The Localization and Regulation of Antennapedia Protein Expression in Drosophila Embryos

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Summary

The homeotic Antennapedia (Antp) gene of Drosophila is required for the normal differentiation of the thoracic segments during embryonic development and metamorphosis. Antibodies to a recombinant Antp protein were used to localize the protein in whole mount embryos. Antp is expressed in the nuclei of cells of the thoracic embryonic epidermis and several segments of the ventral and peripheral nervous systems. Analysis of Antp expression in mutant embryos revealed three levels of Antp regulation by genes of the bithorax complex, pleiotropic homeotic loci, and Antp itself. The distributions of the Antp and the Ultrabithorax (Ubx) proteins in doubly-labeled embryos suggest that the Ubx protein may be one direct negative regulator of Antp gene expression.

Introduction

The genetic control of body segment diversification of Drosophila melanogaster involves the activity of genes within two major complexes, the Antennapedia (ANT-C) (Kaufman et al., 1980) and bithorax (BX-C) (Lewis, 1978). Most loci in these complexes are homeotic, that is, mutations in them result in the derepression of specific segmental identities. Homeotic genes in the AN1-C are required in the head and thorax while BX-C genes are required in parts of the thorax and abdomen. Previous studies have shown that homeotic genes control differentiation of segments by being expressed in position-specific patterns: sites of phenotypic gene function correspond, in general, to sites of gene expression. Therefore two important questions are: one, how is position-specific expression attained and two, what are the molecular functions of the gene products. We have used antibody probes for proteins encoded by the Antennapedia (Antp) homeotic locus, one gene within the AN1-C, to examine issues related primarily to the first of these questions.

Antp has a major role in the thorax both in promoting the thoracic pathway and in preventing the development of antennal or head structures (Struhl, 1981a; Kaufman and Abbott, 1984). Dominant Antp mutations cause adult flies to develop with antennae transformed into mesothoracic legs. However, the dominant mutations have been shown to represent abnormal gene function in the head (Denell, 1973; Duncan and Kaufman, 1975; Struhl, 1981a; Hazelrigg and Kaufman, 1983). Normal head development does not require Antp function (Denell et al., 1981; Struhl, 1981a; Kaufman and Abbott, 1984). Embryos homozygous for null alleles of the locus exhibit transformations of the mesothorax and metathorax into hybrid prothorax-head segments (Wakimoto and Kaufman, 1981; Struhl, 1983; Sato et al., 1985). A phenotype which has been difficult to interpret definitively. One interpretation is that in Antp embryos, parasegment 4 (posterior T1 plus anterior T2) is transformed into parasegment 3, and parasegment 5 is transformed into a parasegment of mixed identity that has characteristics of both thoracic and abdominal segments (Martinez-Arias, 1985). Effects of Antp gene function on posterior T3 (anterior parasegment 6) have also been observed (Sato et al., 1985). In addition, in the absence of BX-C function, activity of Antp in the embryonic abdomen can be detected (Duncan, 1982b; Sato et al., 1985).

Antp is a very large gene, spanning some 103 kb of DNA (Garber et al., 1983; Scott et al., 1983), but RNA processing results in the production of two size classes of mature RNA that are only about 3.5 kb and 5.0 kb in length. Sequences of antisense clones has revealed that only 1.1 kb of each of the RNAs is a protein coding region (Sohnouwly et al., 1986; Laughon et al., submitted). At least four proteins are encoded; alternative RNA splicing gives rise to largely identical coding sequences varying by up to 17 codons (Bermingham, Laughon, and Scott, unpublished data). In situ hybridization of Antp cDNA probes to sectioned Drosophila tissue has indicated a complex spatial pattern of Antp RNA distribution (Levine et al., 1985). The large amount of untranslated DNA and RNA in Antp suggests that most of the sequence functions as regulatory elements controlling the complex pattern of gene expression seen during Drosophila development. There is good correspondence between the segmental structures influenced by Antp and the places where the RNA is expressed.

It has been proposed that cell fates are determined by the array of homeotic genes active within each cell (Lewis, 1985). To test this hypothesis it is necessary to determine where homeotic gene products are expressed. These questions have been addressed with single cell resolution only in studies of one homeotic gene, Ultrabithorax (Ubx), a gene in the BX-C. Ubx protein is expressed at a similar level in all of the cells of some parasegments, but is expressed at different levels in different cells within other parasegments (White and Wilcox, 1984; Beachy et al., 1985). Some of the complexity of the Ubx pattern is due to regulation by other genes in the BX-C (Struhl and White, 1985). Although it is clear that multiple BX-C homeotic genes are expressed in some parasegments, the resolution of in situ hybridization studies has not been adequate.
Expression of β-Galactosidase-Antp Fusion Proteins in E. coli and Production of Specific Antibodies to the Antp-Encoded Domains

