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Transcriptional Activation by the *Antennapedia* and *fushi tarazu* Proteins in Cultured Drosophila Cells

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Summary

Drosophila homeodomain proteins bind to specific DNA sequences in vitro and are hypothesized to regulate the transcription of other genes during development. Using a cotransfection assay, we have shown that homeodomain proteins encoded by the homeotic gene *Antennapedia* (*Antp*) and the segmentation gene *fushi tarazu*, as well as a hybrid homeodomain protein, are activators of transcription from specific promoters in cultured Drosophila cells. Sequences downstream of the *Antp* P1 and *Ultrabithorax* transcription start sites mediate the observed activation. A TAA-rich DNA sequence to which the *Antp* protein binds in vitro is sufficient to confer regulation on a heterologous promoter. The results demonstrate that homeodomain proteins are transcriptional regulators in vivo and that in cultured cells, different homeodomain-containing proteins can act upon a common sequence to modulate gene transcription.

Introduction

The formation and differentiation of body segments in Drosophila is controlled by a network of interacting genes (reviewed in Akam, 1987; Scott and Carroll, 1987; Ingham, 1988). Maternally active genes provide information for establishing the anterior–posterior and dorsal–ventral axes of the embryo (Nüsslein-Volhard et al., 1987). Zygotically active genes interpret and refine the information so that proper segmentation and dorsal–ventral differentiation occur. Segmentation genes are involved in the formation of the repeating segmental pattern in the embryo, and in some cases also affect the differentiation of segments from each other (Nüsslein-Volhard and Wieschaus, 1980). The segmental differentiation process also requires the activities of homeotic genes, which are differentially expressed along the anterior–posterior and dorsal–ventral axes. Homeotic genes appear to integrate information from earlier acting genes and may subsequently regulate developmental pathways by controlling the transcription of as yet unknown target genes. In addition, some homeotic genes are regulators of other homeotic genes (Hafen et al., 1984; Struhl and White, 1985; Carroll et al., 1986; Riley et al., 1987; Bienz and Tremml, 1988).

More than a dozen of the Drosophila segmentation and homeotic genes share a common feature, the "homeobox," an evolutionarily conserved DNA sequence that encodes a 61 amino acid peptide, the homeodomain (reviewed in Gehring and Hiromi, 1986; Gehring, 1987; Scott et al., 1989). The homeodomain has been proposed to be a DNA binding domain on the basis of its similarity to helix-turn-helix regulatory proteins in bacteria (Laughon and Scott, 1984) and yeast (Shephard et al., 1984; Laughon and Scott, 1984). The nuclear location of the homeodomain proteins studied to date is consistent with this hypothesis. In addition, the altered spatial patterns of transcription of some segmentation and homeotic genes observed in Drosophila embryos mutant for other homeobox-containing genes is consistent with the hypothesis that homeodomain-containing proteins are regulators of transcription (Hafen et al., 1984; Harding et al., 1985; Struhl and White, 1985; Carroll et al., 1986; Ingham and Martinez-Arias, 1986; DiNardo and O'Farrell, 1987). In vitro DNA binding studies have demonstrated that Drosophila homeodomain-containing proteins bind to specific DNA sequences within promoter regions of genes regulated by such proteins (Desplan et al., 1985, 1988; Hoey et al., 1988; Laughon et al., 1988; Beachy et al., 1988).

We have designed experiments to test one function of two homeodomain proteins using the Drosophila cultured cell system described in the accompanying paper (Krasnow et al., 1989). The approach makes it possible to study the interactions of a single *trans*-acting factor on a target gene in a simpler in vivo context than that of the developing embryo. Drosophila cultured cells are transiently cotransfected with a protein-encoding plasmid and a reporter plasmid containing a Drosophila promoter sequence coupled to a reporter gene. The effect of the protein produced from one of the plamids on the other is then monitored by changes in reporter gene expression. In the accompanying paper (Krasnow et al., 1989), the regulatory effect of proteins encoded by the *Ultrabithorax* (*Ubx*) homeotic gene are described. Here we report the effect of two homeodomain-containing proteins, encoded by the *Antennapedia* (*Antp*) homeotic gene and the *fushi tarazu* (*ftz*) segmentation gene as well as an *Antp–Ubx* hybrid protein. The *Antp* and *ftz* genes are both members of the Antennapedia Complex (ANT-C), a cluster of regulatory genes that includes at least nine homeobox-containing genes (Kaufman et al., 1980; Mahaffey and Kaufman, 1989) while *Ubx* is a member of the Bithorax Complex (Duncan, 1987). The *Antp*, *ftz*, and *Ubx* proteins contain very similar homeodomains (77% amino acid identity), but have very different functions during development. While *ftz* is required for proper segmentation, *Antp* and *Ubx* specify segmental identities. Because regulatory interactions among homeotic and segmentation genes have been proposed on the basis of genetic experiments, we have used promoter sequences from *Antp* and *Ubx* as targets for regulation by homeodomain-containing proteins in the cultured cell system. We report the abilities of *Antp*, *ftz*, and *Antp–Ubx* proteins to specifically activate transcrip-

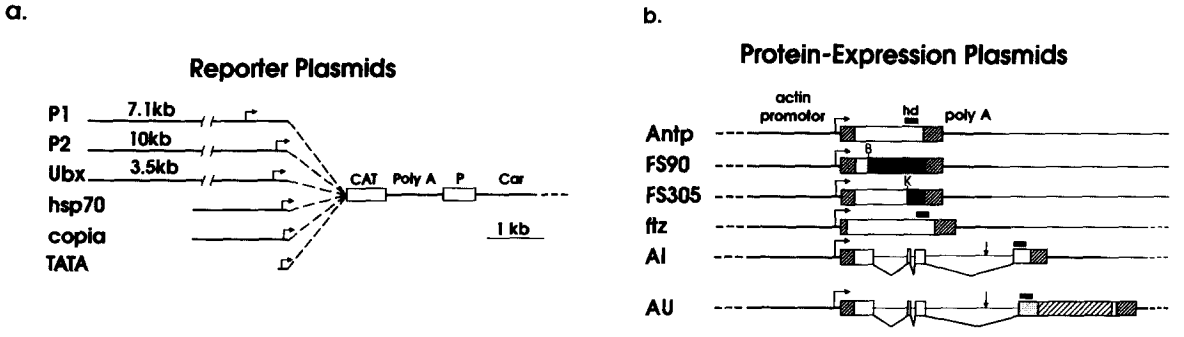


Figure 1. Diagram of Plasmid Constructions

(a) Reporter Plasmids. Reporter plasmids contain the 5′ regulatory regions and 5′ nontranslated sequences from the indicated genes inserted into the vector pC4CAT upstream of the bacterial chloramphenicol acetyltransferase gene. P1 and P2 are DNA fragments from the two *Antp* promoters. TATA is a short fragment from the *hsp70* gene, which contains 40 bp upstream and 90 bp downstream from the transcription start site inserted into the vector phsCaspCAT (for details, see Experimental Procedures). Open boxes indicate the coding sequence of the CAT gene and P element sequences, respectively. Poly(A):SV40 polyadenylation signals. Car: Carnegie 4 P element transformation vector. The horizontal arrows indicate the start of transcription.

