Different patterns of transcription from the two Antennapedia promoters during Drosophila embryogenesis

JOHN R. BERMINGHAM, Jr1*, ALFONSO MARTINEZ-ARIAS2, MATTHEW G. PETITT1 and MATTHEW P. SCOTT1

1Howard Hughes Medical Institute and Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, CO 80309-0347, USA
2Department of Zoology, Downing Street, Cambridge CB2 3EH, UK

*Present address: Howard Hughes Medical Institute, U.C. School of Medicine, 3rd and Parnassus Avenues, U-426, San Francisco, CA 94143, USA

Summary

The homeotic genes of Drosophila control the differentiation of segments during development. Mutations in these genes cause one or more segments to develop structures normally found elsewhere in the organism. Several studies have shown that the spatial patterns of homeotic gene transcription are highly complex, and that these precise patterns of transcription are critical to normal development. The homeotic gene Antennapedia (Antp), a member of the Antennapedia Complex, is required for the correct differentiation of thoracic segments in both embryos and adults. The patterns of total Antp transcript and protein accumulation have been described in detail, but the contribution of each promoter to the overall pattern in embryos has not been reported. We have examined in detail the spatial distribution of transcripts from each of the Antp promoters in both embryo sections and whole embryos by in situ hybridization using promoter-specific probes. We show that the transcripts from each of the two promoters accumulate in distinct, but overlapping patterns during embryogenesis. The results demonstrate that the two Antp promoters are differentially regulated in embryos and provide a basis for examining the regulation of the two promoters and characterizing more fully the function of Antp during embryogenesis. In addition, we have examined the regulation of each of the Antp promoters by genes of the bithorax complex (BX-C). We show that in BX-C- embryos both promoters are derepressed in the abdomen.

Key words: promoters, homeotic genes, spatial expression, parasegments.

Introduction

The segments of the Drosophila embryo differentiate along distinct developmental pathways, providing each segment with a unique set of structures appropriate to its position along the body axis. Genetic analyses have identified several classes of genes required for the normal development of embryonic segments (reviewed in Nüsslein-Volhard et al. 1987; Akam, 1987; Scott and Carroll, 1987; Ingham, 1988). Maternally acting genes are required for the establishment of the anterior-posterior and dorsal-ventral embryonic axes. A set of zygotically active genes also controls pattern formation along the dorsoventral axis. The zygotically active segmentation genes are required to make the correct number of segments, and the correct pattern and orientation of structures within each segment. In addition, some of the segmentation genes affect the identities of segments, i.e. which segments form head or thoracic or abdominal structures. Mutations in the 'gap' segmentation genes, for example, lead to a failure to form particular groups of segments, and, in some cases, the identities of remaining segments are altered as well. Segment-specific differentiation is also controlled by the homeotic genes; mutations in these genes cause one or more segments to follow the developmental pathway characteristic of another segment.

To learn how genes control differentiation, many of the homeotic genes and their products have been characterized. Two important conclusions have emerged from this work. One is that homeotic genes are transcribed in complex spatial patterns that change during development. Second, many homeotic genes encode transcriptional regulators. The array of homeotic genes expressed in a cell, in concert with other regulators, appears to determine the fate of the cell.

Many of the known homeotic genes are clustered in two complexes, the Antennapedia complex (ANT-C; Kaufman et al. 1980) and the bithorax complex (BX-C; Lewis, 1978). Different members of the ANT-C and
BX-C are expressed, and function, in different regions along the anterior–posterior axis of the embryo (reviews, op. cit.). There are also dorsal–ventral differences in where genes are active. The initial expression of the homeotic genes is spatially restricted; the patterns are maintained and refined as development proceeds. It appears that the early patterns of expression of the homeotic genes are regulated by gap, pair-rule, and segment polarity segmentation genes (Ingham and Martinez-Arias, 1986; Duncan, 1986; White and Lehmann, 1986; Riley et al. 1987; Martinez-Arias and White, 1988; Irish et al. 1989). Later in development, the patterns are modified and maintained by interactions among the homeotic genes. The transcription of those homeotic genes that are primarily expressed in more anterior domains appears to be repressed by the action of homeotic genes expressed in more posterior domains (Hafen et al. 1984; Harding et al. 1985; Struhl and White, 1985; Carroll et al. 1986; Wirz et al. 1986; Riley et al. 1987).

Several lines of evidence suggest that the protein products of homeotic genes are transcriptional regulators (reviewed in Gehring, 1985; Levine and Hoey, 1988; Scott et al. 1989; Mitchell and Tjian, 1989). Many homeotic genes contain a conserved DNA sequence, the homeobox (McGinnis et al. 1984; Scott and Weiner, 1984), that encodes a protein motif, the homeodomain, with predicted structural homology to bacterial and yeast DNA-binding regulatory proteins (Laughon and Levine, 1984). Human homeo-box-containing proteins bind specific DNA sequences (Desplan et al. 1985, 1988; Hoy and Levine, 1988; Beachy et al. 1988; Laughon et al. 1988). Several Drosophila homeodomain-containing proteins also regulate transcription in cultured cells or yeast (Jaynes and O’Farrell, 1988; Thali et al. 1988; Fitzpatrick and Ingles, 1989; Han et al. 1989; Samson et al. 1989; Winslow et al. 1989; Krasnow et al. 1989); one has been shown to do so in embryos (Driever and Nusslein-Volhard, 1989) and several mammalian transcription factors have been found to contain homeodomains (Ko et al. 1988; Bodner et al. 1988; Ingraham et al. 1988; Sturm et al. 1988; Scheidereit et al. 1988). These findings strongly support the idea that homeodomain-containing proteins regulate transcription and suggest that the distribution of transcription factors in precise spatial arrays in the developing embryo is a major mechanism of developmental control. Differences between cells in the array of transcription factors they contain presumably lead to differences in developmental fate. Thus, in order to understand the role of homeotic genes in segment differentiation, it is important to understand how the patterns of homeotic gene expression are generated.