λ gt11 (c[83]) lysogens containing the Antp inserts were induced at 42°C for 2 hr and cell lysates were prepared for electrophoresis on a 7.5% SDS-polyacrylamide gel. Part of the gel was stained with Coomassie blue (A), and replicate lanes were transferred to nitrocellulose and probed with affinity-purified antibodies against β-galactosidase (B) and the Antp portion of the hybrid protein (C). The molecular weight markers in (A) are β-galactosidase (116 kd), phosphorylase b (97 kd), bovine serum albumin (66 kd), and ovalbumin (45 kd). The dark arrows indicate the position of the far less abundant full-length products of the λAntp P10 and λAntp S10 fusions; the open arrows indicate the position of the major products of the fusions that lack the homeodomain determinants.

Results

Expression of the Antp Protein in E. coli

In order to generate antibodies directed against the Antp protein, gene fusions were constructed in the λgt11 expression vector to produce the protein in E. coli (Young and Davis, 1983). Two subclones of the Antp pDmG100 cDNA (Scott et al., 1983) were used in gene fusions. One subclone contained 1,298 bp from the PstI site to the 3′ end of the cDNA and the other contained 1,616 bp from the Smal site to the end of the Antp cDNA clone. Appropriate EcoRl linkers were ligated to these fragments to introduce the DNA in the proper reading frame into λgt11 for expression of β-galactosidase hybrid proteins. The phages λAntp P10 and λAntp S10 encode Antp products 242 and 348 amino acids long, respectively, joined to 114 kd of β-galactosidase protein.

Lysogens containing the two Antp fusion phages were induced to express the hybrid β-galactosidase-Antp proteins. Upon thermal induction, fusion proteins migrating with apparent molecular weights of 135 kd and 150 kd were produced (Figure 1a, open arrows) as the major species. These values are in agreement with the protein sizes predicted from the DNA sequence (Schneuwly et al., 1986; Laughon et al., submitted). However, immunological analysis of the proteins produced by the lysogens revealed that the 135 kd and 150 kd bands are in fact not full-length products. Anti-β-galactosidase staining of nitrocellulose blots revealed that much smaller amounts of larger fusion proteins (approximately 150 and 165 kd) were produced in each case (Figure 1b, solid arrows). Antibodies to the homeodomain, a highly conserved 60 amino acid protein domain found in the C terminal part of the Antp protein (Schneuwly et al., 1986; Laughon et al., submitted) and in other homeotic protein products (Scott and Weiner, 1984; McGinnis et al., 1984), were useful for determining whether the C terminal part of the Antp protein is present in the fusion proteins. Antibodies against the N terminal part of the homeodomain (Carroll et al., 1985a) detect the larger fusion proteins, but not the 135 kd and 150 kd major bands (data not shown). Thus, the major products accumulating in the λgt11 Antp lysogens are not full length and result from either incomplete synthesis or rapid in vivo proteolysis of the C terminal parts of the fusion proteins. The β-gal-Antp fusion products migrate on gels much more slowly than predicted from the sequence, probably because of their high proline (10%) content. Similar behavior has been observed for other proteins with high proline content (e.g., Watt et al., 1985; Carroll and Scott, 1985).

Antibodies against Antp Protein

The 135 kd Antp P10 major fusion protein product, which lacks the homeodomain, was purified by aminophenylthio-[β-galactopyranoside] affinity chromatography, anti-β-galactosidase immunoaffinity chromatography, and preparative SDS gel electrophoresis and then used to immunize rabbits. The Antp-specific antibodies were affinity-purified using the λgt11 Antp S10 protein cross-linked to anti-β-galactosidase antibody attached to CNBr-activated Sepharose 4B (Carroll and Scott, 1985). The specificity of the final antibody preparation for the Antp-encoded portion of the two hybrid proteins was demonstrated by immunoblotting. The antibody reacts with all forms of the hybrid proteins containing Antp determinants but not with β-galactosidase or other E. coli proteins (Figure 1c). We have not yet detected Antp products in extracts from Drosophila.
tissues using protein blots. Based upon the known exon composition of all Antp transcripts, the antibody should recognize all forms of Antp antigen. The lack of homeodomain determinants in the purified fusion protein immunogen eliminated the potential reactivity of the antibody with other gene products containing the homeodomain.

Localization of Antp Protein

The Ectoderm: To localize Antp proteins during embryogenesis, fixed whole embryos were stained with anti-Antp antibodies as described in Carroll and Scott (1985). Staining of whole mount embryos allows the detection of Antp protein in many different tissues at all stages of embryogenesis until the cuticle is formed. We have examined Antp protein distribution in the nervous system and ectoderm, but not in the mesoderm.