(b) Protein-expression plasmids. The coding (open boxes) and noncoding (hatched boxes) regions from the *Antp*, *ftz*, and *Ubx* genes were inserted into the expression vector pP_{ac} (for details, see Experimental Procedures). A portion of the Drosophila *actin 5C* promoter and the *actin 5C* polyadenylation signals are indicated (bold horizontal lines). The light horizontal lines indicate pUC18 sequences. The homeodomain is indicated by the solid bars above the constructions. FS90 and FS305 are *Antp* constructs that contain frameshift mutations engineered at the BstXI (B) or Kpn (K) sites. AI ($pP_{ac}AI$) is the *Antp* intron-containing "minigene." The introns are indicated by the lines connecting the open boxes. The vertical arrows indicate the site of truncation of the intron preceding the homeodomain exons. AU ($pP_{ac}AU$) is the *Antp–Ubx* hybrid minigene. In this construct, *Ubx* intron sequences, the *Ubx* homeobox (stippled), and the 3′ noncoding sequence (hatched) have been substituted for the corresponding region in the *Antp* minigene. Some *Antp* sequences remain in this plasmid downstream of the *Ubx* transcription termination sites.

tion of certain promoters and characterize the sequences through which the proteins act.

Results

Gene Activation by the *Antp* Protein

Several Drosophila promoters were screened for possible regulation by the *Antp* protein. The five promoters that were tested in this study were the *Ubx* promoter (Hogness et al., 1985; Saari and Bienz, 1987; Kornfeld et al., 1989), the two *Antp* promoters (Schneuwly et al., 1986; Stroeher et al., 1986; Laughon et al., 1986), the *hsp70* promoter (Karch et al., 1981), and the *copia* transposon promoter (Sinclair et al., 1986). Each of the reporter plasmids used contains a promoter fragment joined to the E. coli chloramphenicol acetyl transferase (CAT) gene (Figure 1a; Thummel et al., 1988; Krasnow et al., 1989). The plasmids were transfected into Drosophila Schneider Line 2 (S2) cells (Schneider, 1972), and CAT activity was assayed 48–60 hr later. All of the tested promoters were detectably active in these transient assays (data not shown; Krasnow et al., 1989).

To determine whether *Antp* protein can affect transcription from any of the candidate target promoters, a plasmid was constructed that directs expression of an *Antp* protein (Figure 1b). In this plasmid, the Drosophila *actin 5C* promoter (Bond and Davidson, 1986) was joined to the *Antp* cDNA G1100 (Laughon et al., 1986). *actin 5C* is active in most or all Drosophila cells. The *Antp* protein–expression plasmid contains the entire *Antp* protein–coding sequence as well as 253 bp of the 5′ nontranslated region, part of the 3′ trailer sequence, and *actin 5C* polyadenylation signals (Figure 1b). When the *Antp* protein–producing plasmid was introduced into S2 cells, *Antp* protein was immunochemically detected in the cell nuclei (Figure 2a), where it also localized in the embryo (Carroll et al., 1986; Wirz et al., 1986). Ten to thirty percent of the cells make detectable *Antp* protein during the period after transfection when CAT activity is assayed (2–2.5 days posttransfection).

It is important for the experiments that both of the cotransfected plasmids enter the same cells, because not all of the cells express the transfected DNA. In control experiments using two different plasmids that express *Antp* and *Ubx* protein, the *Antp* and *Ubx* proteins were detected using fluorescein- and rhodamine-conjugated secondary antibodies, respectively. Cells that produced one protein also made the other (data not shown). Apparently, Drosophila S2 cell cultures, like mammalian cultures (Wigler et al., 1979), contain a subset of cells that exhibit a high probability for productive transfection so that cells that can take up one plasmid take up two or more.

For use in control experiments, frameshift mutations were introduced within the protein-coding sequence of the *Antp* cDNA (which has 378 codons). One frameshift was at codon 90 (FS90) and the other was at codon 305 (FS305; Figure 1b). The FS305 frameshift interrupts the open reading frame at the 11th codon of the homeodomain. Immunolocalization experiments using a polyclonal anti-*Antp* antibody revealed a barely detectable amount of protein in the cytoplasm and nuclei of S2 cells transfected with the FS90 plasmid (Figure 2b). The decrease in detectability is likely due to the loss of antigenic determinants from the truncated protein. *Antp* protein was read-

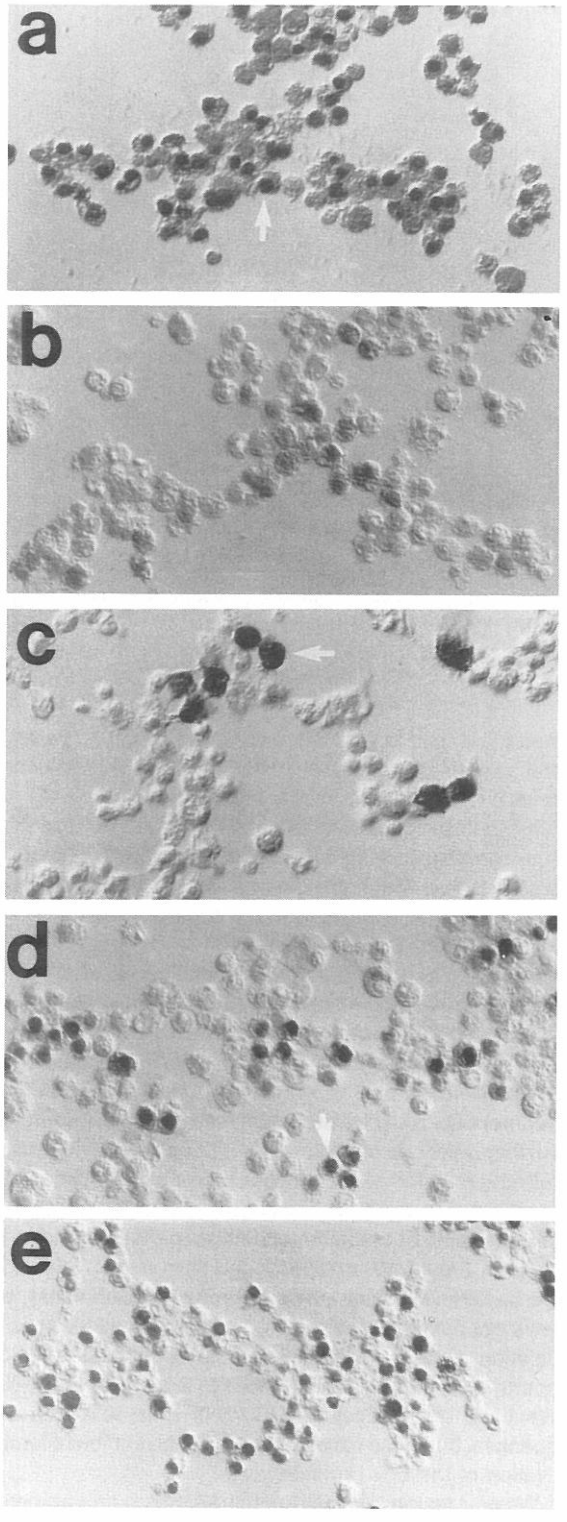


Figure 2. Localization of the *Antp* Protein in Cultured Cells

The cells were transfected with plasmids encoding various forms of the *Antp* protein (Figure 1) and stained with polyclonal anti-*Antp* antibody followed by a horseradish peroxidase–conjugated secondary antibody (see Experimental Procedures).