The Antennapedia (Antp) gene, which contains a homeobox and is located in the ANT-C, plays a major role in the differentiation of the thoracic segments. Dominant and recessive mutations in the Antp gene cause transformations involving thoracic structures. Most Antp mutations are recessive lethals; homozygous mutant embryos display transformations of the second and third thoracic segments toward the first thoracic segment (Denell et al. 1981; Wakimoto and Kaufman, 1981). The first metameric divisions of the embryo are parasegmental rather than segmental (Martinez-Arias and Lawrence, 1985). The precise features of the transformation in Antp~ embryos suggest that parasegment (PS) 4, which consists of the posterior compartment of T1 and the anterior compartment of T2, is transformed into PS 3, and PS 5 into a hybrid with features of both PS 3 and PS 6 (Martinez-Arias, 1986). Certain dominant mutations activate the gene ectopically, resulting in antenna to leg transformations (Denell, 1973; Struhl, 1981; Hazelrigg and Kaufman, 1983; Frischer et al. 1986; Schnewly et al. 1987a,b; Gibson and Gehring, 1988) or a transformation of dorsal head toward dorsal thorax (I. Duncan and E. Lewis, unpublished data; Scott et al. 1983).

The Antp locus has a complex structure that is reflected genetically as intragenic complementation. Molecular analyses have revealed that Antp exons are distributed over a 100 kb region, and that Antp transcripts are initiated from two promoters, P1 and P2, separated by approximately 65 kb (Scott et al. 1983; Garber et al. 1983; Schnewly et al. 1986; Stroehrer et al. 1986; Laughon et al. 1986) (Fig. 1). Transcripts initiated from either promoter contain the same protein-coding exons. Antp mutations that primarily affect one transcription unit apparently can complement Antp mutations that affect the other transcription unit. Many Antp mutations are associated with chromosome rearrangements that have one breakpoint between the P1 and P2 promoters, thereby interrupting the P1 transcription unit (Fig. 1; Scott et al. 1983; Garber et al. 1983). Such mutations complement the recessive allele Antp~ (Kaufman and Abbott, 1984; Abbott and Kaufman, 1986), a mutation in which a transposon is inserted into an exon that is only included in P2 transcripts (e.g. Antp psb, Fig. 1; Scott et al. 1983; Laughon et al. 1986). Thus, mutations that destroy the P1 transcription unit can provide the P2 function that is damaged by the Antp~ mutation, and the Antp~ allele can provide P1 transcription unit function. These results suggest that the Antp gene has at least two separable functions, which correspond to the P1 and P2 transcription units. In addition to the intragenic complementation, somatic clonal analyses suggest that the two promoters have different functions: the P1 promoter is necessary for proper dorsal thoracic development in adults, while the P2 promoter is necessary for proper leg development (Abbott and Kaufman, 1986). The comparative functions of the two promoters in the embryo have not been determined.

In the imaginal discs, the primordia of adult tissues, the two Antp promoters are expressed in different patterns, implying that they are controlled by different regulators. P1 transcripts are located primarily along the anterior margins of the wing and mesothoracic leg imaginal discs, while P2 transcripts are more evenly distributed in these discs. Both promoters are also expressed in the prothoracic and metathoracic leg discs (Jorgensen and Garber, 1987). In embryos, Antp RNA
Expression patterns of the two Antp promoters

has been detected in thoracic ectoderm and mesoderm, as well as in much of the ventral nervous system, using nucleic acid probes (Hafen et al. 1983; Levine et al. 1983; Hafen et al. 1984; Martinez-Arias, 1986; Harding et al. 1985; Wedeen et al. 1986; Ingham and Martinez-Arias, 1986) and antibody probes (Carroll et al. 1986; Wirz et al. 1986). However, the RNA localization studies have only examined expression of the P1 promoter, or the sum of the P1 and P2 contributions, and the antibody probes cannot distinguish between the contributions of the P1 and P2 promoters in wild-type embryos.

Previous studies of Antp expression during embryogenesis have left several important questions unanswered. When and where do Antp P1 and P2 transcripts accumulate in the embryo? Do the expression patterns differ from those of Antp proteins, indicating posttranscriptional regulation? Do homeotic genes of the BX-C, which are known to negatively regulate Antp, act on one or both promoters? In this paper, we compare the separate tissue- and stage-specific expression patterns of the P1 and P2 promoters during embryogenesis. In addition, we describe the expression of the two promoters in embryos lacking the BX-C genes.

Materials and methods

Construction of probes

Two different pairs of Antp promoter-specific probes were used in these experiments. One pair, P1A and P2A, consisted of a 4 kb EcoRI exon A-containing (Fig. 1) fragment from phage A577 (Scott et al. 1983) cloned into pSP64, and a 1.9 kb EcoRI fragment from phage 448 (Scott et al. 1983) containing 657 bp of exon C cloned into pSP64. The other pair of probes, P1B and P2B, was constructed as follows: P1B: a 1216 bp SacI–Acl fragment containing the P1 promoter and 835 bp of exon a was cloned into pGEM2 that had been cut with HindIII, partially filled in to accept the Acl sticky end, and then cut with SacI. After cutting with XhoI, this subcloned DNA served as a template to produce T7-generated transcripts containing 784 bases of exon A. The template for P1B DNA probes was a gel-purified XhoI–Acl fragment from a separate subclone of this region. P2B: a 1901 bp HindIII–ClaI fragment containing the P2 promoter and 1283 bp of exon C was cloned into pGEM-1 that had been cut with HindIII and Acl. After digestion with AvaiI, this subcloned DNA served as a template to produce T7-generated transcripts containing 796 bases of exon C. For DNA probes, we isolated the AvaiI–XhoI fragment containing the same 796 bp of P1 sequence plus about 10 bp of vector sequence.