After fertilization, the Drosophila zygote undergoes 13 nuclear divisions as a syncytium before cell membranes form (Foe and Alberts, 1983). At the completion of cellularization, gastrulation begins as longitudinal and transverse folds appear (Turner and Mahowald, 1977). The ventrolateral coil along the ventral furrow invaginates to form the presumptive mesoderm. The ventral germ band then elongates such that the posterior-most cells move along the dorsal surface nearly to the point of the transverse cephalic furrow, with cells moving ventrally around the sides of the embryo at the same time. During early germ band extension, when segment primordia are three to four cells wide, there is no detectable expression of Antp protein or any reactive antigen within any cells (Figure 2e). The specificity of the Antp antibodies is also demonstrated by the absence of staining in Antp- mutant embryos (Figure 3e).

While the germ band is extended, as a cycle of cell divisions is complete in the ectoderm and when the segment primordia are about eight cells wide, a low level of Antp protein becomes detectable in the presumptive thoracic region in the anterior ventrolateral region of the embryo (Figure 2b). The initial pattern of Antp expression is not uniform across the region; it appears to be strongest at the anterior edges of two separate regions (arrows). The anterior-most patch of staining does not extend as far dorsolaterally as the more posterior patch (Figure 2c). The cells within the anterior portion of the second patch are more heavily stained than the most anterior cells. The position of the Antp-containing cells in the entire patch, as
determined by measurement of egg length and cell counting, extends from approximately the posterior compartment of the labial segment through the anterior compartment of the first abdominal segment. This pattern is surprising since Antp is not known to be required in the labium or the first abdominal segment. This pattern is transient (see below) and is also reflected in patterns of RNA expression when investigated using promoter-specific RNA probes (A. Martinez-Arias, personal communication). The most heavily stained cells are within the posterior prothorax/anterior mesothorax, a region also designated parasegment 4 (Martinez-Arias and Lawrence, 1985).

As the germ band begins to shorten, after segments have appeared, the thoracic ectodermal staining becomes stronger ventrolaterally (Figure 2d). It can be seen that the Antp protein is predominantly nuclear (Figure 2d). From the segment boundaries visible at this stage, it is apparent that cells in the posterior prothorax (T1), in all of the mesothorax (T2), and in all of the metathorax (T3) express Antp protein. Certain Antp transcripts (from the most 5' Antp promoter) have been shown to accumulate to the highest level in parasegments 4 and 5 in the extended germ band (Martinez-Arias, 1986). We observed that Antp is expressed in these parasegments as well as anterior parasegment 6 (posterior T3). Reexamination of Antp RNA distribution indicates that the region interpreted to comprise only parasegment 5 may also include posterior T3 (see Figure 2; Martinez-Arias, 1986).

As the germ band shortens to return to the ventral surface, the Antp pattern changes in the ectoderm and protein appears in the nervous system. In the ectoderm, Antp protein expression persists in ventrolateral cells of posterior T1 and most of T2, but diminishes in posterior T3 (Figure 2e). This correlates well with changes observed in Antp RNA distributions (Martinez-Arias, 1988). One can also see the beginnings of Antp expression in the ventral nervous system at this stage (Figure 2e, arrows). The level of Antp expression in the ectoderm decreases throughout germ band shortening. Only a small amount of protein is detected in subsets of thoracic ectodermal cells after the head segments have involuted, at 14–16 hr of embryogenesis, mostly at the posterior T1/anterior T2 border (solid arrow, Figure 2f). By this time, sensory cells within the thoracic segments express Antp at high levels (open arrows, Figure 2f). The pattern of disappearance of Antp protein from the ectodermal cells (Figure 2f) is uneven, in contrast to the uniformity of staining at an earlier stage (e.g., Figure 2e) suggesting that subcompartmental cell differentiation has occurred by 14–16 hr.

**Antp Protein in the Embryonic Nervous System**

Antp protein expression in the nervous system begins during early neurogenesis. While the germ band is beginning to shorten, ten pairs of patches of staining are visible in the neurogenic region (Figure 3a, see also Figure 2e, arrows). At this stage, the levels of staining are rather uniform within the nuclei of the thoracic and abdominal neural cells. Antp protein is visible in the ventral nervous system from the posterior portion of the prothoracic gan-
tion to the anterior portion of the seventh abdominal ganglion (Figure 3b). As the ventral nervous system condenses, the level of Antp protein steadily increases in the posterior prothorax, the anterior mesothorax, and a portion of the anterior metathorax (Figure 3c), leading to a pattern in which these thoracic cells are intensely labeled (Figure 3d). The cells in the seven most anterior abdominal segments remain weakly labeled. In segments anterior and posterior to the ten stained segments (including segment A8) (Figure 3d), no Antp antigen is detectable in the ventral nervous system. The detectable amount of Antp antigen is variable from cell to cell, especially in the abdominal segments (Figures 3b, 4a). Antp protein appears to be in all of the cells of parasegment 4 but in only some of the cells of the more posterior compartments. The specificity of the antibody for Antp proteins can be addressed by examining the level of staining in Antp\(^{\alpha} \beta\) homozygous embryos. The Antp\(^{\alpha} \beta\) mutation behaves genetically as a null allele (Kaufman and Abbott, 1984). There is no labeling of any cells in the mutant embryos with the anti-Antp antibody (Figure 3e).