(a) Protein produced from a plasmid that encodes the full-length *Antp* protein. Note the nuclear localization (arrow). The background staining in the cytoplasm is slightly higher than usual in this photograph.

(b) Protein produced from the FS90 plasmid. Weak staining is visible in some cells.

(c) Protein produced from the FS305 plasmid. *Antp* protein is expressed at high levels but is not localized to the nuclei (arrow).

(d) Protein produced from the *Antp* minigene plasmid ($pP_{ac}Al$). The protein is localized to the cell nuclei (arrow).

(e) Protein produced from the hybrid *Antp–Ubx* plasmid ($pP_{ac}AU$).

ily detected in cells transfected with the FS305 plasmid, but in contrast to the full-length protein the FS305 protein was found at high levels in both the cytoplasm and the nuclei (Figure 2c).

When an *Antp* protein–producing plasmid was cotransfected with the *Ubx* reporter plasmid, the full-length *Antp* protein was found to stimulate the *Ubx* promoter about 30-fold (Figure 3). The *Antp* P1 promoter was stimulated 10- to 12-fold in six early experiments and from 3- to 7-fold in seven more recent experiments. The reason for the apparent decrease in stimulation was not determined. The FS90 construct did not significantly affect the activity of any promoter tested, indicating that *Antp* protein mediates the observed regulatory effects. The plasmid encoding full-length *Antp* protein did not significantly alter the level of expression from the *Antp* P2, *hsp70*, or *copia* promoters (Figure 3a), indicating that the effect is promoter-specific. The FS305 results show that this truncated protein, which lacks the homeodomain, has no significant effect on the *Ubx* promoter, although it may retain some activity on the *Antp* P1 promoter (Figure 3).

The *Antp* Protein Activates Transcription

We performed RNAase protection analyses to determine if the proper transcriptional start site is utilized in S2 cells and if the changes in CAT activity observed upon transfection of the *Antp* plasmid are due to a change in mRNA levels. Total RNA from S2 cells was harvested 48 hr after transfection and hybridized to a uniformly labeled radioactive antisense RNA probe that is complementary to the region surrounding the predicted *Ubx* transcription start sites (Figure 4a; Saari and Bienz, 1987; Kornfeld et al., 1989). Upon treatment with RNAase A and RNAase T_1, a protected fragment is observed (Figure 4b, lane 4) with the size predicted for an mRNA transcript initiating at the previously mapped embryonic *Ubx* start sites (Saari and Bienz, 1987; Kornfeld et al., 1989). No protected fragments were detected using RNA from mock transfected S2 cells, cells transfected with the *Antp* protein–producing plasmid alone, or the pP_{Ubx}–CAT reporter plasmid alone (Figure 4, lanes 1–3). The basal level of transcription from the pP_{Ubx}–CAT plasmid is apparently below the level of detection of this experiment. The results indicate that *Antp* protein activates transcription from the plasmid copies of the *Ubx* promoter. However, no transcription of the cellular *Ubx* gene was detected in the presence or absence of *Antp* protein, possibly because the cellular copy is not transcriptionally competent in S2 cells. Alternatively, because the endogenous gene is present in only two copies per cell its transcripts may be below the level of detection.

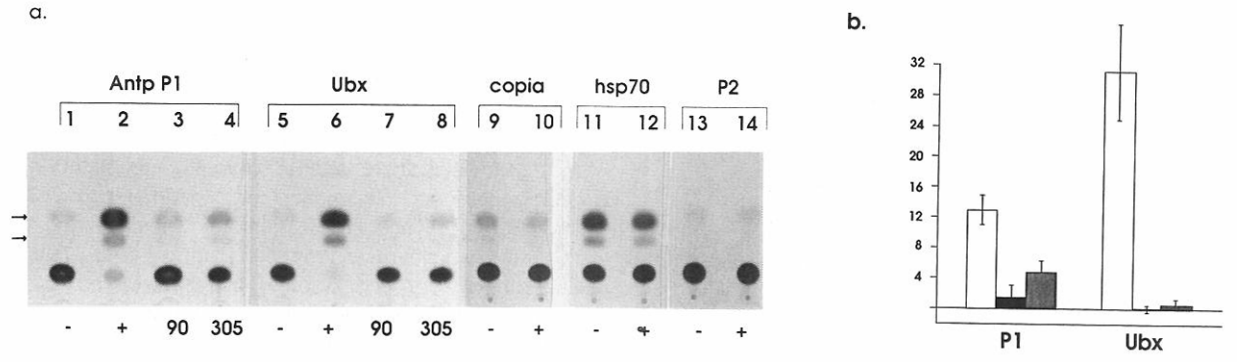


Figure 3. *Antp*-Dependent Stimulation of CAT Activity

(a) Results of the CAT assay. The promoter-CAT construct is indicated at the top. The notation at the bottom refers to the protein producing plasmid. −: the actin expression vector (pP_{ac}) with no insertion of coding sequences. +: the actin vector with the insertion of the full-length *Antp* protein. 90: the FS90 plasmid. 305: the FS305 plasmid (Figure 1). Arrows point to the acetylated forms of chloramphenicol. The full-length *Antp* protein causes a dramatic increase in CAT activity produced from the *Antp* P1 (lane 2) and *Ubx* (lane 6) promoters, while no significant changes are observed from the *copia* (lane 10), *hsp70* (lane 12), or *Antp* P2 (lane 14) promoters. The basal level transcription from the *hsp70* promoter was that obtained without heat shock. No stimulation is observed from the FS90 plasmid (lanes 3 and 7). The FS305 plasmid may cause some stimulation of the *Antp* P1 promoter (lane 4) but does not affect the *Ubx* promoter (lane 8).

(b) Quantitation. The numbers on the abscissa are the fold activation over the basal level CAT activity obtained during the actin expression vector without insertion of a protein-encoding sequence. Where necessary, CAT assays were repeated to obtain data in the linear range of the assay (from about 5% to 60% conversion). The data are from two separate experiments. Open bars: full-length *Antp* protein. Solid bars: FS90. Stippled bars: FS305. The standard deviations are indicated.

