybrid of PS3 and PS6 (Martinez-Arias 1986; originally described as a transformation of T2 and T3 toward T1, Wakimoto and Kaufman 1981). *Antp* mRNA and protein are confined largely to thoracic regions of the embryo (PS4, PS5, and anterior PS6), but lower levels are seen in PS3 and the abdominal segments (Levine et al. 1983; Martinez-Arias 1986; Carroll et al. 1986; Wirz et al. 1986). In addition to epidermal and mesodermal expression, *Antp* products are found in cells of the ventral and peripheral nervous systems (VNS and PNS, respectively).

The *Antp* locus is >100 kb in length. Transcription initiates at two independently regulated promoters separated by ~65 kb (Laughon et al. 1986; Schneuwly et al. 1986; Strocher et al. 1986). Promoter 1 (P1) and promoter 2 (P2) have overlapping, but clearly distinct, spatial and temporal patterns of expression in embryos and imaginal discs (Jorgensen and Garber 1987; A. Martinez-Arias, J. Bermingham, and M. Scott, unpubl.). In this paper we describe a study of the regulatory elements of P2. The P2 transcription pattern in embryos has been examined in detail by A. Martinez-Arias, J. Bermingham, and M. Scott (unpubl.). P1- and P2-specific probes (portions of exon A and exon C, respectively, Fig. 1a) were used for in situ hybridization experiments. While the germ band is elongated, P2 transcripts are found in the epidermal ectoderm in PS3, PS4, and PS5 (at approximately equal levels), in the mesoderm of PS4 and PS5, and in PS3–PS14 of the VNS, with higher levels in PS3–PS5 than in more posterior regions. P2 transcripts are also found in cells that are probably PNS cells.

Several loci encoding potential *trans*-acting regulators of the *Antp* gene have been identified by genetic analysis (Lewis 1978; Duncan 1982; Struhl 1982, 1983; Capdevila et al. 1986). Direct examination of *Antp* mRNA and protein distributions in embryos mutant for BX-C genes, *Polycomb*, gap genes, and *fushi tarazu* (*ftz*, a pair-rule segmentation gene) show that the products of these loci are required, directly or indirectly, for normal *Antp* expression (Hafen et al. 1984; Harding et al. 1985; Carroll et al. 1986; Ingham and Martinez-Arias 1986; Wedeen et al. 1986; Wirz et al. 1986; Harding and Levine 1988). Other segmentation genes are expected to be involved in specifying the initial sites and levels of *Antp* expression. Thus, each of the *Antp* promoters is likely to be associated with a series of regulatory elements through which the products of segmentation and homeotic genes act.

We have begun a study of regulatory elements that di-

rect position-specific transcription of *Antp* P2 using promoter-reporter gene fusions and P-element-mediated transformation of flies. When 10 kb of *Antp* sequence was fused to the *Escherichia coli lacZ* gene and introduced into flies, the pattern of β -galactosidase expression in embryos closely resembled the endogenous *Antp* P2 expression. By successive deletion of sequences upstream of the cap site, we have begun to localize elements required for particular aspects of the *Antp* P2 pattern of expression. These experiments provide a way to study the mechanisms by which known regulators activate, repress, or maintain expression of *Antp*, and are also a step toward characterizing previously unidentified regulators.

Results

Antp protein expression from P2

Antp mutations were used to examine the pattern of protein produced solely from P2 transcripts and to map roughly the locations of regulatory elements upstream of P2. The same protein-coding exons are common to both P1 and P2 transcription units. In previous reports, the protein pattern resulting from the contributions of both promoters was described (Carroll et al. 1986; Wirz et al. 1986). *Antp* mutations caused by chromosome breaks in the region between P1 and P2 have an interrupted P1 transcription unit, but the P2 transcription unit is intact and the coding exons are unaffected (Fig. 1a). In these mutant embryos, *Antp* protein comes only from P2 transcripts.

The *Antp^{s2}* mutation (Scott et al. 1983) is associated with a chromosome inversion that has one breakpoint about 35 kb upstream of the P2 start site and another at a distant site on the chromosome (Fig. 1a). In previous work in our laboratory (Carroll et al. 1986), the polyclonal antibody used did not detect *Antp* protein in *Antp^{s2}* homozygous embryos. A sensitive monoclonal antibody specific for *Antp* protein (generously provided by D. Brower) has allowed the detection of P2-derived *Antp* protein in *Antp^{s2}* homozygous embryos. We have used immunofluorescence detection of the protein to facilitate double-label experiments (see below), although yolk autofluorescence sometimes interferes with the signal. The pattern of *Antp* protein in approximately one-quarter of the embryos produced by a balanced *Antp^{s2}* stock is distinctly different from that of wild-type embryos (Fig. 2). At stage 12 (stages according to Campos-Ortega and Hartenstein 1985), wild-type embryos (Fig. 2a) have much higher levels of *Antp* protein in the epidermis of PS4 and PS5 than are seen in *Antp^{s2}* homozygotes (Fig. 2b). At stage 15, wild-type embryos have *Antp* protein in the VNS at high levels from the posterior prothorax (pT1) through the anterior meta-thorax (aT3) and at lower levels in pT3 through aA7 (anterior seventh abdominal segment) and in aT1 (Fig. 2c; Carroll et al. 1986; Wirz et al. 1986). In *Antp^{s2}* homozygotes, the overall amount of *Antp* protein is significantly lower than that in wild-type embryos, making detection of the *Antp* protein more difficult. However, relatively