In addition to the ventral nervous system Antp protein pattern, there are peripheral nervous system cells that express Antp. These are visible as thin discontinuous strips of cells in thoracic and abdominal regions of the embryo during germ band shortening (Figure 3f, arrows). The precise number, identity, and arrangement of these peripheral neural cells is presently under study, but it appears that only a subset of the peripheral neural cells produce Antp protein at a detectable level.

Since we were able to study Antp expression with clear resolution in the ventral nervous system, we applied the antibody probe to the study of Antp protein regulation. We considered three levels of Antp regulation: one, control by homeotic genes, such as BX-C loci; two, control by pleiotropic regulators of homeosis, such as Polycomb; and three, autogenous control of Antp. We find evidence for all three levels of regulation from examining Antp expression in mutants.

## Regulation of Antp Protein Expression

### The Spatial Relationship of Antp and Ubx Protein Distributions in the Ventral Nervous System of Wild-Type and Mutant Embryos

In addition to Antp, the Ultrabithorax (Ubx) gene is involved in controlling thoracic development (Lewis, 1978). It has been shown that the Ubx protein accumulates at the highest levels in the posterior metathorax and anterior first abdominal segment of the ectoderm and ventral nervous system (White and Wilcox, 1984, 1985; Beachy et al., 1985). There is also expression of Ubx protein at a low level in the posterior mesothorax and at a moderate level in the anterior portions of the second through seventh abdominal segments. It has been proposed that homeotic genes function in a combinatorial manner, the fate of a segment depending on the array of genes active within it (Lewis, 1978, Struhl, 1982, Kaufman and Abbott, 1984, Sato et al., 1985). Therefore, an important question is exactly how the spatial distributions of different homeotic gene products are related.

To compare the spatial distribution of Antp protein to that of Ubx protein, we doubly-labeled whole mount embryos with rabbit anti-Antp antibody and a mouse monoclonal antibody that detects the Ubx proteins (White and Wilcox, 1984). In the ventral nervous system, Antp is expressed at a high level in posterior T1 and most of T2, and at a moderate level in part of T3 (Figure 4a). Ubx protein, examined in the same embryo, is expressed in the posterior part of T2 at a moderate level, and at a high level in parasegment 6 (the posterior part of T3 and the anterior part of A1) (Figure 4b). The parts of the nervous system where each protein is at high levels do not overlap (Figures 4c, d); there is generally an inverse relationship between the levels of the Antp and Ubx proteins. However, there are clearly cells in which both proteins are expressed. The presence of Ubx protein is correlated with a reduced amount of Antp, but not necessarily with the complete absence of Antp protein. In the more posterior parts of the abdomen, Ubx expression is largely or completely confined to the anterior compartments of the abdominal segments, whereas Antp expression appears to be expressed at a low level within a subset of the nuclei throughout the segment (Figure 4a).

### Antp Expression in Homeotic Mutants

Previous studies showed Antp expression to be under the influence of the BX-C genes (Hafen et al., 1984; Harding et al., 1985). The ability to study Antp and Ubx protein distributions simultaneously in whole mount embryos led us to further investigate the role of BX-C genes in Antp gene regulation. In Ubx\(^{-}\) embryos, Antp expression in the ventral nervous system increases in posterior T2, all of T3, and anterior A1 such that the level of Antp protein reaches that found in wild-type embryos in parasegment 4 (compare Figures 4a and 4e). Thus, the areas where Ubx is normally expressed at a high level exhibit increased levels of Antp protein in the absence of Ubx protein(s), in agreement with the view that Ubx is a regulator of Antp expression (Hafen et al., 1984).

Some mutations at the bithoraxoid (bxd) locus cause an increase in Ubx expression in anterior T3 (compare Figure 4b to Figure 4g; Beachy et al., 1985; White and Wilcox, 1985b). If Antp expression is responding to Ubx protein levels, one would expect that the increased expression of Ubx in anterior T3 in a bxd\(^{-}\) embryo would reduce Antp expression. This is indeed the case. The expression of Antp protein in a bxd\(^{-}\) mutant in anterior T3 is reduced to about the level of Antp protein in the abdominal segments (compare Figure 4f to Figure 4a).