Binding of the *Antp* Protein to *Ubx* Sequences

To identify *cis*-acting sequences that respond to the *Antp* protein, we focused on the *Ubx* promoter because it gave the highest levels of induction. The effect of the *Antp* protein could be mediated directly by *Antp* protein bound to the *Ubx* sequences or by transcription factors that are activated in some way by *Antp* protein and that bind to specific *Ubx* DNA sequences. We have used *Antp* protein produced in S2 cells to determine if the protein can bind to DNA sequences responsive to this protein in cotransfection assays. The *Antp* protein was produced in S2 cells stably transformed with a plasmid containing the Drosophila *hsp70* promoter joined to the G1100 cDNA. The details of the protein production and extract preparation will be reported elsewhere (S. Hayashi et al., unpublished data). From the extract, *Antp* protein was immunoprecipitated using a polyclonal anti-*Antp* antibody, and the immunoprecipitate was incubated with end-labeled DNA fragments of the pP_{Ubx}–CAT plasmid. After washing, the DNA fragments that were bound by the *Antp* protein immunoprecipitate were displayed on a polyacrylamide gel (Figure 5). At a high salt concentration (275 mM KCl), only some DNA fragments were bound to *Antp* protein. Three of the bound fragments are from the *Ubx* sequences, and one fragment is from the vector. The *Ubx* fragments that were bound by *Antp* protein are shown schematically in Figure 6. Binding of the *Antp* protein to the four fragments on the *Ubx* promoter was confirmed using a gel mobility shift assay (see Figure 7 for fragment D; data for fragments A, B, and C, are not shown).

Mapping the Target DNA Sequences

To define the elements that are required for activation in the cultured cells, deletions were constructed so as to eliminate one or more of the fragments of the *Ubx* promoter that bind to the *Antp* protein in vitro. The results of one experiment are shown in Figure 6. The deletions caused no significant change in the basal level of CAT activity, indicating that the deletions have not removed sequences required for basal level expression. The only exception is deletion 4, which eliminates the normal transcription start site and results in a drastic reduction of CAT activity (Figure 6).

The most important *Ubx* sequence for induction by the *Antp* protein is downstream from the transcription initiation site in the 5′ nontranslated region. Deletions 1–3 have little or no effect on activation by *Antp*, while deletion 6, which removes part of the 5′ nontranslated region (i.e., fragment D), reduces activation to a low level (Figure 5). Further deletions of upstream sequences in combination with the deletion of fragment D cause no additional loss of stimulation by *Antp* protein (deletions 6–8). In three other repeats of the same experiment, the stimulation of deletion 8 by *Antp* protein ranged from 0.5- to 1.5-fold of the basal level. Using an immunoprecipitation assay, we have not detected *Antp* binding to any *Ubx* sequences in deletion 8, even though this construct contains a region bound by the *Ubx* protein (Figure 5; Beachy et al., 1988). We have also detected binding of *Antp* to vector sequences, but these sites appear not to be sufficient for activation of the *Ubx* promoter.

We next tested the ability of the *Antp*-binding fragments to stimulate gene expression when joined to the deletion 8 construct at a position 625 bp upstream of the *Ubx* transcription start. The deletion 8 construct is quite unresponsive to the *Antp* protein, but addition of fragment D upstream in either orientation restores activity to the level obtained when fragment D is in its normal downstream position (Figure 6; compare 8D+ and 8D− with deletion 3). Little or no activation is seen when *Antp* protein–binding

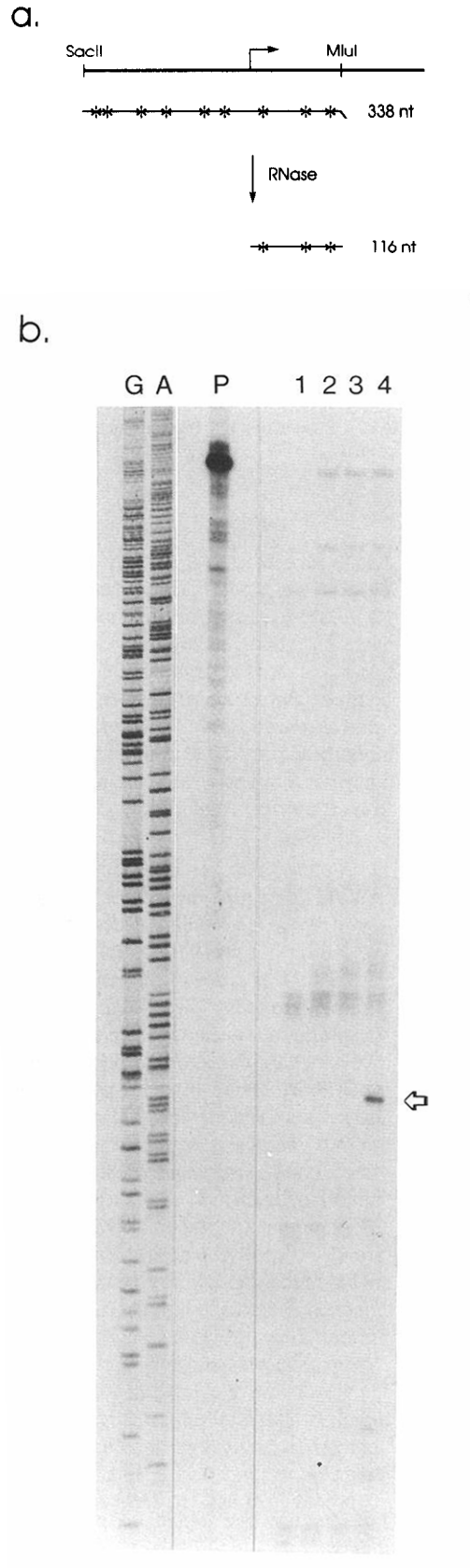


Figure 4. RNA Protection Analysis of the *Ubx* Promoter

(a) A diagram of the 5′ end of the *Ubx* mRNA. The horizontal arrow indicates the approximate position of transcription initiation. A 116–120 RNAase-resistant nucleotide fragment is expected after hybridization of the labeled probe and RNAase digestion.

(b) Total RNA from S2 cells transfected with carrier plasmid only (lane 1), the *Antp* protein–producing plasmid (lane 2), the pP_{Ubx}–CAT plasmid (lane 3), or both the protein-producing plasmid and the *Ubx* reporter plasmid (lane 4) was hybridized to the continuously labeled RNA probe described above (a). After digestion with RNAase A and T_1, an approximately 116 bp fragment is observed only in cells transfected with both the protein-producing and reporter plasmids (lane 4, arrow). The size of the protected fragment corresponds to the size predicted for proper transcriptional initiation (Saari and Bienz, 1987; Kornfeld et al. (1989). Two weakly protected bands are also observable in lane 4 and probably correspond to RNAase cleavage within an AT-rich region of the *Ubx* mRNA. G, A: dideoxy sequencing reaction used as size markers. P: nascent RNA probe.

fragments A or B are inserted at a position at the upstream end of the deletion 8 construct. Therefore, the inability of fragments A and B to respond to *Antp* is not merely due to their normal positions, distant from the *Ubx* promoter. It is possible that fragments A and B bind *Antp* in vitro, but with only a low affinity in vivo, and are consequently not as important for gene activation. We have also tested the activity of a fragment from the *Antp* P1 5′ nontranslated region (+115 to +606) that binds the *Antp* and *Ubx* proteins in vitro (S. Hayashi, unpublished data; Beachy et al., 1988). When added to the deletion 8 construct, this fragment also restored a substantial amount of the *Antp*-dependent CAT activity, suggesting that target sequences responsible for activation of the P1 promoter also lie downstream of the transcription start site and that they can function to activate a different promoter. The deletion 4 construct lacks the *Ubx* transcription start site and has very low basal promoter activity, but is nonetheless stimulated by the *Antp* protein (Figure 6). Deletion 4 retains fragment D, so it is possible that a cryptic promoter on the plasmid can function and is responsive to activation by *Antp* mediated by fragment D.