The INTRON probe, containing intron sequences between P1 and P2 was constructed as follows: R4.6 is a 4.6 kb EcoRI fragment derived from phage 448 (Scott et al. 1983) and contains the P2 promoter and 663 bp of exon C (Laughon et al. 1986). R4.6p-35 is a deletion subclone of R4.6 generated for sequencing; it lacks the P2 promoter and exon C sequences, and contains a BamHI site, introduced during deletion construction, at position 520 in the sequence of Laughon et al. (1986), a 3.8 kb EcoRI–BamHI fragment consisting of the entire R4.6p-35 insert was cloned into pGEM-2 that had been cut with BamHI and EcoRI. After digestion of the construct DNA with EcoRI, it was used as a template to produce T7-generated RNA transcripts. The HB probe was a 1.7 kb EcoRI fragment isolated from phage A2015 and the D-G probe template was a 3.1 kb EcoRI fragment isolated from phage A77 (Scott et al. 1983). The cDNA template was a 1.7 kb fragment containing the sequence between the BafI site at position 144 and the BgII site at position 904 in the Antp sequence (Laughon et al. 1986). This fragment contains part of exon D, all of exons E, F, and G (long form; see Bermingham and Scott, 1989), and part of exon H.

RNAs were labelled by the incorporation of 35S-ribonucleotides during in vitro transcription using the T7 RNA polymerase. Preparation of embryo sections, hybridization and autoradiography were performed as described by Ingham et al. (1985). Sections were exposed for 3 to 8 days.

Digoxigenin-labelled DNA probes used for whole-mount in situ hybridization were labeled using the Boehringer-Mannheim 'Genius' kit. Digoxigenin conjugated dUTP is incorporated during random primed DNA synthesis. In some cases, the DNA template was digested with frequently cutting restriction enzymes to reduce the sizes of the labeled fragments (C. Oh and B. Edgar, personal communication). Whole-mount in situ hybridization was performed essentially as described by Tautz and Pfeifle (1989), with some modifications. After treatment of embryos with heptane and formaldehyde, either while they are in methanol or while they are in 70% ethanol, they are treated with 0.3% hydrogen peroxide for 2–4 min. This step greatly reduces background (T. Yeh and D. Andrew, personal communication).

Results

Antp transcript-specific probes

For in situ hybridization to Antp transcripts, we used labelled RNA to probe sectioned material and labelled DNA to probe whole embryos. Two different pairs of promoter-specific probes were used (Fig. 1, and see Materials and methods); they differ both in size and in their sequence to other sequences. These probes are called P1A, P2A, P1B, and P2B. In addition, the following probes were used and are illustrated in Fig. 1: a probe designated HB, which contains the Antp homeobox sequence, that should detect both P1 and P2 transcripts; the D–G probe, which contains genomic sequence from exons D through G, that should detect both P1 and P2 transcripts; the INTRON probe, which contains sequence located between the P1 and P2 promoters, that should detect P1-derived transcripts only; and the cDNA probe, which contains the entire protein-coding sequence from exons E through H, as well as some noncoding sequence from exons D, E, and H.

Initiation of Antp transcription at the blastoderm stage

P1 promoter

Antp P1 promoter-specific transcripts are first detected early in nuclear cycle 14 (Foe and Alberts, 1983; stage 5 of Campos-Ortega and Hartenstein, 1985), as the blastoderm cells form. P1-specific transcripts are detectable in a ring encircling the blastoderm embryo between about 40% and 55% egg length at the ventral surface, where 0% is the posterior end of the egg (Fig. 2A). The ring is not uniform in width, being somewhat narrower at the dorsal surface, broader at
Antennapedia gene structure and probes used for in situ hybridization. Antp transcripts are initiated at two promoters, P1 and P2. Exon C is spliced out of P1 transcripts but retained in P2 transcripts. Transcripts initiated at either promoter use the same protein-coding exons, E through H. The alternative 3' end processing of Antp transcripts is indicated by the stippled region in exon H. The coordinates shown (in kb) are from Scott et al. (1983). The positions of the Antp^73b inversion breakpoint and the Antps^1 insertion are shown. Antp^73b is one of several inversion mutations at the Antp locus that separate the P1 promoter from the coding exons, yet complement the Antps^1 allele (Abbott and Kaufman, 1986). Probes: The genomic locations of DNA fragments used to make probes for in situ hybridization are shown above the scale. Filled regions of the rectangles indicate exon sequence. Open regions indicate intron or nontranscribed sequence. The stippled portion of probe HB corresponds to sequence that is included in exon H only when the downstream polyadenylation signal is used. The cDNA probe used for whole-mount in situ hybridization is not shown (see Materials and methods).