Figure 1. *Antp-lacZ* fusion genes used for P-element-mediated transformation. (a) *Antp* gene structure. The exons are lettered A–H, as in Laughon et al. (1986). Exons D–G range in size from 39 to 777 bp with introns of 152–381 bp. The ATG for the 1134-bp open reading frame is indicated and is located in exon E. Transcription start sites for P1 and P2 are shown. Transcript structures are drawn above the gene map; two alternate polyadenylation sites, separated by 1.5 kb, can be used for transcripts from each promoter. The breakpoints of three mutant alleles are indicated by arrows on the map. The homeo box is found in exon H. (b) Regions of the P2 and 5'-flanking sequence used in fusion genes. Constructions are named as shown. The 3' end of each *Antp* fragment is 200 bp downstream of the start site. *Antp* DNA sequences were inserted into the polylinker of the vectors diagramed in (c). (c) Transformation vectors. Details of plasmid constructions are given in Materials and methods. (P) P element sequences; (Adh) *Drosophila* alcohol dehydrogenase gene fragment containing an initiator ATG; (hs neo) the neomycin resistance gene driven by the *Drosophila hsp70* promoter; (white) the *white* gene of *Drosophila*; (lacZ) the *E. coli lacZ*-coding region; SV40 poly(A) site, a fragment of the SV40 virus genome containing a polyadenylation signal.

high levels of expression are seen in the T1, T2, and T3 ganglia, with lower levels apparent in nine abdominal ganglia (Fig. 2d). The PNS cells that stain with the anti-*Antp* antibody in *Antp^{s2}* homozygotes appear to be a subset of those seen in wild-type embryos (Fig. 2c,d).

The pattern of *Antp* protein in the mutant embryos agrees well with the pattern of P2 RNA expression seen in in situ hybridization experiments (A. Martinez-Arias, J. Bermingham, and M. Scott, unpubl.). Thus, the mu-

tant protein distribution is likely to result from close to normal expression of the P2 transcription unit, and lack of P1 function does not appear to alter the P2 expression pattern. In *Antp^{73b}* mutant embryos, which have a chromosome inversion separating P1 from P2 with a breakpoint located ~45 kb upstream of the P2 start site (Fig. 1a; Garber et al. 1983; Scott et al. 1983), the distribution of *Antp* protein is very similar to that in *Antp^{s2}* embryos. One difference is evident: *Antp* expression in the ven-

Figure 2. *Antp* protein expression in wild-type embryos and in mutant embryos lacking P1 function. Embryos were stained with a monoclonal anti-*Antp* antibody and a rhodamine-conjugated secondary antibody. Anterior is at the left, and ventral is down in all photographs. (a) *Antp* expression in a wild-type stage 12 embryo. A high level of epidermal expression (arrowhead) is seen in PS4 (indicated by open arrow) and PS5. (b) Protein production from *Antp* P2 in a stage 12 *Antp^{s2}/Antp^{s2}* embryo. (c) Wild-type *Antp* expression in the VNS and PNS. Thoracic segment boundaries are indicated, and the arrow marks PNS expression in A4. (d) *Antp* VNS and PNS expression in an *Antp^{s2}* homozygote. All *Antp* protein derives from P2 transcripts. The level of *Antp* protein in PS3 of the wild-type developing VNS (a) is lower relative to PS4 and PS5 than in *Antp^{s2}* homozygotes. (e) Protein production from *Antp* P2 in an embryo homozygous for the *Antp^{73b}* mutation. The arrow indicates the ventrolateral epidermis, where *Antp* protein is present in *Antp^{73b}* homozygotes but not in *Antp^{s2}* homozygotes (Fig. 2d).

trolateral epidermis of T2 and T3 is seen in *Antp^{73b}* embryos but is absent from *Antp^{s2}* embryos (Fig. 2e). The *Antp^B* chromosome has an inversion with a breakpoint ~10.5 kb upstream of the P2 cap site. In *Antp^B* embryos, the pattern again is nearly identical to that of *Antp^{s2}*. However, a very low level of *Antp* protein can be (barely) detected in the two neuromeres anterior to the first thoracic neuromere, the labial (Lb) and maxillary (Mx) or subesophageal ganglia S2 and S3 (data not shown). Thus,

sequences that control expression in the thoracic epidermis may be located between the *Antp^{73b}* and *Antp^{s2}* breakpoints (–45 and –35 kb), and elements that repress *Antp* expression in the Lb and Mx neuromeres may be located between 10 and 35 kb upstream of the P2 start site. Our first goal was to construct *Antp* P2–*lacZ* fusions that would direct expression of β -galactosidase in a pattern resembling the P2-derived protein pattern observed in the mutant embryos.

Construction of *Antp* P2–*lacZ* fusion genes and generation of transformed lines

Sequences encompassing the P2 transcription initiation site were joined to an *Adh-lacZ* reporter gene (the initiation codon and a short 5' leader sequence from the *Drosophila* alcohol dehydrogenase (*Adh*) gene attached to the *E. coli lacZ*-coding region). Fusion genes with 10, 5.6, 4, 2, and 0.6 kb of 5'-flanking DNA and 200 bp of DNA downstream of the cap site were constructed in P-element transformation vectors (Fig. 1b,c). Constructs were made in a vector containing the neomycin resistance gene driven by the *hsp70* promoter (C4hsneo) or the CaSpeR vector containing the *white* gene (Fig. 1c; Materials and methods).

A number of independent lines containing each fusion gene were generated by P-element-mediated transformation (Table 1; Materials and methods; Rubin and Spradling 1982; Spradling and Rubin 1982). Transformant line designations indicate the number of kilobases of DNA upstream from the P2 start site, the site of fusion to *Adh-lacZ* downstream of the start (d9 is a deletion ending 200 bases downstream), and the line number.

β -Galactosidase expression in transformant lines

The patterns of β -galactosidase expression in embryos carrying P2 fusion genes were examined using an X-gal assay for enzyme activity or by indirect immunofluorescence with an anti- β -galactosidase antibody (Materials and methods). Of the three lines with 10 kb of *Antp* P2 5'-flanking DNA fused to the *lacZ*-coding region (Table 1), 10d9.1 had the highest level of β -galactosidase expression in X-gal assays and was used for more detailed analyses.