An *Antp* Binding Site Can Confer *Antp*-Dependent Activation upon a Heterologous Promoter

When various deletions of fragment D were tested for binding to *Antp* protein in a mobility shift assay using *Antp* protein partially purified from cultured cells, nucleotides +224 to +298 were found to be required (data not shown). Results of a mobility shift assay using a 75 bp double-stranded oligonucleotide containing the sequence +224 to +298 demonstrate *Antp* sequence-specific DNA binding (Figure 7). After incubation with the *Antp* extract, at least four protein–DNA complexes are observed in the presence of a 1000-fold excess of poly(dI–dC) and a 12-fold excess of salmon sperm DNA (Figure 7, open arrows). Incubation with a monoclonal anti-*Antp* antibody further retards the mobility of two of the complexes (Figure 7, lanes 3 and 4; open arrows), while a nonspecific IgG has no effect (Figure 7, lanes 6 and 7). Furthermore, addition of unlabeled oligonucleotide at a 10- to 300-fold excess successfully competes for the formation of the complexes

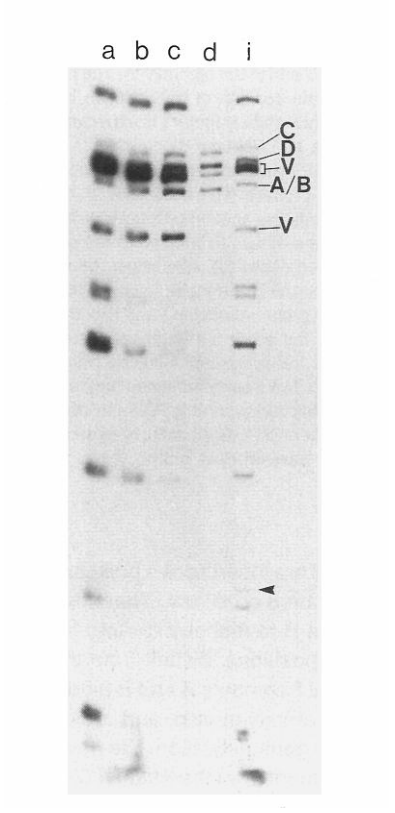


Figure 5. Immunoprecipitation DNA Binding Assay

The pP_{Ubx}–CAT plasmid was cleaved with HinfI, end-labeled, and incubated with an *Antp* protein immunoprecipitate. The complexes were washed with 200 (a), 225 (b), 250 (c), and 275 (d) mM KCl in binding buffer (see Experimental Procedures), phenol extracted, and displayed on a 5% polyacrylamide gel. i: input DNA. Fragments A/B, C, D contain DNA sequences from the *Ubx* upstream region. Fragments labeled V are from the C4CAT vector and contain P element sequences. The identities of the retained fragments were confirmed by digestion with a second enzyme. The positions of fragments A–D are shown in Figure 6. A and B are mapped to the same fragment in this assay, but subsequent mobility shift assays revealed that *Antp* binds to both of the fragments (data not shown). The arrowhead marks a fragment from the *Ubx* 5′ nontranslated region (+59 to +173) that contains a binding site for the *Ubx* protein Ubx-A; Beachy et al., 1988). There is no apparent binding of this fragment to the *Antp* protein in this assay.

(Figure 7, lanes 8–11). The same region of DNA is protected by the *Antp* protein in DNAase I protection experiments (S. Hayashi et al., unpublished data). The protected nucleotides correspond to the "Ubx-B" region bound by the *Ubx* proteins (Beachy et al., 1988), suggesting that *Antp* and *Ubx* bind to the same sequences in vitro.

The 75 bp oligonucleotide was joined to a truncated *hsp70* promoter (essentially a TATA box and an initiation site) to test whether the oligonucleotide could confer *Antp*-dependent gene activation on a heterologous Drosophila promoter. The sequence of the oligonucleotide is indicated in Table 1. The truncated *hsp70* promoter contains 40 bp upstream of the transcription start site and 90 bp of the 5′ nontranslated region joined to the CAT gene; its basal level CAT activity was not affected by a cotransfected plasmid that encoded the *Antp* protein (Table 1). When the oligonucleotide was inserted upstream of the

Table 1. Activation of a Heterologous Promoter

Insertion	Fold Change in Basal Level[a]	Induction by *Antp*	Induction by *ftz*
hsCaspCAT	–	1.1 ± 0.75[b]	1.0 ± 0.17
+[c]	1.4 ± 0.66	6.4 ± 3.2	19.1 ± 9.3
–	0.90 ± 0.15	8.9 ± 3.9	16.7 ± 4.9
– – +	1.1 ± 0.13	33.4 ± 4.5	22.6 ± 3.5

[a] Basal level CAT activity obtained without heat induction, using the FS90 plasmid.

[b] Data presented are the mean and standard deviation of two or three duplicate transfections. In at least three separate experiments, very similar results were obtained.

[c] + or – indicates orientation and number of oligonucleotide(s) in phsCaspCAT relative to transcription initiation.

The sequence of the oligonucleotide is: 5′-TAATAATCGTTCAAATC-GTTAAAACCATAAAAATAATAATAATTGCAATAACAATAAACATA-GTAATAATG-3′ (+ orientation), with the addition of a 5′ GATC extension at each end.

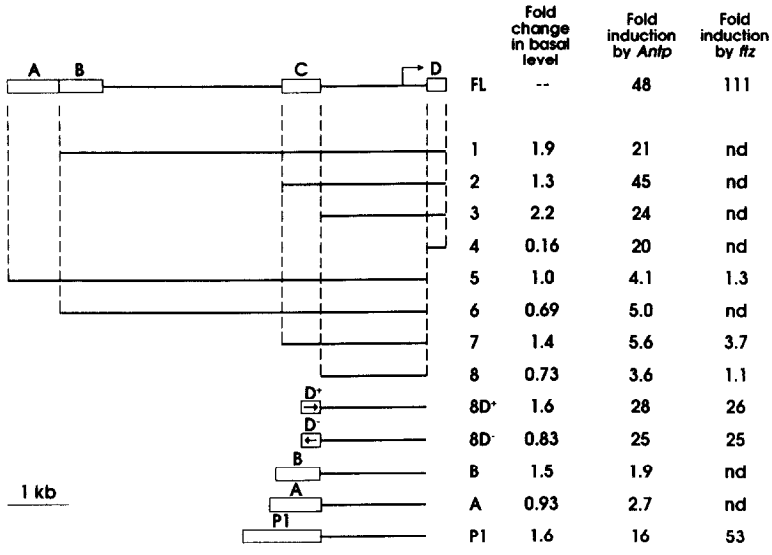
truncated *hsp70* promoter, CAT activity was increased 6- to 9-fold over the basal level in the presence of *Antp* protein (Table 1). About the same stimulation was observed when the oligonucleotide was inserted in either orientation relative to the start of transcription. When three copies of the oligonucleotide were inserted, more than 30-fold stimulation was observed (Table 1). Therefore, a relatively short sequence from the *Ubx* 5′ nontranslated region allows a normally nonresponsive heterologous promoter to be activated by the *Antp* protein.