We have examined Antp expression in other BX-C homeotic mutants. Mutations at the abdominal-A (abd-A or iab-2) (Lewis, 1978; Karch et al., 1985) locus of the BX-C transform the second through seventh abdominal segments into segments resembling the first abdominal segment (Sanchez-Herrero et al., 1985; Karch et al., 1985) and result in an increase in Ubx protein expression throughout the A2–A7 segments (Struhl and White, 1985). Antp expression in abd-A\(^{-}\) embryos appears to be essentially unaffected (data not shown). However, the additional absence of the Ihtb gene (i.e., in a Ubx\(^{-}\), abd-A\(^{-}\) double mutant) results in high levels of Antp expression from
Figure 4. Spatial Relationship of the Distribution of the Antp and Ubx Proteins: The Level of Ubx Protein is Inversely Related to the Antp Protein Level

(a) Wild-type embryonic nervous system labeled with anti-Antp antibody and detected with fluorescein-conjugated secondary antibody. Antp expression is high in cells of the posterior prothorax, the anterior mesothorax and metathorax, and a smaller subset of anterior metathorax cells.

(b) Same embryo as (a) labeled with a monoclonal antibody to the Ubx protein and detected with a rhodamine-conjugated secondary antibody. Ubx expression occurs in posterior cells of the mesothorax, and in anterior cells of the first seven abdominal segments. Note that in the thorax, high levels of Ubx expression occur within regions where Antp expression is low.

(c) and (d) High magnification views of the embryonic nerve cords shown in (a) and (b) respectively. Inspection of the packing of the respectively labeled cells indicates that the high levels of each product are in different regions of the thoracic ganglia. The cells of the posterior portion of the mesothorax (pT2) are indicated.

(e) Antp expression in a homozygous Ubx^{28B} embryo. Antp expression is at high levels over the regions where Ubx is normally at high levels. Antp expression at high levels extends from posterior T1 through anterior A1.

(f) Antp expression in a homozygous bxd^{113} embryo. Antp protein expression is greatly reduced in anterior T3, the site of new Ubx expression in the bxd mutant (see Figure 5g).

(g) Ubx expression in a bxd^{113} embryo. Ubx expression increases in anterior T3.

posterior T1 to anterior A7 (compare wild type in Figure 5a to Figure 5b). One interpretation of these observations is that abd-A^{+} is a negative regulator of both Antp and Ubx. In the absence of only abd-A^{+}, both Antp and Ubx are on in posterior abdominal segments, but Ubx keeps Antp at a low level. The absence of Abdominal-B (Abd-B) gene (or lab-7; Lewis, 1978; Karch et al., 1985) function results in transformations of abdominal segments 5–8 into segments resembling the fourth abdominal segment (Sanchez-Herrero et al., 1985) and an increase in Ubx expression in the eighth abdominal segment (Struhl and White, 1995). Antp expression is not detectably affected by the absence of the Abd-B gene (data not shown). The absence of all BX-C functions (i.e., the Df(3R)P9 deletion which includes Abd-B) affects Antp expression in the same manner as the absence of just Ubx and abd-A (Figure 5c). This is surprising because Abd-B does negatively influence Ubx expression in segment 8 (Struhl and White, 1985). The effects of the BX-C mutations on Antp and Ubx expression in the nervous system are summarized in Figure 6, and illustrate that Antp expression is controlled in the posterior embryo by the loci of the BX-C.

The Sex combs reduced (Scr) locus of the ANT-C is required for normal labial and prothoracic segment development (Kaufman et al., 1980; Wakimoto and Kaufman, 1981; Sato et al., 1985). Scr is required in the prothorax and thus appears to be the next most anteriorly acting homeotic gene to Antp (Harding et al., 1985; Kuroiwa et al., 1985). In embryos homozygous for the strong Scr^{117} mutation (Wakimoto and Kaufman, 1981), Antp protein expression is normal. The anterior limit of Antp protein expression remains unchanged (data not shown).

The activities of the homeotic genes of the ANT-C and BX-C are regulated in part by a number of pleiotropic regulatory loci (Lewis, 1978; Ingham and Whittle, 1980; Duncan and Lewis, 1982; Duncan, 1982a; Struhl, 1982; Ingham, 1984; Dura et al., 1985; Struhl and White, 1985). These loci affect the fate of segments normally under the control of both complexes. Mutations at the maternal ef-
flect extra sex combs (esc) locus and in the Polycomb (Pc) locus result in embryos with all segments resembling the eighth abdominal segment (Struhl, 1981a; Lewis, 1978; Duncan and Lewis, 1982; Denell and Frederick, 1983). Antp protein expression is depressed in all segments of the nervous system in Pc (Figure 5d) and esc- (Figure 5e) embryos, to a weaker than wild type abdominal segment level of expression. An@ expression also extends into the presumptive head segments (Figure 5d). These results are in agreement with the previously reported effects of Pc on Antp transcript distribution (Wedeen et al., 1988).