Fig. 1. Antennapedia gene structure and probes used for in situ hybridization. Antp transcripts are initiated at two promoters, P1 and P2. Exon C is spliced out of P1 transcripts but retained in P2 transcripts. Transcripts initiated at either promoter use the same protein-coding exons, E through H. The alternative 3' end processing of Antp transcripts is indicated by the stippled region in exon H. The coordinates shown (in kb) are from Scott et al. (1983). The positions of the Antp^73b inversion breakpoint and the Antps^1 insertion are shown. Antp^73b is one of several inversion mutations at the Antp locus that separate the P1 promoter from the coding exons, yet complement the Antps^1 allele (Abbott and Kaufman, 1986). Probes: The genomic locations of DNA fragments used to make probes for in situ hybridization are shown above the scale. Filled regions of the rectangles indicate exon sequence. Open regions indicate intron or nontranscribed sequence. The stippled portion of probe HB corresponds to sequence that is included in exon H only when the downstream polyadenylation signal is used. The cDNA probe used for whole-mount in situ hybridization is not shown (see Materials and methods).

P2 promoter

The expression pattern of the Antp P2 promoter alone has been inferred from studies of mutant embryos. Antp protein in embryos lacking a functional P1 transcription unit is distributed in cells that are a subset of those making Antp protein in wild-type embryos (Boulet and Scott, 1988), suggesting that the two promoters are expressed in different patterns in embryos, as they are in imaginal discs. However, the analysis of protein in these mutants may not truly reflect the P2 transcription pattern for two reasons: First, mutations disrupting the P1 transcription unit may affect P2 function as well. Second, Antp transcripts could be subject to posttranscriptional regulation. Another limitation is that it is difficult to detect the Antp protein at the blastoderm stage.

Antp P2 promoter-specific transcripts are first detected at roughly the same time as P1 transcripts, at the blastoderm stage, but the two classes of transcripts are expressed in different patterns. P2 transcripts initially accumulate in a ring three or four cells wide,
Fig. 3. Whole-mount in situ hybridization. (A, B) The P2 pattern (probe P2B) in two different optical horizontal sections of the same cellular blastoderm stage embryo. Arrowheads indicate the locations of specific parasegments. B shows the ventral surface of the embryo to indicate the extent of P2 expression around the circumference of PS 6. (C) P2 expression in an early gastrula. Most of the PS 6 cells expressing P2 at high levels invaginate at the ventral furrow (arrows). (D–F) cDNA pattern in the lateral ectoderm. At stage 11 (panel D) Antp expression is detected in a small cluster of cells on the lateral surface of each segment from PS 4 to PS 14. The cells are approximately at the center of each parasegment and may be the primordia of peripheral neurons. P2 probes detect a similar pattern of RNA at stage 11, but P1 probes do not (data not shown). During stages 12 (panel E) and 13 (panel F), ectodermal or subectodermal staining remains in a subset of cells within each segment from T2 through A9. A similar pattern is seen with P2 probes, but not with P1 probes. (G) Horizontal view of a stage 14 embryo probed with the cDNA probe. The arrows indicate two patches of cells that express Antp. (H, I) Two horizontal, optical sections of the same stage 16 embryo probed with the cDNA probe. H shows Antp expression in mesodermal cells surrounding one of the midgut constrictions (arrow). The dark, diffuse signal at about T2 is due to ventral nervous system expression, which is out of focus.
Expression patterns of the two Antp promoters

centered around 56% egg-length (Figs 2B, 3A, and 4A). These cells lie in PS 4, three or four cells anterior to the primordium of PS 6. The position of PS 6 was determined using the expression of the Ultrabithorax (Ubx) homeotic gene as a reference (Fig. 4A; Akam and Martinez-Arias, 1985). The segmentation gene fushi tarazu (ftz) is expressed in PS 2, 4, 6, 8, 10, 12 and 14 (Carroll et al. 1988; Lawrence and Johnston, 1989).
ftz expression was also used to localize the *Antp* P2 transcript in adjacent serial sections (data not shown).

Two other bands of P2 expression appear at the cellular blastoderm stage in the primordia of PS 6 and 14. The PS 6 band is limited to a strip of cells at the ventral surface of the blastoderm embryo (Figs 2B and 3B). This band probably corresponds to the mesodermal primordium in PS 6, since most of the staining cells invaginate as part of the ventral furrow (Fig. 3C). Occasionally, very faint staining is seen at the dorsal surface of PS 6, although the dorsal signal is always much weaker than the ventral one (data not shown). The PS 14 stripe appears to encircle most or all of the embryo, being centered at about 12% egg length ventrally and 18% egg length dorsally. In whole embryos, the signal is strongest in horizontal views (Fig. 3A) and is only very weak in sagittal views (Fig. 2B). Embryo sections often contain the PS 4 signal with only one of the other two signals, probably due to the plane of sectioning.

**P1 intron-containing transcripts**

The P2-specific probes could hybridize to unprocessed P1 transcripts, as well as to P2 transcripts, giving a misleading 'P2-specific' pattern. Serial sections of late cellular blastoderm (stage 5) embryos were hybridized with the P1B probe, the P2B probe, or the INTRON probe. The INTRON probe produced a signal like that produced by the P1B probe, but slightly weaker (data not shown). The similarity of the P1B and INTRON patterns suggests that the pattern of P1 transcript accumulation seen in the embryo is not due to spatially controlled RNA processing. The P1 pattern is barely detectable, if at all, on sections hybridized with the P2B probe, indicating that the P2 pattern is principally due to bona fide P2 transcripts.