The *ftz* Protein also Activates the *Ubx* Promoter

Antp, *Ubx*, and *ftz* contain highly similar homeodomains and might therefore act on similar or identical *cis*-acting elements. We found that the *ftz* protein also stimulates CAT activity from the *Ubx* promoter (Figure 6). The induction ranged from 10- to 100-fold in different experiments. The *ftz* gene is required for normal *Ubx* mRNA levels in embryos as well (Ingham and Martinez-Arias, 1986). When the fragment D is eliminated from the pP_{Ubx}–CAT plasmid, essentially all stimulation is lost (Figure 6, deletion 8). As with the *Antp* protein, a significant portion of the stimulatory effect can be recovered when the D fragment is joined upstream of deletion 8, or when a fragment from the *Antp* P1 5′ nontranslated region (+115 to +606) is inserted (Figure 6). A bacterial *ftz*-β-*gal* fusion protein has been demonstrated to bind in vitro to DNA sequences 3′ to the P1 transcription start site (Laughon et al., 1988). As with *Antp*, *ftz* can activate the heterologous *hsp70* promoter containing the 75 bp insertion (Table 1). The accompanying paper (Krasnow et al., 1989) demonstrates that this same region is also required for *Ubx* protein–dependent *Ubx* promoter activation in a transient assay. Therefore, *Antp*, *ftz*, and *Ubx* proteins all act via the same region near the *Ubx* promoter to activate transcription.

A *Ubx* Homeodomain Can Substitute for the *Antp* Homeodomain

The above results suggest that *Antp*, *ftz*, and *Ubx* recognize the same *cis*-acting elements in the *Ubx* promoter.



	Fold change in basal level	Fold induction by *Antp*	Fold induction by *ftz*
FL	--	48	111
1	1.9	21	nd
2	1.3	45	nd
3	2.2	24	nd
4	0.16	20	nd
5	1.0	4.1	1.3
6	0.69	5.0	nd
7	1.4	5.6	3.7
8	0.73	3.6	1.1
8D+	1.6	28	26
8D-	0.83	25	25
B	1.5	1.9	nd
A	0.93	2.7	nd
P1	1.6	16	53

Figure 6. Deletion Analysis of the *Ubx* Upstream and Nontranslated Region

The arrow indicates the start of transcription. Fragments that have been found to bind the *Antp* protein in vitro are indicated as open boxes and are labeled A through D (for the exact nucleotide positions of the fragments, see Experimental Procedures). The deletions were designed to eliminate one or more of the *Antp* binding fragments and are labeled 1 through 8. The *Antp* binding fragments were also inserted at position −625 in deletion 8 and are indicated as 8D, 8B, etc. + and − refer to the orientation of fragment D relative to the start of transcription. The data in column 1 are the fold changes in the basal level CAT activity obtained for each separate deletion cotransfected with the FS90 plasmid. The level of induction obtained with the *Antp* and *ftz* protein is that measured over the basal level activity of each deletion. P1 is an insertion of a fragment of the *Antp* P1 5′ nontranslated region (+115 to +606). The data show that fragment D is required for a significant portion of the activity obtained with the full-length pP_{Ubx}–CAT construct. Insertion of fragment D in deletion 8, in either orientation, restores much of the original activity. Each experiment was repeated at least three times with similar results. nd: not determined.

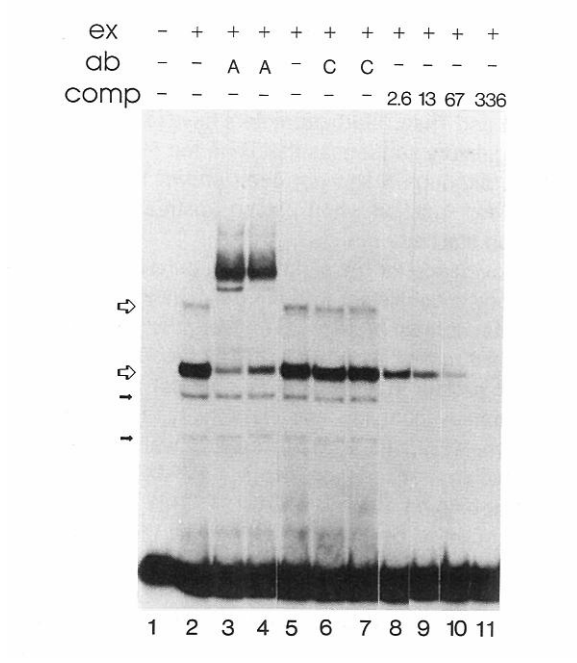


Figure 7. Gel Mobility Shift Assay

Nuclear extracts (approximately 0.2 μg of protein) from an *Antp*-producing S2 cell line were incubated with 1 ng of a radiolabeled 75 bp oligonucleotide from the *Ubx* nontranslated region (see Table 1 for nucleotide sequence) in the presence of 1 μg of poly(dI–dC) and 12.5 ng of sheared salmon sperm DNA. Several bands of decreased mobility are observed after incubation with the *Antp* protein extract (lane 2, arrows). When the extract was preincubated with a monoclonal anti-*Antp* antibody (A) (1:2700, lane 3; 1:8100, lane 4), the mobility of the *Antp* protein–DNA complexes is further retarded (lanes 3 and 4). Preincubation with a control immunoglobulin (C) (1:2700, lane 6; 1: 8100, lane 7) caused no change in the mobility of the protein–DNA complexes. The addition of increasing amounts of unlabeled oligonucleotide effectively competed for *Antp* binding to the labeled oligonucleotide (lanes 8 to 11). ex: protein extract from *Antp*-producing S2 cells. ab: antibody. comp: nanograms of unlabeled competitor oligonucleotide. Open arrows: *Antp*-specific protein DNA complexes. Solid arrows: protein–DNA complexes not recognized by anti-*Antp* monoclonal antibody.

This prompted us to ask whether the homeodomain from the *Ubx* protein can substitute for the homeodomain of the *Antp* protein. The primary sequence of the *Antp* and *Ubx* proteins are quite different except for the homeodomain, a YPWM sequence a short distance upstream of the homeodomain, an M repeat (Wharton et al., 1985), and a MXSYF sequence at or near the N termini (Schneuwly et al., 1986; Stroeher et al., 1986; Laughon et al., 1986; Wilde and Akam, 1987; Weinzierl et al., 1987; Kornfeld et al., 1989). A plasmid encoding a hybrid protein containing the N-terminal 295 amino acids from the *Antp* protein and the *Ubx* homeodomain and C-terminal 29 amino acids was constructed. Because the homeodomain and C termini of *Antp* and *Ubx* are encoded in the 3′ exons of the genes, and the reading frame of each gene is interrupted at the same position in the codon by the intervening introns (Schneuwly et al., 1986; Stroeher et al., 1986; Laughon et al., 1986; Kornfeld et al., 1989), it was possible to use RNA splicing to join the different protein-coding exons.