Mutations of the Antp Locus

The Antp gene has two promoters, one (P1) for a 103 kb transcription unit and one (P2) for an overlapping (nested) 36 kb transcription unit. Transcripts initiating at both promoters contain the same protein coding exon sequences, but have different untranslated 5' leader sequences (Schneuwly et al., 1986; Laughon et al., submitted). In situ hybridizations to sectioned embryos with transcription unit-specific probes have revealed that the promoters are expressed with similar, but slightly different, spatial patterns during embryogenesis (A. Martinez-Arias et al., unpublished data). The P1- and P2-derived transcripts are found in roughly equal amounts in embryo RNA (Laughon et al., submitted). The antibody probe used here does not discriminate between Antp proteins derived from one transcription unit or the other.

Many Antp alleles cause lethality at late embryogenesis or larval stages. Included in this group of alleles are null alleles such as AntpW23 (an apparent point mutation, see Figure 4e) and Antp2, which is associated with a chromosome inversion that interrupts the P1 transcription unit, but leaves the smaller P2 transcription unit and the coding exons apparently intact. The Antp2 breakpoint is about 35 kb upstream of the internal P2 promoter (Scott et al., 1983; Laughon et al., submitted). Therefore, in Antp2 homozygotes we would expect to observe only the Antp protein that is encoded by transcripts from the P2 transcription unit. Surprisingly, no Antp protein can be detected in Antp2 homozygous embryos. Antp protein is also undetectable in embryos homozygous for five other

**Figure 5. Regulation of Antp Expression by Other Homeotic Genes**

(a) Wild-type embryo stained with anti-Antp antibody. Expression of Antp extends from posterior T1 to anterior A7. High levels of protein are in posterior T1, anterior T2, and anterior T3.

(b) Homozygous Ubx109 (Ubx-, abd-A-) embryo. High levels of Antp expression now extend from posterior T1 to anterior A7.

(c) Homozygous Df(SR)P9 (Ubx-, abd-A-, Abd-B') embryo. High levels of Antp expression still extend to anterior A7 in the absence of abd-B.

(d) Homozygous PolycombRE embryo. Antp expression is at low levels throughout the CNS, including the brain (large arrow) and A8 (eighth small arrow).

(e) Embryo from esc2/esc10 parental cross. Antp expression is at low levels throughout the CNS; note pairs of large nuclei visible in each segment (small arrows).

**Figure 6. Summary of the Spatial Regulation of Antp Expression in the Ventral Nervous System**

Schematic diagram of the patterns of Antp and Ubx protein distribution in wild-type and mutant embryonic nerve cords. Segments are indicated on the left, parasegments on the right. The protein products diagrammed are indicated at the top of the figures, the genotypes studied are shown at the bottom. Black areas represent high levels of expression, medium shading represents moderate levels of expression, and the lighter areas weak expression. Ubx and abd-A regulate the level of Antp in the abdominal segments. abd-B modulation of Ubx expression leads to repression of Antp in anterior T3.
alleles: Antp<sup>3B</sup>, Antp<sup>6</sup>, Antp<sup>x</sup>, Antp<sup>C</sup>, and Antp<sup>Cx</sup> (data not shown), all of which are dominant alleles associated with chromosome rearrangements that interrupt the Pl transcription unit and leave the P2 unit apparently intact. Heterozygotes carrying the dominant mutations have head-to-thorax transformations. Flies homozygous for the dominant mutations die as embryos or larvae, and have T2 to T1 transformations, like homozygous Antp<sup>2b</sup> or Antp<sup>x10</sup> flies (Wakimoto and Kaufman, 1981). The breakpoint of the Antp<sup>Ca</sup> inversion is just within the Pl unit, about 65 kb upstream of the P2 promoter (Scott et al., 1985). Thus, a lesion in the Pl unit, even at a considerable distance from the P2 promoter, prevents both the Pl and P2 units from producing protein at detectable levels.

Discussion

The Antp Protein Is Located in the Nucleus

In all of the tissues examined, the Antp protein is localized in the nuclei of Antp-expressing cells. The molecular function of the Antp proteins is unknown, but it has been hypothesized that by virtue of their homeodomains they may bind DNA-binding proteins (Laughon and Scott, 1984; Laughon et al., 1985). The nuclear location of Antp protein is consistent with this hypothesis and extends previous observations on the localization of homeodomain-containing products. The antigens recognized by antibodies against the Ultrathorax (White and Wilcox, 1984; Beachy et al., 1985), engrailed (DiNardo et al., 1985), and fushi tarazu (Carroll and Scott, 1985) protein products, each of which contains a homeodomain, are all located in the nuclei of the cells in which they are expressed.