The 10d9.1 insert was crossed into an *Antp^{s2}* background for direct comparison of the β -galactosidase pattern to the P2 protein pattern, using double labeling with anti-*Antp* and anti- β -galactosidase antibodies (Materials and methods). In a stage 15 embryo, the *Antp* and β -galactosidase patterns are very similar (Figs. 3a,b,e,f): strong staining in the thoracic segments and a lower level of staining in nine abdominal segments. (Note that *Antp* protein, located within the nucleus, gives a sharper

signal than β -galactosidase, which is found throughout the cell.) In a stage 12 *Antp^{s2}/Antp^{s2}* embryo, during germ-band retraction, protein produced from the endogenous P2 promoter is present in the developing nervous system in 12 connected patches (Figs. 2b and 3c). Anti- β -galactosidase staining also detects 11 or 12 patches (Fig. 3d), although connecting lines of stain are not always visible (Fig. 5a). Two stripes of epidermal β -galactosidase expression are visible in the ventrolateral region of each segment, with the highest levels in T2 and T3. As the germ band continues to retract, β -galactosidase expression in the VNS appears between the ventral ends of these stripes (Fig. 3h). *Antp* protein in *Antp^{s2}* homozygotes of the same stage is confined predominantly to the developing VNS (Fig. 3g). The epidermal β -galactosidase expression from 10d9.1 is similar to the *Antp* protein pattern seen in *Antp^{73b}* homozygous embryos (Fig. 2e).

In 10d9.1 transformant embryos, β -galactosidase is produced in cells that are probably in the PNS (Fig. 3f). Double-label experiments indicate that the 10d9.1 fusion is expressed in many of the same PNS cells that contain *Antp* protein produced from P2 transcripts (Fig. 3e,f). Presently, we do not know whether there is an exact cell-for-cell correspondence between the two patterns.

Although the overall 10d9.1 and endogenous P2 patterns are very similar, some differences can be detected. The earliest β -galactosidase pattern in 10d9.1 embryos consists of 11 or 12 patches of equal intensity in the developing VNS (Fig. 5a). At later stages, the three thoracic neuromeres are stained more strongly than abdominal neuromeres (Figs. 3b and 4a). The equivalence of early neuromere staining agrees with *in situ* data (A. Martinez-Arias, J. Bermingham, and M. Scott, unpubl.) but differs from the *Antp* protein pattern observed in *Antp^{s2}*, *Antp^{73b}*, and *Antp^B* homozygous embryos. This may indicate that post-transcriptional controls play a role in the distribution of *Antp* protein produced by P2.

A second difference is that β -galactosidase expression in head regions of the embryo is seen in all three transformant lines containing 10d9 *Antp-lacZ* fusions. The staining is first detectable near the cephalic furrow, and the expressing cells move anteriorly as head involution occurs. Because *Antp* RNA and protein are not normally seen in the head primordia, elements required to prevent P2 expression in this region are apparently lacking from the 10-kb construct.

Although the levels of β -galactosidase are lower in 10d9.2 and 10d9.3 than in 10d9.1, the patterns of expression are nearly identical to 10d9.1 (not shown). The results described thus far indicate that sequences within 10 kb upstream and 200 bp downstream of the P2 cap site are sufficient to direct a good approximation of the *Antp* P2 expression pattern.

Effects of 5' deletions on the expression of *Antp* P2 fusions

Having demonstrated that a 10-kb region upstream of P2 is sufficient for many aspects of normal P2 expression,

Table 1. Transformant lines

Construction	No. of lines	Vector ^a	Linkage ^a
10d9	3	C4hsneo	X (3)
5.6d9	1	C4hsneo	III (1)
4d9	8	C4hsneo (1)	III (1)
		CaSpeR (7)	II (4)
			III (3)
2d9	12	CaSpeR (12)	II (3)
			III (9)
0.6d9	10	CaSpeR (10)	X (2)
			II (6)
			III (2)

^a Numbers in parentheses indicate the number of separate lines.

Figure 3. Direct comparison of 10d9.1 fusion gene expression to *Antp* P2 expression. *Antp*^{s2} homozygous embryos containing the 10d9.1 fusion were double-labeled with polyclonal anti- β -galactosidase and monoclonal anti-*Antp* antibodies. Staining was visualized using fluorescein-conjugated anti-rabbit and rhodamine-conjugated anti-mouse secondary antibodies. Anterior is to the left for all embryos. Ventral side is down in *c–f* and face on in *a, b, g*, and *h*. (*a*) *Antp* protein in the VNS of an *Antp*^{s2} homozygote (ventral view). (*b*) β -Galactosidase expression in the VNS of the same embryo shown in *a*. (*c*) *Antp* expression in a stage 12 *Antp*^{s2} homozygous embryo. (*d*) β -Galactosidase expression in the same embryo as *c*. β -Galactosidase is present in the ventrolateral region of the epidermis in each segment, with the highest levels in T2 and T3 (T3 stain indicated by open arrow). (*e*) PNS cells producing *Antp* protein in an *Antp*^{s2} homozygous embryo. (*f*) β -Galactosidase expression in the same embryo shown in *e*. A number of cells can be identified that appear to express both *Antp* protein and β -galactosidase (arrows). (*g*) Ventral view of a stage 12 *Antp*^{s2} homozygous embryo showing *Antp* expression in the developing VNS. (*h*) Same embryo as in *g* showing β -galactosidase staining. Note epidermal expression of β -galactosidase at positions where *Antp* is not detected (open arrow).

we sought to map the locations of specific *cis*-acting regulatory regions. The effects of successive deletions of *Antp* P2 5'-flanking sequences were examined in transformant lines with 5.6, 4, 2, or 0.6 kb of upstream DNA (all with 200 bp of downstream sequences) joined to

Adh-lacZ. In the single line with a 5.6-kb fusion (5.6d9.1), the β -galactosidase pattern, as determined by X-gal staining of embryos, appears identical to that of 10d9.1, except that PNS expression is not detectable (data not shown). The nine 4d9 lines also lack detectable

Figure 4. β -Galactosidase expression from fusion genes containing 10, 4, 2, and 0.6 kb of P2 5'-flanking DNA. Anterior is to the left, and the ventral surface faces downward except when a ventral view is shown. (a) Expression of β -galactosidase in the VNS of a 10d9.1 embryo (ventral view). (b) β -Galactosidase expression from a fusion gene with 4 kb of P2 upstream DNA (4d9.5, ventral view). (c) β -Galactosidase expression in the VNS of a 2d9.7 embryo (ventral view). Two cells at the edges of each thoracic neuromere produce high levels of β -galactosidase (arrows indicate examples). (d) β -Galactosidase expression from a fusion gene with 4 kb of P2 upstream DNA (4d9.5, lateral view). Note expression in the Lb and Mx neuromeres (labeled arrows). (e) 0.6d9.3 embryo stained with anti- β -galactosidase antibody. β -Galactosidase expression in the VNS is at barely detectable levels. (f) Ventrolateral view of an untransformed embryo stained with anti- β -galactosidase antibody. The bright signal in the center of the embryo is due to nonspecific staining of the yolk (arrow).