**Transcripts containing exons D through G**

During nuclear cycle 14, the probes D–G and cDNA, which both hybridize to both P1 and P2 transcripts, detect transcripts only in a P2-like pattern (Fig. 2C), although the signal from the D–G probe is detected somewhat later in the cycle than the P2-specific signal, and it remains weak (data not shown). Because both the cDNA and D–G probes contain only sequences close to the 3' end of the *Antp* gene, this result suggests that complete P2 transcripts appear before complete P1 transcripts. The P1 primary transcripts are about 64 kb longer than the P2 primary transcripts (100 kb versus 36 kb), and, therefore, if transcription at both promoters begins at about the same time, then the lag in appearance of P1 transcripts containing exons D–G is probably due to the extra time it takes to transcribe the P1 transcription unit relative to the P2 transcription unit.

**Antp expression during gastrulation and early germ band elongation**

Gastrulation and germ band elongation occur rapidly after blastoderm cell formation, and are accompanied by extensive changes in the patterns of *Antp* expression. Adjacent sections of the same embryos were probed with P1- or P2-specific probes (Fig. 4).

**P1 promoter**

During gastrulation and early germ band elongation, the posterior limit of P1 expression on the ventral side of the embryo is at approximately 17% egg length, thus presumably including PS 12 (Fig. 4C). P1 transcripts are also detected in PS 5 of the forming mesoderm (data not shown). In stage 8 embryos, midway through germ band elongation, the P1 promoter is expressed principally in the ectoderm of PS 4, the ectoderm and mesoderm of PS 5 and anterior PS 6, and more weakly in the ectoderm from posterior PS 6 through PS 12 (Fig. 4E, G).

**P2 promoter**

During gastrulation and germ band elongation, P2 transcripts continue to be present at high levels in PS 4. PS 6 expression is only detected in the cells that invaginate as part of the ventral furrow (Fig. 3C), although ectodermal PS 6 expression is detected early during germ band elongation. As the pole cells move dorsally, so do the ventral PS 14 cells that express P2 (Fig. 4D, open arrow). The dorsal PS 14 expression is hard to follow and may simply disappear. As the germ band begins to elongate, P2 transcripts are detected in PS 3, as well as at low levels in some ectodermal cells of each PS from 5 through 14. P2 is strongly expressed in the ectoderm and mesoderm of PS 4 and in the mesoderm of PS 6 (Fig. 4D). In stage 8 embryos, the P2 promoter is strongly expressed in the ectoderm of PS 3 and PS 4, and in the mesoderm of PS 4 and PS 6 (Figs 4E and 4F, H). Additionally, the P2 promoter is expressed more weakly in the ectoderm and mesoderm of PS 5 and in the ectoderm from PS 6 through PS 14, with stronger signals in the even-numbered parasegments (see also Ingham and Martinez-Arias, 1986). For comparison, a stage 8 embryo probed with both a P2 probe and a *Ubx* probe is shown in Fig. 4B, confirming the identification of PS 4 and PS 6 as the locations of the strongest mesodermal P2 expression. The patterns of hybridization seen with the cDNA probe continue to resemble the P2 pattern during the early stages of germ band elongation.

**Antp expression in extended germ-band embryos**

The germ band begins to retract at the end of stage 11, approximately 7 h 20 min after fertilization. During stage 12, the germ band shortens, and the ventral nerve cord separates from the epidermis (Campos-Ortega and Hartenstein, 1985). At this stage, the homeobox probe detects a pattern that is the sum of P1 and P2 transcription patterns, suggesting that the two promoters contribute similar amounts of RNA to the pattern (for example, see wild-type embryos in Fig. 7).

**P1 promoter**

During stages 11 and 12, RNAs from P1 are found at high levels in most epidermal cells of PS 4, PS 5, and
Expression patterns of the two Antp promoters

Fig. 5. Promoter-specific expression after germ band shortening. (A, B) Alternate horizontal sections of a stage 15 or 16 embryo probed with P1A (panel A) or P2A (panel B). Arrowhead indicates the T2 segment. The posterior limit of P1 expression in the nerve cord is A7. P2 expression in the nerve cord extends from the T1 neuromere through the A9 neuromere. Subectodermal expression of P2 is visible in every segment from T2 to A9 (not visible in this section).

(C, D) Adjacent sagittal sections of a stage 16 embryo showing P1 (panel C) and P2 (panel D) expression. Arrowheads indicate the approximate locations of the T2 neuromeres. (E) Sagittal section of an embryo probed with the HB probe. The arrowhead indicates the approximate location of the T2 neuromere. (F, G) Adjacent sagittal sections of a stage 17 embryo probed with P1A (panel F) or P2A (panel G). Arrowheads indicate the locations of the T2 neuromeres.

anterior PS 6, as well as the mesoderm of PS 5 and anterior PS 6 (Martinez-Arias, 1986; Figs 2D and 7A,B). P1 transcripts are detected in the developing VNS during stage 11 in PS 4 and 5. During stage 12, transcripts from P1 decrease in the epidermis and increase in the nervous system (Fig. 2G).
Fig. 6. Promoter-specific expression in the mesodermal derivatives. (A, B) Alternate horizontal sections of a stage 14 embryo probed with either P1 (panel A) or P2-specific (panel B) probes. Solid arrows indicate visceral mesoderm expression. Open arrow in B indicates ventral nerve cord expression in A8/A9. P1 expression in the gap between T1 and T2 (panel A) corresponds to cells at or near the anterior spiracle, as described previously (Martinez-Arias, 1986).
(C, D) Adjacent sections of a stage 14 embryo probed with P1- (panel C) or P2-specific (panel D) probes. Solid arrows above the gut indicate expression in the visceral mesoderm. The P1 and P2 patterns overlap, but the P2 pattern extends farther anteriorly and posteriorly. P1 is expressed in somatic muscles of T3 (panel C). P2 expression is visible in clusters of cells below the epidermis in every segment from T2 to A9 (panel D). The open arrow in D indicates a signal that is also seen with both the cDNA and P2 probes in whole embryos (data not shown). The identity of these cells has not been determined unambiguously.