A control plasmid was first constructed using *Antp* genomic DNA to demonstrate that a protein product could be produced from an intron-containing "minigene" plasmid. The construct contains the G1100 cDNA 5′ sequences joined to genomic sequences containing exons E through G, a truncated version of the 10 kb intron that precedes the homeodomain exon (exon H), and the entire *Antp* homeodomain and C terminus, inserted into the actin ex-

Table 2. Promoter Activation by a Hybrid Homeodomain Protein

Protein-Expression Plasmid	Reporter Plasmid		
	UbxCAT[a]	hsCaspCAT75[b]	P1CAT
Antp[c]	48.6 ± 5.3[d]	36.5 ± 4.7	6.8 ± 1.5
AI	42.6 ± 7.1	28.5 ± 4.0	ND
AU	64.5 ± 11.3	32.9 ± 7.1	4.9 ± 2.3
YE12	47.0 ± 11.3	ND	ND
FS305	ND	ND	1.9 ± 0.27
Ubx[e]	ND	ND	0.27 ± 0.07

[a] The basal level CAT activity was that obtained using the pP_{ac} (for P1) or the FS90 (for *Ubx*) control plasmid. The use of pP_{ac} or FS90 gave essentially the same basal activity (see Figure 3b). The numbers indicate the fold activation over the basal level activity.

[b] The reporter plasmid is the truncated heat shock plasmid (see Figure 1) containing three insertions of the oligonucleotide from the *Ubx* promoter (Table 1).

[c] Protein expression plasmids are as labeled in Figure 1b. YE12 is an *Antp* protein isoform encoded by the *Antp* cDNA YE12 (see text for details).

[d] The data presented are the mean and standard deviation of two or three reactions. In at least three separate experiments, very similar results were obtained.

[e] The pP_{ac}UbxIb protein-expression plasmid (Krasnow et al., 1989). ND: not determined in the above experiment.

pression plasmid (Figure 1b; for details of the construction see Experimental Procedures). This minigene construct produces apparently normal *Antp* protein in cultured cells: the protein accumulates in nuclei (Figure 2d), comigrates on a polyacrylamide gel with the protein produced from the cDNA construct (data not shown), and stimulates all promoters that have been tested at levels equivalent to those observed with the cDNA-encoded *Antp* protein (Table 2, and data not shown). Although we have not confirmed that the correct splice donors and acceptors are used, the proteins from the *Antp* minigene and from the cDNA-containing plasmid appear to function identically. Different versions of the *Antp* protein are produced in the embryo as a result of alternative splicing (Bermingham and Scott, 1988; Stroeher et al., 1988), and at least some alternatively spliced forms could be made from the minigene construct in our cells. We have tested one protein isoform. A cDNA encoding an alternative form of *Antp* protein that is missing four amino acids N-terminal to the homeodomain (YE12; Bermingham and Scott, 1988) gave activation of pP_{Ubx}–CAT equivalent to the activation observed using the plasmid encoding the longer *Antp* protein form (Table 2).

To construct the hybrid *Antp–Ubx* minigene, exon H and part of the preceding intron from *Antp* were replaced with a genomic fragment that contains the upstream intron sequence, the homeobox, and the C-terminal coding regions from the *Ubx* gene (Figure 1b). Protein produced from this construct is also located in nuclei (Figure 2e) and functions in the cotransfection experiments in a manner essentially identical to the full-length *Antp* protein on all promoters tested (Table 2). In four independent experiments, where a 3- to 7-fold stimulation of the *Antp* P1 promoter was observed, the *Antp–Ubx* hybrid protein activated the P1 promoter at levels roughly equal to the full-length *Antp* protein. The FS305 plasmid, which caused a mild stimulation of the P1 promoter in early experiments, did not significantly activate the P1 promoter in the hybrid protein experiments, possibly because the overall levels of activation are lower. The full-length *Ubx* protein represses the P1 promoter (Table 2; Krasnow et al., 1989), so the hybrid protein appears to function like *Antp*, even though it contains the *Ubx* homeodomain and C-terminal sequences.

Discussion

Antp and *ftz* Act as Transcriptional Regulators

We have shown that in cultured Drosophila cells the homeodomain proteins encoded by the *Antp* and *ftz* genes can activate transcription of the *Antp* P1 and *Ubx* promoters. Recent studies have demonstrated transcriptional activation of other promoters by the *ftz* protein (Jaynes and O'Farrell, 1989; Fitzpatrick and Ingles, 1989; Han et al., 1989). Although the most relevant *cis*-acting sequence in the *Ubx* promoter region lies downstream of the transcription start site and could conceivably affect post-transcriptional events, the sequence also functions when placed in either orientation upstream of the transcriptional start site of the *Ubx* promoter or of a truncated *hsp70* promoter and therefore has properties of an enhancer. While relatively uncommon, the positioning of enhancer elements in transcribed regions has been reported (Gillies et al., 1983; Hayashi et al., 1987). In addition, in vitro transcription studies of the *en* (Soeller et al., 1988) and *Ubx* (Biggin and Tjian, 1988) promoters have identified important regulatory sequences that lie in the 5′ nontranslated region, although it has not been shown that these elements can function when placed upstream of the transcription start site.

The evidence for transcriptional regulation obtained in this study is consistent with the previously proposed role for homeodomain proteins as transcription factors in vivo. The yeast mating type gene *MATα2* produces a homeodomain protein that binds to an operator and represses transcription (Johnson and Herskowitz, 1985; Hall and Johnson, 1987). In addition, several well-characterized mammalian transcription factors, PIT-1/GHF-1 and an octamer-binding protein (OCT-2, OTF-2) have recently been shown to contain homeodomains (Ko et al., 1988; Müller et al., 1988a; Scheidereit et al., 1988; Ingraham et al., 1988; Bodner et al., 1988), which suggests that homeodomain-containing proteins from Drosophila, yeast, and mammals, and presumably other homeodomain proteins, constitute a class of transcription factors. Although the *trans*-activation that we observe could conceivably be indirect, the correspondence between the DNA sites bound in vitro and the regulatory sequences defined by the cotransfection experiments indicate that the *Antp* and *ftz* proteins activate transcription by binding to these sites.

Gene Interactions in Cultured Cells and in the Embryo

We have used the cell culture system as an alternative to the living embryo to study gene regulation because of advantages such as the opportunity to study a specific gene interaction in a single cell type, the rapidity of the assays,

and the ability to accurately quantitate levels of gene expression. In the embryo, molecular analysis of regulatory interactions is more difficult because a multiplicity of *trans*-acting regulators act through many *cis*-acting elements to modulate the activity of a given gene.

To what extent do interactions in cultured cells mimic those observed during development? In Drosophila embryogenesis, one can find examples of gene interactions similar to those observed in this study. For example, normal levels of *Ubx* mRNA in early embryos are dependent on the expression of *ftz* (Ingham and Martinez-Arias, 1986), and *ftz* activates *Ubx* transcription in the cultured cells. In embryos that lack *Ubx* function, *Antp* transcription and protein production is derepressed in regions where *Ubx* protein would normally be found (Hafen et al., 1984; Harding et al., 1985; Carroll et al., 1986), which suggests that *Ubx* plays a direct role in the repression of *Antp* transcription. In the cell culture system, the *Ubx* protein has been found to repress transcription from the *Antp* P1 promoter (Table 2, Krasnow et al., 1989).