PNS expression, as do all lines with shorter 5'-flanking regions. The VNS pattern in 4d9 lines is very similar to 10d9.1 (cf. Fig. 4, b to a), except that the 4d9 lines have strong nervous system staining in the two segments anterior to the first thoracic segment, the Lb and Mx neuromeres (Fig. 4d). Lb and Mx β -galactosidase expression is detectable in 10d9 lines but at much lower levels. Correspondingly, *Antp* protein is seen at low levels in the Lb and Mx neuromeres in *Antp^B* mutant embryos, which also have ~10 kb of *Antp* DNA upstream of P2. The pattern of β -galactosidase seen in 4d9 embryos during germ-band shortening consists of 11 or 12 connected patches (Fig. 5b). The head expression seen with the 10d9 fusions is also seen with all of the shorter constructs examined (Fig. 5).

In the 12 transformant lines with fusions that contain 2 kb of DNA sequence upstream of the P2 start site, β -galactosidase is detected in the ganglia of T1, T2, and T3. The pattern of strongly staining cells appears dif-

ferent from the patterns seen with 4-, 5.6-, and 10-kb fusions (Fig. 4c; cf. to Fig. 4b). In 2d9 lines, strong β -galactosidase staining is seen in cells lying at the outer edges of each thoracic hemisegment. In line 2d9.7, a low level of β -galactosidase expression resembling the pattern characteristic of larger fusion constructs is detectable (Fig. 4c). Expression in the developing VNS becomes apparent during germ-band shortening (Fig. 5c). Prior to the appearance of VNS expression, a series of 10 or 11 stripes is seen in embryos from each of the 2d9 lines (Fig. 5d). These stripes are located more laterally (extending from the amnioserosa) than the VNS patches and do not correspond to the epidermal expression seen in 10d9.1 transformants. The striped pattern persists during germ-band shortening, becoming a series of lateral spots that vary in relative intensity from one line to another (Fig. 5c).

β -Galactosidase expression in transformant lines carrying a construct with only 0.6 kb of P2 upstream DNA

appears to be more variable from line to line than with longer constructs. Two consistent features can be identified among the 10 lines obtained: In many lines, a striped pattern is present in stage 12 embryos, which is similar to that seen in 2-kb fusion lines (Fig. 5e), and VNS expression resembling that of 2-kb or longer constructs is not observed (Fig. 4e). Two lines have uniform levels of β -galactosidase throughout the VNS (not shown), and in the remaining lines, little or no β -galactosidase is detectable in the nervous system (cf. untransformed embryo stained with anti- β -galactosidase, Fig. 4f). Therefore, 0.6 kb is not sufficient for expression in an *Antp* P2 pattern.

Expression of *Antp* P2-lacZ fusion genes in mutant embryos

Antp expression is regulated negatively by the BX-C genes *Ubx* and *abd-A* (Hafen et al. 1984; Harding et al. 1985; Carroll et al. 1986). In *Df(3R)P9* embryos lacking all BX-C function (Lewis 1978; Sanchez-Herrero et al. 1985), *Antp* expression in PS6–PS12 (pT3–aA7) increases to the level found in PS4 and PS5 (pT1–aT3). It has not been determined previously whether this effect results from derepression of *Antp* P1, *Antp* P2, or both promoters. To ask whether endogenous P2 is derepressed in *Df(3R)P9* embryos, *Antp* protein expression was examined in embryos homozygous for both *Antp*^{73b} and *Df(3R)P9*. As is described above, the *Antp*^{73b} mutation interrupts the P1 transcription unit; in homozygous mutant embryos, only P2 transcripts contribute to *Antp* protein production, and the pattern of P2 expression is similar to that of *Antp*^{s2} mutants (Fig. 2e). The *Antp*^{73b} *Df(3R)P9* double-mutant embryos have a high level of *Antp* protein in the first through the ninth abdominal segment (A1–A9), as well as in the thoracic segments (Fig. 6a; cf. the *Antp*^{73b} pattern in Fig. 2e). This result indicates that in wild-type embryos, BX-C products repress P2 function in PS6–PS14 (pT3–aA9). In previous studies in which both P1 and P2 expression were examined, derepression was only observed in PS6–PS12. Presumably, only P1-derived protein was detected, and P1 is not derepressed in PS13–PS14 (pA7–aA9) in BX-C[−] embryos. The failure to detect an increased level of *Antp* protein in PS13 and PS14 may have been due to the lower sensitivity of the polyclonal antibody used relative to the monoclonal antibody used here. The results suggest that a gene, or genes, other than those of the BX-C, keeps P1 expression at low levels in PS13 and PS14.