P2 promoter
During stage 11, the patterns of P2 expression in the epidermis are different from the patterns of P2 expression in the developing nervous system. In the epidermis, expression is restricted to a subset of cells in PS 3, PS 4 and PS 5 ventrally. Laterally, P2 transcripts are detected in a small cluster of epidermal or subepidermal cells in the center of each PS from 4 to 14. The pattern is visible with the cDNA probe in Fig. 3D (arrows). These cells may correspond to the primordia of sensory organs, since slightly later P2 transcripts are detected in cells clearly located just below the epidermis (e.g. Fig. 6D). In the nervous system, transcripts from P2 accumulate in a subset of the neural cells within each parasegment from PS 3 to the anus (Fig. 7D,E). During stage 12, P2 transcripts also decrease in the epidermis and increase in the nervous system (Fig. 2H).

Antp expression after germ-band retraction
Soon after the end of germ-band retraction, head involution and dorsal closure of the embryo commence. Later, the gut encloses the yolk sac to form a closed tube. The final stages of embryogenesis are highlighted by constrictions of the midgut and contraction of the ventral nerve cord (Campos-Ortega and Hartenstein, 1985).

P1 promoter
After germ band shortening, transcription in the epidermis decreases to undetectable levels (Fig. 2J), except in a few cells in anterior PS 4, including or adjacent to the primordium of the anterior spiracle (Figs 2J and 6A,C). In the mature nervous system, P1 continues to be expressed at high levels in PS 4, at lower levels in PS 5, and at very low levels in PS 6–12 until hatching (Figs 2J and 5A,C,F).

The differentiation of somatic and visceral muscles during and after germ band shortening allows identification of specific muscle sets expressing Antp. At the shortened germ band stage, all somatic muscles of T3 express P1 (Martinez-Arias, 1986; Fig. 6C). In the visceral mesoderm, at least two isolated groups of cells around the anterior midgut express P1 (Fig. 6A,C). At stage 17, P1 is also expressed in cells in or near the aorta (data not shown; Campos-Ortega and Hartenstein, 1985). In horizontal views of whole embryos probed with the P1B probe, the pattern is a narrow band of
Fig. 7. Regulation of P1 and P2 by the BX-C. Arrowheads indicate the boundary between PS 3 and PS 4 and, in some cases, the boundary between PS 12 and PS 13. (A, B) Bright-field (panel A) and dark-field (panel B) images of a sagittal section of a stage 11 embryo probed with the P1B probe. (C) Sagittal section of a Df(3R)P9 homozygote (lacking the BX-C) probed with the P1B probe. (D, E) Section adjacent to the one in A and B probed with the P2B probe. (F) Section adjacent to the one in C (i.e. BX-C−) probed with the P2B probe. (G, H) Sagittal section of a stage 11 embryo probed with the HB probe. Note that the pattern generated by the HB probe is approximately the sum of the patterns generated by the P1 and P2 probes.
cells posterior to the lymph glands and just below and perpendicular to the dorsal midline.

**P2 promoter**

After germ band shortening, P2 transcription decreases to very low levels in the epidermis (Figs 2K and 6D). P2 is active in groups of cells below the epidermis within every segment from T1 to A8/A9 (Figs 5B and 6B,D, cDNA pattern shown in Fig. 3F). Based upon their location in the subepidermal cleft, these cells probably correspond to precursors of sensory organs (Campos-Ortega and Hartenstein, 1985). In the mature nervous system, P2 is expressed at a uniform low level in a subset of neurons in each parasegment from PS 3 to PS 14 throughout the later stages of development (Fig. 5B,D,G). Most or all of the somatic muscles of T2 and T3 express P2 (Fig. 6D). In the visceral mesoderm, P2, like P1, is expressed in a group of cells around the anterior midgut. The P2 pattern is broader than the P1 pattern, and overlaps it (Fig. 6B,D). The cDNA pattern in the visceral mesoderm is shown in Fig. 3 (G and H). The Antp expression in the visceral mesoderm cells appears to be responsible for the formation of the gut constriction (Fig. 3H) in that this constriction does not form in Antp~ embryos (R. Reuter and M.P.S., submitted). Like P1 transcripts, P2 transcripts are detected in or adjacent to cells of the aorta in stage 17 embryos. The identities of the cells in this area expressing both P1 and P2 transcripts has not been determined unambiguously, nor is it known whether P1 and P2 transcripts are present in the same cells.

Hybridizations with the HB, D–G, and cDNA probes give signals that are the sum of the P1 and P2 patterns after germ band retraction, again suggesting the two promoters contribute comparable amounts of RNA to the total pattern (Figs 5E and 7G,H).

**Antp expression in embryos that lack bithorax complex functions**

In embryos lacking the entire BX-C, Antp expression is derepressed in the abdomen. Antp transcripts, as detected by cDNA probes that hybridize to both P1 and P2 transcripts, are present at high levels from the second thoracic segment through most of the abdominal segments (Hafen et al. 1984; Harding et al. 1985). Antp protein is abundant in the nerve cord of BX-C~ embryos from pT1 through aA7 (PS 4–12; Carroll et al. 1986; Wirz et al. 1986). These observations indicate that genes in the BX-C negatively regulate Antp expression, either directly or indirectly. BX-C regulation of P2 has previously been examined in two somewhat indirect ways. First, in BX-C~ flies that carry a lacZ gene under the control of the P2 promoter, β-galactosidase expression is derepressed posteriorly through PS 14 (Boulet and Scott, 1988). These P2–lacZ fusions, however, may not contain all the cis-acting control elements required by the endogenous P2 promoter for proper regulation by BX-C genes or may respond abnormally. Second, Antp P2-derived protein is expressed in PS 3–14 in BX-C~ embryos (Boulet and Scott, 1988). However, this result was obtained from embryos that in addition to lacking the BX-C also carried the dominant Antp72b mutation, which was necessary to eliminate the P1 contribution to the protein pattern. Conceivably the P2 expression pattern in these embryos could be affected by the Antp72b mutation. A direct examination of P2 transcripts in BX-C~ embryos that are wild-type for Antp is therefore preferable. It has not been reported whether the P1 promoter is regulated by BX-C genes.