The Dynamics of Antp Protein Expression and Its Correlation with Sites of Gene Function and RNA Expression

In the ectoderm, the Antp protein distribution changes rapidly. Initially, Antp expression is detected over an eight compartment region extending from the posterior labial to the anterior first abdominal segment. Later, as segments form, Antp proteins are found in five compartments from posterior T1 to posterior T3; the proteins then gradually disappear from the ectoderm. The initial distribution of Antp protein was unexpected, as no function of Antp has been established for the development of the posterior labium, anterior T1, or anterior A1 compartments. The longer lasting, higher level of expression in posterior T1 through posterior T3 correlates with the compartments where Antp<sup>+</sup> is known to be required (Wakimoto and Kaufman, 1981; Struhl, 1983; Sato et al., 1985; Struhl, 1981a, 1982; Kaufman and Abbott, 1984). In addition to the thoracic ectodermal expression of Antp, there is a high level of Antp expression in thoracic ganglia of the ventral nervous system. Although the complexities of cell divisions and movements within the nervous system make observations difficult, it appears that the nervous system pattern of Antp expression does not exactly correspond to the initial ectodermal pattern in that cells within the posterior T2 ganglia and T3 ganglia do not express Antp at high levels.

There is no clear indication of a discrepancy between the spatial patterns of Antp HNA and protein. The RNA described as being in parasegments 4 and 5 is probably also in anterior parasegment 6, judging from the relative widths of the two parasegment signals (Martinez-Arias, 1985), in agreement with our results. However, the time at which the protein was detectable was much later than when transcripts have been observed. Antp protein was not detectable until the germ band extension stage of embryogenesis, like Ubx protein, whereas transcripts are detectable at the cellular blastoderm stage (Levine et al., 1983). The discrepancy is likely to reflect the time needed to process the very long Antp transcripts, and possibly a system of translational control as well. The large 5' leaders of Antp mRNAs (Schneuwly et al., 1986; Laughon et al., submitted), may be involved in control of translation.

The Regulation of Antennapedia Expression

Examination of Antp expression in the nervous system in embryos homozygous for a variety of mutations has revealed three main features of Antp regulation: one, the level of Antp expression is negatively regulated by the more posteriorly active genes Ubx and abd-A; two, Antp expression is influenced by the more globally acting extra sex combs and Polycomb loci; and three, normal embryonic expression of Antp protein depends upon the integrity of elements up to 65 kb upstream of the smaller transcription unit and the coding exons.

Antp protein levels are modulated by the activity of BX-C genes, and exhibit an inverse relationship to the level of Ubx expression (summarized in Figure 6). The level of Antp in the more posterior abdominal segments is also influenced by the abd-A gene, as can be seen by the dramatic effects of removal of abd-A product in a Ubx, abd-A double mutant. It is surprising that the Abd-B locus has no effect on Antp expression, since it does affect Ubx expression in segment A8 (Struhl and White, 1985). Other genes, outside of the BX-C, must act to repress Antp expression in A8.

In the absence of csc or Polycomb products, Antp and Ubx (Beachy et al., 1985; White and Wilcox, 1985b; Struhl and White. 1985) patterns of expression degenerate to an improper spatial pattern with low levels of expression extending through most segments of the nerve cord, including the head (Figure 5d). Thus, the esc and Pc products are required in all segments (not just the more posterior regions) for proper homeotic gene activity. It has been found that Antp RNA is initially derepressed in most cells in Pc<sup>-</sup> embryos (Wedeon et al., 1986). The low level of Antp protein observed in esc<sup>-</sup> and Pc<sup>-</sup> embryos may be due to the eventual repression of Antp expression by the derepressed expression of Ubx, abd-A, and possibly other negative regulators of Antp (Struhl and White, 1985).

Proper embryonic expression of Antp protein depends upon the integrity of elements far upstream from the coding exons of the locus. We could not detect protein expression in embryos homozygous for the six chromosome rearrangement mutations analyzed, even though the P2 unit is apparently intact in each case. The five dominant alleles must, at some later point in development, express Antp product(s), since the alleles do affect head development. Two hypotheses are suggested by these results.
One is that cis-acting elements required by the smaller P2 transcription unit are located as far as 65 kb upstream of the P2 promoter. The second is that Antp protein may itself be required for initiating or maintaining P2 transcription unit activity, and that protein (or RNA) made using the P1 promoter stimulates protein production by the P2 unit. In the absence of a functional P1 unit, in cis or in trans, the P2 unit would fail to produce protein. A less likely possibility is that the P2 unit transcripts are not translated into protein in either wild-type or mutant embryos.