To determine whether an *Antp* P2 fusion gene is subject to the same controls as the endogenous promoter, the 10d9.1 chromosome was transferred into a *Ubx*[−] background [*Ubx*¹, (Bender et al. 1983) or *Ubx*^{6.28} (Beachy et al. 1985)], a *Ubx*[−]*abd-A*[−] background [(*Df(3R)Ubx*¹⁰⁹ (Karch et al. 1985)], and a *Ubx*[−]*abd-A*[−]*Abd-B*[−] background [*Df(3R)P9* (Lewis 1978)]. In *Ubx*[−] embryos, *Antp* protein (P1 plus P2) expression in PS6 (pT3–aA1) increases to the levels seen in PS4 and PS5 (Carroll et al. 1986; Fig. 6c). β -Galactosidase expression driven by 10d9.1 in the same embryo is at a high level in

Figure 5. Expression of β -galactosidase in stage-12 embryos from 10-, 4-, 2-, and 0.6-kb fusion genes. Anterior is to the left, and ventral is down in all photographs. Each embryo shows 'head' expression, as described in the text (open arrows). (a) Expression of β -galactosidase in the developing VNS of a 10d9.1 stage 12 embryo. (b) 4d9.5 embryo. (c) 2d9.7 embryo. Small spots of β -galactosidase staining are visible on the lateral sides of the embryo (small arrows). (d) 2d9.7 embryo at a slightly earlier developmental time than c. (e) 0.6d9.7 embryo.

Figure 6. Expression of the 10d9.1 fusion gene in embryos mutant for BX-C genes, *ftz*, and *Antp*. Anterior is to the left, and ventral surface is seen face on. (a) *Antp*^{73b}*Df(3R)P9* homozygous embryo stained with anti-*Antp* antibody. A high level of *Antp* expression is seen from T1 through A8/9. (b) *Antp*^{w10}/*Antp*^{w10} embryo stained with anti- β -galactosidase antibody. (c) *Antp* protein expression in a *Ubx*¹/*Ubx*¹ embryo. High-level expression extends through aA1. (d) β -Galactosidase expression in the same embryo shown in c. The A1 neuromere expresses β -galactosidase at a level equal to that of the thoracic neuromeres. (e) *Antp* expression in a *Df(3R)Ubx*¹⁰⁹ homozygous embryo. *Antp* protein is at high levels through aA7. (f) The same embryo as e stained with anti- β -galactosidase antibody. β -Galactosidase expression is at approximately equal levels from T1 through A7. (g) A *ftz*^{w20}/*ftz*^{w20} embryo stained with anti-*Antp* antibody. Thoracic (T) and abdominal (A) segments of the VNS are labeled. The severe disruption of the normal pattern makes precise assignments of segment identities uncertain. (h) β -Galactosidase expression in the embryo pictured in g; labeling as in g.

A1 (posterior PS6), as well as in the three thoracic segments of the VNS (Fig. 6d). Therefore, the loss of *Ubx* function derepresses the 10-kb *P2-lacZ* fusion in the A1 neuromere, i.e., in cells where *Ubx* protein is normally at high levels.

In a *Ubx*⁻ *abd-A*⁻ [*Df(3R)Ubx*¹⁰⁹] mutant background, the thoracic level of β -galactosidase expression from 10d9.1 extends from T1 through A7 (Fig. 6f). This corresponds to *Antp* protein expression in the same mutant embryo [Fig. 6e; Carroll et al. 1986]. In *Df(3R)P9* homozygous embryos that lack all BX-C function, high-

level β -galactosidase expression is seen all the way back through A8 and A9 (data not shown). This is the extent of high-level expression that is observed with the endogenous *P2*-derived protein in the *Antp*^{73b} *Df(3R)P9* experiment described above. Therefore, it appears that the 10-kb of *Antp* sequence contains the requisite *cis*-acting elements for responding to three homeo-domain-containing proteins. The 2d9.13 transformant was also crossed into *Df(3R)Ubx*¹⁰⁹ and was expressed at high levels from T1 through A7 (not shown). Therefore, the 2-kb *Antp* sequence contains elements that mediate re-

pression by at least *Ubx* and *abd-A*.

A third gene that has been shown to regulate *Antp* is the pair-rule segmentation gene *ftz*. In the absence of *ftz* function, the embryo is divided into 7 parasegments instead of 14; the even-numbered parasegments do not form (Wakimoto and Kaufman 1981). *ftz* is required to activate P2 (but not P1) transcription in PS4, where both *ftz* and P2 are expressed; the high level of P2 transcripts normally seen in PS4 at the late blastoderm stage is not seen in *ftz*⁻ embryos (Ingham and Martinez-Arias 1986). The distribution of P2 transcripts in *ftz*⁻ embryos at later stages of development has not been reported.

The 10d9.1 P2-*lacZ* fusion behaves similarly to the endogenous gene in its response to the absence of *ftz* function. *Antp* protein (from P1 and P2) in *ftz*⁻ embryos is found at higher levels in the 'T1' and 'T3' neuromeres than in the remaining segments of the VNS. The VNS structure often is disrupted badly, and the levels of *Antp* protein in the abdominal region of the VNS occasionally appear as high as in the 'thoracic' neuromeres (Fig. 6g). In the VNS, β -galactosidase from 10d9.1 is expressed at high levels in the two thoracic neuromeres and sometimes at nearly equivalent levels in abdominal neuromeres (Fig. 6h). At early stages, the β -galactosidase pattern is also similar to the total *Antp* protein pattern (not shown).

Hiromi and Gehring (1987) have presented evidence that the *ftz* protein, which contains a homeo domain, regulates its own expression. To ask whether P2 is regulated by *Antp* protein in an analogous autoregulatory loop, we have crossed the 10d9.1 construct into an *Antp*⁻ background. *Antp* protein is undetectable in *Antp*^{w10} homozygous embryos, even with the monoclonal antibody (Carroll et al. 1986; A.M. Boulet, unpubl.). The absence of *Antp* protein does not affect the overall pattern of β -galactosidase expression from the 10d9.1 fusion gene (Fig. 6b). However, the pattern of stained cells within each neuromere appears different from that in wild-type embryos. This may indicate that VNS differentiation is altered in the absence of *Antp* protein. Further experiments will be required to determine whether *Antp* plays a role in the development of VNS structure in the embryo. Thus, there is no evidence that *Antp* protein is essential for activating P2 in the VNS, and the 10d9.1 pattern is not merely a reflection of *Antp* protein distribution. Therefore, it is unlikely that the derepression of 10d9.1 due to the loss of *Ubx* or *abd-A* function is mediated by derepression of endogenous *Antp* expression.