**P1 promoter**

In extended germ-band embryos that lack BX-C function, P1 transcripts are expressed at high levels from PS 4 to PS 12 (Fig. 7C). Therefore genes of the BX-C negatively regulate P1 expression. However, P1 transcripts in BX-C~ embryos, as in wild-type embryos, are not detected in PS 13 and PS 14. The apparent absence of P1 transcripts in these parasegments suggests that either P1 is repressed by other genes in PS 13 and PS 14, or that, unlike P2, P1 does not contain the regulatory sequences required for activation in those parasegments.

**P2 promoter**

In BX-C~ embryos, P2 transcripts are expressed from PS 3 through PS 14, further posterior than P1 expression (Fig. 7F). Derepression of the P2 promoter is more subtle than derepression of the P1 promoter and is difficult to document convincingly. However, embryo sections displaying putative P2 derepression can be distinguished from sections of wild-type embryos on the basis of slightly more intense labelling of the nerve cord. Such identifications are possible only with good sagittal sections, and were confirmed as BX-C~ embryos by the ectopic P1 expression seen in adjacent serial sections. The P2 derepression may be less extreme than the P1 derepression for several possible reasons. First, P2 may be expressed more strongly in the nervous system in wild-type embryos, so its derepression is less noticeable. Second, P1 could be repressed in more cells or tissues than P2. Third, within a given cell, P1 could be repressed more strongly than P2.

**Discussion**

How do Antp and other homeotic genes control segment differentiation? Many of the homeotic genes encode transcription factors that presumably activate and/or repress different sets of ‘target’ genes that in turn generate the morphology of the fly. Because the ANT-C and BX-C homeotic genes are expressed in specific domains within the embryo, cells in different parts of the embryo may activate or repress distinct sets of target genes, or control the synthesis of particular ratios of target gene products, thus directing cells along particular developmental pathways. Ectopic expression of Antp or Dfd results in segment transformations (Denell, 1973; Struhl, 1981; Hazelrigg and Kaufman, 1983; Frischer et al. 1986; Schnewly et al. 1987a,b; Gibson and Gehring, 1988; Kuziora and McGinnis,
Expression patterns of the two Antp promoters

Therefore the position-specific expression patterns of the homeotic genes constitute a crucial aspect of their function in development. The initial patterns of expression of many homeotic genes are fairly simple, but as development proceeds, they become extremely complex: different tissues, and different cells within these tissues, express various combinations of homeotic gene products (e.g. Carroll et al. 1988). Such dynamic expression patterns result from complicated regulatory interactions, including interactions among the homeotic genes themselves.

In this paper, we have shown that the two Antp promoters are expressed in distinct spatial patterns during embryogenesis, as is summarized in Fig. 8. The patterns are tissue specific as well as position specific, and in several cases it is apparent, even with the limited resolution of in situ hybridization, that only some of the cells within a segment are expressing Antp. The observations are important for two reasons: First, the two Antp promoters must respond to different regulatory inputs during embryogenesis. Second, Antp transcripts with different 5' untranslated sequences are made in different places during development. The two types of Antp transcript could have different stabilities and/or could be translated with different efficiencies.

The Antp P1 and P2 promoters perform different functions

Antp plays multiple roles in development. The phenotypes of somatic clones of Antp cells, the intragenic complementation among Antp mutations, and the distinct lethal periods for individuals carrying different Antp mutations suggest that the two transcription units have different functions. The P1 transcription unit is required for anterior spiracle eversion and dorsal thoracic development, while the P2 transcription unit is required for embryonic viability and leg development (Kaufman and Abbott, 1984; Abbott and Kaufman, 1986).

The distributions of P1 and P2 transcripts corroborate, in part, the genetic inferences about promoter-specific functions. P1 transcripts are observed in or near the developing anterior spiracles during embryogenesis, while P2 transcripts are not (Fig. 6). P1 transcripts accumulate in those parts of the wing discs destined to become dorsal thorax (Jorgensen and Garber, 1987). However, both P1 and P2 RNAs accumulate in both dorsal and ventral tissue (Jorgensen and Garber, 1987). The presence of P1 or P2 transcripts in tissues that are not affected by specific mutations that alter the P1 or P2 transcription unit suggest that Antp can be expressed in places where it does not function, or that the effects of Antp in these tissues are subtle and therefore have not yet been observed.

The Antp phenotypes that have been observed are likely to be just the most obvious external effects of the loss of Antp function. There are severe limitations to what it has been possible to learn about the detailed phenotypes of Antp mutants, particularly in internal tissues such as the nervous system. It is generally impossible, presently, to recognize the transformation of the fates of one or a few cells to those of similar cells. The complexity of the expression patterns suggests that Antp participates in a very large number of developmental decisions, only some of which can currently be recognized and interpreted.