The enormous size and complex pattern of expression of the Antp locus suggest that there are multiple mechanisms through which other genes may control the time, place, and level of Antp activity. Transcription and RNA processing are clearly both important levels of control that affect Antp protein production. Having learned of some of the genes, such as Ubx and Abd-A, that control Antp in trans, it will be important to find out the molecular mechanisms that underlie the regulatory interactions among the genes.

Experimental Procedures

Gene Fusions

The Antp cDNA clone pDmG1100 has been described previously (Scott et al., 1983) and completely sequenced (Laughon et al., submitted). A 1298 bp PstI–EcoRI fragment and a 1816 bp Smal–EcoRI fragment were purified, single-stranded ends were made blunt with T4 DNA polymerase, and EcoRI 10-mer linkers that had been phosphorylated with T4 polynucleotide kinase were ligated to the fragments. After ligation, the DNA was digested with EcoRI and the fragments were repurified. The fragments were subcloned into pmC8 and cloned plasmid DNA was digested with EcoRI to recover and purify the linkered fragments. The DNA was digested with EcoRI and the fragments were repurified. The fragments were subcloned into pmC8 and cloned plasmid DNA was digested with EcoRI to recover and purify the linkered fragments. Each fragment was ligated into the unique EcoRI site of λgt11 at the 3' end of the lacZ gene (Young and Davis, 1985). Ligated DNA was packaged and the phage were plated on RY1090 and scored for colorless plaques. The DNA from phages yielding colorless plaques was purified and analyzed for inserts of the Antp fragments in the proper orientation. The construction was checked by Allen Laugon for correct reading frame usage by DNA sequencing insert junction fragments.

Expression and Purification of β-Galactosidase–Antp Hybrid Proteins in E. coli

Lysogens of λgt11 Antp P10 and λgt11 Antp S10 were established in RY1090 (Young and Davis, 1983). Hybrid proteins were induced, extracted, and purified essentially as described elsewhere (Carroll and Scott, 1985).

Production, Purification, and Assay of Antibodies

Rabbits were immunized with 600 μg of soluble Antp10 antigen in complete Freund's adjuvant on day 0 and again with 200 μg of antigen days 14 and 21 (and monthly thereafter) in incomplete adjuvant. All injections were subcutaneous at multiple sites. Immunizations with gel-purified SDS-denatured Antp P10 antigen (200 μg each) were intramuscular in incomplete Freund's adjuvant. Rabbits were bled on day 28 and about monthly thereafter until the final exsanguination.

Antisera were absorbed on β-galactosidase-Sepharose 4B as described (Carroll and Scott, 1985). The flow-through fraction from the β-galactosidase column of the Antp P10 serum was affinity-purified on a column containing the Antp S10 hybrid antigen crosslinked to anti-β-galactosidase antibody that was coupled to CNBr-activated Sepharose 4B. Affinity columns containing the Antp P10 antigen did not yield antibody that was useful for immunofluorescence. The procedures for constructing the affinity columns are described elsewhere, as are the immunoblotting procedures for assaying antibody specificity (Carroll and Scott, 1985).

Immunofluorescence

Whole Drosophila embryos were dechorionated, permeabilized in heptane, fixed in formaldehyde, and devitellinized in heptane/methanol as described (Karr and Alberts, 1986). Embryos were stained with primary anti-Antp antibody and monoclonal anti-Ubx antibody (White and Wilcox, 1984) and secondary fluorescein-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-mouse Ig as described for other antibodies (Carroll and Scott, 1986). Ubx-Δ2 is a null mutant that does not produce detectable Ubx protein (Beachy et al., 1985). Abd-A mutant embryos were generated from the abd-AΔ11 stock (Sanchez-Herrero et al., 1985) or as progeny of DfP1; Ubx1/1M3 parents. Abd-B embryos were derived from the Abd-BΔ11 stock (Sanchez-Herrero et al., 1985) Ubx-Δ2 double mutants were derived from the Df(3R)109 stock, which is a deletion of the BXC to the ib-5 region (Karch et al., 1985). It is possible that Df(3R)109 has some negative cis effect on abd-B esc-ε embryos were derived from a cross of esc-ε/esc-α trans-heterozygotes generated as described (Struhl and White, 1985). Embryos mutant for the Ubx, Ubx-Δ2, abd-A, Pc, and esc-ε loci were unambiguously identified on the basis of their altered Ubx staining and then inspected for their Antp pattern. The zygotic mutants (and the zygotic effect of Pc) were constructed by crossovers of heterozygotes. For the Scharz mutation, several hundred stained embryos were inspected, all of which exhibited the wild-type pattern.

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