Discussion

Homeotic gene expression is activated and maintained through the action of a network of regulatory genes—including several cases that we have discussed—which controls pattern formation in the *Drosophila* embryo. In general, eukaryotic genes expressed in complex spatial and temporal patterns have correspondingly complex regulatory regions. Control elements are often distrib-

uted over several kilobases of DNA and may be found both upstream and downstream of the transcription initiation site. Many of these elements possess the properties of enhancers in that they act at a distance and can activate a heterologous promoter. The patterns of homeotic gene expression are the most complex of any of the known genes in the *Drosophila* regulatory network and, therefore, may result from the largest number of regulatory inputs. The large size of the homeotic genes may reflect the need to integrate signals from a large number of regulators.

We have roughly mapped several elements that direct expression of a homeotic gene in cells in specific positions and at the correct times of development. The control elements of *Antp* P2 that we have localized are found both >4 kb and <2 kb from the P2 start site. We have also presented evidence that additional elements are located >10 kb upstream of the cap site, perhaps as distant as 35 kb. The action of enhancers from distant sites of the DNA has been documented in many cases. For example an immunoglobulin enhancer has been found to function over >17 kb (Wang and Calame 1985). The control region of another homeotic gene, *Ubx*, extends over at least 35 kb upstream of the regulated transcription unit (Bender et al. 1985; Hogness et al. 1985). In keeping with these mutation-mapping studies, gene fusions with up to 8 kb of upstream *Ubx* sequence joined to *lacZ* have only some correct aspects of *Ubx* expression (Bienz et al. 1988). Therefore, the large regions necessary for correct *Antp* P2 expression are within the size ranges for other large and complex genes.

Important control elements are located close to the transcriptional start site in many simpler and smaller genes. Otherwise lethal mutations in the *ftz* segmentation gene can be complemented with a fragment 10.5 kb long, containing ~6 kb of upstream sequence (Hiromi et al. 1985). Mutations in another regulatory gene called *extra sex combs* can be rescued with a 12-kb fragment (Frei et al. 1985); the amount of upstream control sequence in the fragment has not been reported. All of the sequences necessary for the function of the *rosy* (xanthine dehydrogenase) gene are contained in an 8-kb fragment containing ~3.5 kb of upstream sequence (Rubin and Spradling 1982; Keith et al. 1987). A 3.3-kb DNA fragment provides everything needed for function of the ribosomal protein gene *rp49* (Kongsuwan et al. 1985); this fragment contains 2.1 kb of upstream sequence. These examples show that *Antp* is likely to have a more complex *cis*-acting element structure than many genes that are expressed in simpler spatial and temporal patterns but may well be typical, in its regulatory complexity, of homeotic genes.

Correspondence between the normal P2 pattern and the β -galactosidase patterns

The DNA sequence extending from 10 kb upstream to 200 bp downstream of the *Antp* P2 cap site is capable of directing β -galactosidase expression in a spatially and temporally regulated pattern in *Drosophila* embryos.

This pattern correlates well with *Antp* protein expression in *Antp* mutant embryos in which only the P2 transcription unit is intact.

The major discrepancy between the gene fusion expression pattern and the normal *Antp* P2 pattern is in the head primordia, where the fusion constructs are expressed at high levels in cells that do not make *Antp* mRNA and protein. Because head expression is observed in almost all lines, it is unlikely to be due to vector sequences (it is seen with both C4hsneo and CaSpeR constructions) or to position effects. The β -galactosidase expression in the head region could be due to the absence of sequences that normally repress *Antp* P2 transcription in these cells.

Locations of cis-acting elements acting on P2

Deletions from the 5' end of the 10-kb sequence have allowed us roughly to map a number of regulatory elements of *Antp* P2. Sequences necessary for PNS expression lie between 4 and 10 kb upstream of the P2 start site. (The single 5.6-kb line allows only a tentative placement of the 3' boundary at 5.6 kb.) Sequences involved in controlling the relative levels of expression in specific cells of the thoracic ganglia are located between -2 and -4 kb. The lack of proper VNS expression in 0.6-kb lines indicates that elements essential for generating the pattern of P2 transcription in the VNS may be located between 0.6 and 2 kb upstream of the cap site.

The neural-specific regulation of *Antp* P2 is reminiscent of the neural-specific regulatory element of the *ftz* segmentation gene (Hiromi et al. 1985; Hiromi and Gehring 1987) and an element of the dopa decarboxylase gene specific for the central nervous system (Beall and Hirsh 1987). Like *Antp*, the *Ddc* gene contains at least two separate elements required for nervous system expression (Beall and Hirsh 1987). Element I is located between -83 and -59 bp, with respect to the transcription start site. A second element is found between -2.2 and -0.8 kb. In *Antp* P2, sequences within the region from -4 to -2 kb regulate expression in specific cells of each neuromere. Sequences located between -2 and +0.2 kb are required for VNS expression in the basic P2 pattern, high levels in the thoracic neuromeres, and lower levels in the abdominal neuromeres. Further characterization of the *Antp* P2 elements will be required to determine whether they share other properties of previously identified tissue or cell-type-specific elements.

The 4d9 transformant lines have strong β -galactosidase expression in the Lb and Mx neuromeres. Expression of β -galactosidase in the Lb and Mx segments can also be detected in 10d9 lines, as can analogous expression of *Antp* protein in *Antp^B* mutant embryos, but at much lower levels. Although Wirz et al. (1986) detected *Antp* protein in these neuromeres, other anti-*Antp* antibodies reveal no staining anterior to PS3 (Carroll et al. 1986; this paper). In addition, *Antp* mRNAs are not detectable anterior to PS3 (Levine et al. 1983; A. Martinez-Arias, J. Bermingham, and M. Scott, unpubl.). Lb and Mx expression with only 10 kb (the 10d9 fusions) or 10.5 kb

(*Antp^B*) of P2 upstream sequence indicates that elements upstream of -10 kb normally repress Lb and Mx expression. Because 4-kb fusions have a higher level of Lb and Mx expression than 10-kb fusions, there may be additional sequences between -10 and -4 kb that contribute to full repression. β -Galactosidase is not detected in the Lb and Mx neuromeres in 2d9 lines, indicating that there is a sequence between -2 and -4 kb required for Lb and Mx expression.