The pair of Antp promoters provide Antp with regulatory flexibility

It is becoming clear that the complicated expression patterns of the homeotic genes result from large arrays of cis-acting regulatory elements. In many cases, these
regulatory elements are likely to be binding sites for proteins that regulate homeotic gene expression. For example, the transcription of the Ultrabithorax (Ubx) gene is controlled by regulatory elements that act over great distances on the Ubx promoter (Hogness et al. 1985; Bender et al. 1985; Peifer et al. 1987). It is likely that Antp is also regulated by a large number of regulatory elements as well, but, in contrast to Ubx, two promoters must be regulated. It is not yet clear whether any cis-acting control elements act on both promoters, or whether each promoter has its own set of regulatory sequences. Many genes containing multiple promoters are expressed in complex temporal and position-specific patterns. In Drosophila, these genes include Alcohol dehydrogenase (Adh) (Benyajati et al. 1983), caudal (cad) (Modzik and Gehring, 1987), and hunchback (hb) (Tautz et al. 1987; Schröder et al. 1988). In each case, the different promoters are utilized in different stage- and tissue-specific patterns. At least one other homeotic gene, Abdominal B, uses multiple promoters (Sánchez-Herrero and Crosby, 1988; DeLorenzi et al. 1988; Kuziora and McGinnis, 1988a).

**Genes that differentially regulate the Antp promoters**

The different patterns of expression of the Antp promoters suggests that they are controlled by different sets of genes. Homeotic gene expression is regulated, directly or indirectly, by maternally active genes, segmentation genes, and other homeotic genes. Several of these genes appear to differentially regulate the two promoters. The results of in situ hybridizations of Antp promoter-specific probes to mutant embryos suggest that the maternally active gene oskar regulates P2 but not P1 (Irish et al. 1989). The zygotically active gap segmentation genes Krüppel, hunchback, and knirps also regulate Antp expression (Harding and Levine, 1988). Krüppel activates P1 but not P2, while hunchback activates P2 but not P1 (Irish et al. 1989). Antp expression is altered in embryos mutant for knirps, but the effect may be indirect, resulting from regulatory interactions among the gap genes themselves (Harding and Levine, 1988). The pair-rule gene fushi tarazu (ftz) activates P2 but not P1 (Ingman and Martinez-Arias, 1986). The transcription factor DTF-1 activates the P2 promoter in vitro, but probably not P1 (Perkins et al. 1988). Therefore although many regulators of Antp remain to be identified, there is already ample evidence for promoter-specific regulation.

Both P1 and P2 are derepressed in embryos carrying mutations in the BX-C. However, in BX-C- embryos P1 is derepressed only back through PS 12 while P2 is derepressed through PS 13–14. Therefore an activator of P2 is absent from PS 13 and 14, or an additional negative regulator of P2 exists in PS 13 and 14. Recently, a hybrid Deformed-Ubx protein, containing a Ubx homeodomain, has been expressed under heat shock promoter control in embryos and shown to activate P1, but not P2, in the head (M. Kuziora and W. McGinnis, personal communication). Antp is not activated by Deformed protein under these conditions so the effect on Antp is presumably due to the binding of the hybrid protein to the Antp gene through the Ubx homeodomain. These results suggest that Ubx acts differentially upon the two Antp promoters.

Surprisingly, Antp transcripts are not detected in PS2, where ftz and hb, both activators of Antp P2 in some cells, are expressed. This observation suggests that a negative regulator of Antp P2, perhaps bicoid, prevents Antp expression in PS 2 (Irish et al. 1989).

The Antp P1 and P2 transcripts may be translated with different efficiencies

Recently it has been shown that a polyclonal anti-Antp antisemum (Carroll et al. 1986) mainly detects protein translated from P1 transcripts, while a more sensitive monoclonal anti-Antp antisemum detects protein translated from both P1 and P2 transcripts (Boulet and Scott, 1988). These results indicate that P1-derived Antp protein is more easily detected than P2-derived Antp protein. Three plausible explanations for this observation are: (1) P1 transcripts are much more abundant than P2 transcripts. Two observations indicate that this is not the case. First, probes that hybridize to both P1 and P2 transcripts do not predominantly detect the P1 pattern (for example, see Fig. 2). Second, S1 nuclease protection analysis of RNA from 4–8 h embryos reveals that transcripts from the two promoters are present at similar steady-state levels (Bermingham and Scott, 1988). (2) P1 transcripts produce protein different from that produced from P2 transcripts. Although alternative RNA splicing permits four different (but closely related) Antp proteins to be made, there is no detectable linkage between alternative splicing and promoter usage (Bermingham and Scott, 1988; Strocher et al. 1988). (3) P1 transcripts are translated more efficiently than are P2 transcripts. AUG codons followed by short open reading frames upstream of a larger open reading frame have been implicated in translational efficiency and regulation (Thireos et al. 1984; Hinnebusch, 1984; Hinnebusch, 1988). Therefore, it may be relevant that P1 transcripts contain 8 upstream AUGs while P2 transcripts contain 15 upstream AUG’s (Schniewly et al. 1986; Strocher et al. 1986; Laughon et al. 1986).

The authors wish to thank Dietherd Tautz and C. Pfeifle for communicating their method for whole-mount *in situ* hybridization before publication; Joan Hooper, Tammie Yeh and Debbie Andrew for useful technical advice; Rolf Reuter for the use of some of his *in situ* hybridization data; and D. Andrew and John Tamkun for making useful suggestions on the manuscript. M.P.S. is an investigator of the Howard Hughes Medical Institute. The work was supported by N.I.H. grant no. 18163.

**References**


Expression patterns of the two Antp promoters


(Accepted 29 March 1990)