Other differences between 10d9.1 β -galactosidase expression and endogenous P2 expression may simply reflect differences in protein stability between β -galactosidase and *Antp* protein. The β -galactosidase expression in the ventrolateral epidermis of 10d9 transformant lines (Fig. 3h) is paralleled by *Antp* protein in *Antp^{73b}* embryos but not in *Antp^{s2}* or *Antp^B* embryos. If DNA located between the breakpoints of *Antp^{73b}* (-45 kb) and *Antp^{s2}* (-35 kb) is responsible for the epidermal expression seen in *Antp^{73b}*, then the similarity of the 10d9 pattern to the *Antp^{73b}* pattern is not easily explained. One possibility is that two separate control elements are required for ventrolateral epidermal expression. One element, located within 10 kb of the P2 start site, may direct a low level of ventrolateral expression, and the second element, located between -45 and -35 kb, may act as an enhancer. In *Antp^{s2}* and *Antp^B*, the level of protein in ventrolateral cells could be too low to detect with antibody staining. However, if β -galactosidase is more stable than *Antp* protein, a low level of transcription in the 10d9 lines may result in the accumulation of detectable levels of β -galactosidase, even in the absence of the upstream enhancer. A second possibility is that the ventrolateral expression in *Antp^{73b}* embryos is due to the foreign promoter that is juxtaposed to *Antp* by the inversion (Frischer et al. 1986; Schneuwly et al. 1987). Post-transcriptional controls acting only on the endogenous gene products may also contribute to differences between endogenous and fusion gene expression.

The 0.6d9 and 2d9 constructs are expressed in early striped patterns that are not seen either with the longer constructs or in the course of normal *Antp* expression. The stripes appear at the extended germ-band stage, when the segment polarity genes are expressed in one stripe per segment. Short *Ubx-lacZ* fusions are expressed in similar stripes (Bienz et al. 1988). In that case, the stripes have been shown to be coincident with, and dependent upon, the striped expression pattern of the segment polarity gene *engrailed*. Many segmentation genes, including *engrailed* (Fjose et al. 1985; Poole et al. 1985), encode proteins that contain homeo domains, and the striped patterns seen with both *Ubx* and *Antp* P2 may be due to the presence of homeo domain binding sites in the constructs used and to inappropriate action upon the sites by segmentation gene products. In the intact genes, homeo domain binding sites might mediate regulation by only appropriate *trans*-acting regulators. The 4d9 constructs do not give the early striped pattern, suggesting that sequences between -4 and -2 kb block the anomalous striped expression.

Many of the regulatory sequences inferred from the

deletion analysis probably interact with protein factors whose identity is presently unknown. In contrast, some of the genes in the known regulatory network, including *ftz* and the BX-C genes, clearly act, directly or indirectly, on *Antp* P2 sequences.

P2 responses to trans-acting regulators: a target of action of homeo-box-containing genes

We have begun to localize sequences that mediate the response to the known *trans*-acting regulators of *Antp*. It is not known whether interactions with the products of genes such as *ftz*, *Ubx*, and *abd-A* are direct. In embryos that lack *ftz* function, the 10-kb construct is expressed in a pattern similar to that of the endogenous *Antp* gene, although the relative contributions of P1 and P2 to the endogenous pattern are not known. This provides further evidence that the 10d9 construct is responding to the normal regulators of *Antp*. In the absence of all BX-C function (*Ubx*⁻, *abd-A*⁻, and *Abd-B*⁻, i.e., in *Df(3R)P9* homozygotes), the endogenous P2 promoter is expressed at high levels in A1 through A9. Based on the known domains of BX-C gene function [reviewed in Duncan 1987], this result suggests that in wild-type embryos, *Ubx* negatively regulates P2 expression in A1, *abd-A* negatively regulates *Antp* in A2–A7, and *Abd-B* acts negatively in A8 and A9. The expression of β -galactosidase from the 10d9.1 and 2d9.13 *Antp* P2 fusion genes is altered in the absence of the *Ubx*, *abd-A*, and *Abd-B* gene products. The results with the 2-kb constructs indicate that *Antp* P2 sequences susceptible to negative regulation by *Ubx* and *abd-A* lie between 2 kb upstream and 200 bp downstream of the cap site. The 0.6d9 constructs are not expressed differentially in the thoracic versus abdominal segments, suggesting that a target of *Ubx* and *abd-A* action lies between 2 and 0.6 kb upstream of P2. More refined mapping of *cis*-acting elements, together with biochemical analyses, should permit a full understanding of how homeo domain-containing proteins control other homeotic genes.

Materials and methods

Drosophila strains

The *rosy*⁵⁰⁶ stock used for embryo injection was provided by A. Spradling. *Df(1)w,67cly* flies (obtained from V. Pirrotta, formerly *67c23y*) contain a deletion of the *white* gene, thus producing white-eyed flies (Goldberg et al. 1982). The mutant alleles used were *ftz*^{w20} [no detectable *ftz* protein due to a 4.9-kb insertion within the transcription unit (Weiner et al. 1984; Carroll and Scott 1985)], *Antp*^{w10} [no detectable *Antp* protein (Carroll et al. 1986; A.M. Boulet, unpubl.)], *Df(3R)Ubx*¹⁰⁹ [a deletion removing both *Ubx* and *abd-A* gene function (Karch et al. 1985)], *Ubx*¹ [no detectable *Ubx* protein in embryos due to a Doc element insertion in the 5' exon (Bender et al. 1983; Weinzierl et al. 1987)], *Ubx*⁶²⁸ [no detectable *Ubx* protein due to a deletion of 32 bp in the 5' exon, which causes a frameshift and premature termination of translation (Beachy et al. 1985)], and *Dp(3;1)P68; Antp*^{73b} *Df(3R)P9,red/In(3LR)Cx,Sb* (provided by I. Duncan).

Plasmid constructions

The *C4neo-lacZ* vector was constructed as follows: The *Hind*III fragment of *cosPneo* (Steller and Pirrotta 1985) containing the neomycin resistance gene, driven by the *hsp70* promoter and the 5' P element end, was joined to *Hind*III-digested Carnegie 1 (Rubin and Spradling 1983), which provided the 3' P element end, as well as pUC8 sequences. This plasmid was called *C4neo*. An *Eco*RI fragment containing the alcohol dehydrogenase (*Adh*) gene initiator ATG joined to the *lacZ*-coding region, followed by a polyadenylation site from SV40 (provided by C. Thummel), was blunt-ended and *Sall* linkers were added. The *Sall* fragment was then inserted into the *Sall* site of *C4neo* to create *C4neo-Adh-lacZ*, or simply *C4neo-lacZ* (Fig. 1c). *C4neo-Antp-lacZ* plasmids were produced by inserting restriction fragments containing 10, 5.6, 4, 2, or 0.6 kb of sequences upstream and 200 bp of sequences downstream of the P2 cap site [the downstream site resulted from a deletion generated for DNA sequencing (Laughon et al. 1986)] into polylinker sites adjacent to the *Adh-lacZ*-coding region. Constructions of 4, 2, and 0.6 kb in *CaSpeR* (a gift from V. Pirrotta) were produced by transferring the appropriate *Antp-Adh-lacZ* portion from a *C4neo-lacZ* plasmid to the *CaSpeR* vector. In both vectors, the direction of *Antp* transcription is opposite that of the *white* or *neo* marker gene, and the 3' end of *Adh-lacZ* is adjacent to the P-element end.

Germ-line transformation

Host embryos, *ry*⁵⁰⁶ or *Df(1)w, 67cly*, were injected with a solution of 100 μ g/ml π 25.7wc helper DNA (Karess and Rubin 1984) and 350–400 μ g/ml *C4neo-Antp-lacZ* or *CaSpeR-Antp-lacZ* DNA. Transformed G₁ progeny were identified by their ability to survive selection on G418 after heat shock treatment (Steller and Pirrotta 1985) or by restoration of eye color. The chromosome linkage of each insert was determined by following the segregation of the marker in consecutive crosses with balancer lines (Table 1). Appropriate balancer chromosomes were used to establish transformant stocks for the lines that would not survive as homozygotes. With *CaSpeR* transformants, it was possible in many cases to create homozygous lines by selecting flies with a darker eye color.

Blot analyses of genomic DNA were used to check the integrity of P element inserts, to identify lines likely to have inserts at the same site (lines derived from the same injected parent), and to estimate insert copy number.

Analysis of β -galactosidase expression patterns

Embryos from each transformant line were examined for β -galactosidase activity using an X-gal assay (adapted from Simon et al. 1985). Embryos in small plastic baskets were dechorionated in 50% bleach and fixed in a 1 : 1 mixture of 4% paraformaldehyde [in 100 mM PIPES (pH 6.9), 2 mM EGTA, 1 mM MgSO₄] and heptane for 20–25 min. Embryos were then rinsed with heptane, drained well, rinsed twice with 1 $\times$ PBS, 0.1% Triton X-100, transferred to 1.5-ml tubes, and soaked in 1 $\times$ PBS, 0.1% Triton X-100 for 1.5–2 hr to remove paraformaldehyde. After a brief incubation in X-gal assay buffer [10 mM Na phosphate (pH 7.2), 150 mM NaCl, 1 mM MgCl₂, 3.1 mM K ferrocyanide, 3.1 mM K ferricyanide, 0.1% Triton X-100], embryos were stained in assay buffer plus 0.2% X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) at room temperature for 10 min to several hours. Embryos were mounted in 1 $\times$ PBS, 10% glycerol.

The expression of β -galactosidase in representative lines for each fusion construct was examined by indirect immunofluorescence (adapted from Carroll and Scott 1985). Embryos were

dechorionated in 50% bleach and fixed directly in a 1 : 1 mixture of 4% paraformaldehyde and heptane for 10–13 min. After removing the aqueous phase, absolute methanol was added and embryos were devitellinized by shaking for 1–2 min. Embryos were rinsed three times with methanol, once with 50% methanol, 0.5× PBS, 0.05% Triton X-100, and several times in PBSBT (1× PBS, 0.1% Triton X-100, 1% BSA). Antibody staining was carried out according to the procedure of Carroll and Scott (1985). Primary antibodies were polyclonal anti- β -galactosidase (provided by P. Riley and S. Carroll), monoclonal anti-*Antp* (4C3, provided by D. Brower), and monoclonal anti-*Ubx* (FP3.38, White and Wilcox 1984). Secondary antibodies were fluorescein-conjugated goat anti-rabbit IgG (Cappel) and rhodamine-conjugated goat anti-mouse IgG (Boehringer-Mannheim). Embryos were mounted in 430 μ g/ml *p*-phenylenediamine, 8.6% glycerol, and 43 mM Tris (pH 8.8) and photographed on Kodak Technical Pan film, which had been hypersensitized in a Lumicon Hyper Kit 1200 (Lumicon; Livermore, California) for 15 days at 35°C.

To examine the effect of *ftz*, *Antp*, *Ubx*, *Ubx* plus *abd-A*, and *Ubx* plus *abd-A* plus *Abd-B* mutations on the expression of the 10d9.1 construct, flies were generated that contained both the 10d9.1 insert and a third chromosome carrying the mutation. Embryos from these flies were assayed or stained by immunofluorescence, as described above. Mutant embryos were identified by morphology (*ftz*⁻) or by alterations in the *Antp* protein pattern visualized by *Antp* antibody staining (Carroll et al. 1986). To study the effect of deletion of the BX-C on expression from P2, *Dp(3;1)P68;Antp^{73b}Df(3R)P9* flies were first outcrossed to produce females heterozygous for the BX-C duplication *Dp(3;1)P68*. These females were then mated to males from the original stock. One-sixteenth of the progeny of the second cross should be BX-C⁻ and lack P1 function.

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