There are, however, several cases of gene interactions that are observed in cultured cells but have no precedent in the embryo. For example, there is no detectable regulation of *Ubx* transcription by *Antp* protein in the embryo (Struhl and White, 1985; Bienz and Tremml, 1988). In addition, the *Antp* P2 promoter, which has not been shown to respond to any regulator in cultured cells, is negatively regulated by *Ubx* in vivo (Boulet and Scott, 1988) and activated by *ftz* in vivo (Ingham and Martinez-Arias, 1986). The lack of a P2 response is unlikely to be due to the use of an insufficiently large P2 DNA fragment because the 10 kb fragment tested has been shown to direct a fairly normal P2 pattern of expression when introduced into flies as a *lacZ* fusion (Boulet and Scott, 1988). Therefore, the S2 cells may lack factors needed for regulating P2. Conversely, the absence of *ftz* function does not appear to alter the initial blastoderm stage pattern of *Antp* P1 promoter expression in embryos (Ingham and Martinez-Arias, 1986), but *ftz* is capable of activating P1 in cultured cells (Figure 6, and data not shown).

The ability of *Antp*, *ftz*, and *Ubx* proteins to activate the *Ubx* promoter in cultured cells may be because these proteins have similar homeodomains and bind to the same DNA sequence. In the embryo, all of these genes are expressed in tightly controlled temporal and spatial patterns, which restricts the possible interactions among the genes and their products. Also, the accessibility of *cis*-acting sequences to homeodomain proteins may depend on the particular kind of cell or tissue type. For example, at the blastoderm stage *ftz* is regulated by the homeobox-containing segmentation gene *even-skipped* (*eve*) (Carroll and Scott, 1986), and not vice versa (Harding et al., 1986), whereas in the nervous system, *ftz* activates *eve* expression (Doe et al., 1988). Also, the expression of *Ubx* protein is dependent on the presence of *ftz* protein in some, but not all, cells in the nervous system (Doe et al., 1988).

An *Antp–Ubx* Hybrid Protein Is Similar in Function to *Antp* Protein

The experiments using a hybrid protein consisting of the *Antp* N-terminal 295 amino acids coupled to a *Ubx* homeodomain and C terminus demonstrate that the *Ubx* sequence can substitute for the *Antp* homeodomain and C terminus. The hybrid *Antp–Ubx* protein functioned in a manner similar to the full-length *Antp* protein on all promoters tested. The two homeodomains differ at 7 of 61 amino acids, and the 29 C-terminal amino acids of *Ubx* are completely different from the 23 C-terminal amino acids of *Antp*, but the differences apparently do not affect the ability of the hybrid to function like the full-length *Antp* protein on the targets tested. We have not, however, characterized the hybrid protein biochemically. In another case, a hybrid *Ubx–abdominal A* homeotic gene has been shown to be developmentally active (Rowe and Akam, 1988; Casanova et al., 1988).

Ubx negatively regulates the P1 promoter, but the *Antp–Ubx* hybrid protein positively regulates P1, which indicates that a region other than the homeodomain or C terminus is responsible for determining whether a protein bound to DNA near a promoter will act positively or negatively on transcription. The full-length *Antp* and *Ubx* proteins appear to bind in vitro to identical or similar *cis*-acting elements in the *Ubx* 5′ nontranslated region, so it is likely that the hybrid protein binds to these sites as well. This is supported by the result that the *hsp70* promoter, which contains the 75 bp oligonucleotide as the only *Antp* binding site, is also activated by the hybrid protein.

Sequence Requirements for Gene Regulation and DNA Binding by Homeotic Proteins

In a recent compilation of 87 homeodomain sequences from many organisms (Scott et al., 1989), it became clear that most of the sequences could be grouped into ten classes, with another set of sequences not yet placed into any class. The full meaning of the classes is not clear at present, but one possibility is that the proteins in each class bind to similar DNA sequences. *Antp*, *ftz*, and *Ubx* have closely related homeodomains and are all in the *Antp* class. In the results presented here and in the accompanying paper (Krasnow et al., 1989), we have shown that all three proteins can act upon the same short sequence to activate transcription.

The sequence required for *Antp*, *ftz* and *Ubx* activation of the *Ubx* promoter is found within a 75 bp region of the *Ubx* 5′ nontranslated region. The 75 bp element contains several repeats of the sequence TAA or TAAT and is bound by the *Antp* and *Ubx* proteins in vitro. The consensus $(TAA)_4$ is sufficient for binding a *Ubx* protein in vitro (Beachy et al., 1988). The sequence TAA or TAAT is also found in the consensus binding sequences of a number of homeodomain-containing proteins that have been studied, including *Antp* (Müller et al., 1988b; Hayashi and Scott, unpublished data), *even-skipped* (*eve*) (Hoey et al., 1988), *engrailed* (*en*) (Desplan et al., 1988), *ftz* (Laughon et al., 1988; and unpublished data), and *bicoid* (*bcd*) (Driever and Nüsslein-Volhard, 1989). The *en*, *eve*, and *bcd* homeodomains are not in the *Antp* class of homeodomains and have somewhat (*en*, Desplan et al., 1988; *eve*, Hoey et al., 1988) or quite (*bcd*, Driever and Nüsslein-Volhard, 1989) different DNA sequence preferences from the *Antp* class homeodomains studied here. However, *ftz* can bind to an *en* protein consensus binding sequence,

and its binding can be competed by the addition of TAA oligomers (Desplan et al., 1988). Thus, the TAA or TAAT sequence may be a common element for homeodomain sequence recognition by proteins from the *Antp*, *en*, *eve*, and *bcd* classes. However, because the sequence surrounding the TAA or TAAT appears to be different for each class, proteins from different classes may have different binding specificities. The mammalian PIT/GHF-1 and OCT proteins, which are members of yet another (POU) class of homeodomain proteins (Scott et al., 1989), are only distantly related to the Drosophila *Antp* class homeodomain proteins. PIT and OCT bind, respectively, to the related sequences T/AT/ATATNCAT (Bodner and Karin, 1987; Nelson et al., 1988) and ATTTGCAT (Staudt et al., 1986; Wirth et al., 1987). These consensus binding sites appear to be very different from those of the *Antp* class proteins. Like *ftz*, the octamer binding protein (OCT-2, OTF-2) can bind to the *en* protein consensus sequence, but with apparently diminished affinity (Ko et al., 1988; Scheidereit et al., 1988). The importance of binding specificity among the *Antp* class proteins is currently unresolved, but important functional differences may result from differences in binding site preferences.

The Specificity of Action of Homeotic Genes

If different morphological structures are ultimately the result of the activity of different homeotic genes, how is specific transcriptional regulation of target genes achieved? One possibility is that subtle differences in the affinities of homeodomain proteins for target sequences are important but are difficult to detect in vitro: Competitive or cooperative interactions among different homeodomain proteins for the same *cis*-acting sequences could then determine the level of transcriptional activity of the target genes. Interactions with non-homeodomain proteins (which may themselves be differentially expressed) could also alter the specificity of the protein–DNA interactions. For example, the yeast α2 homeodomain protein binds in vitro to operator sequences with an approximately 50-fold greater affinity in the presence of a ubiquitous cell protein (Keleher et al., 1988). A similar factor that might influence binding specificity may not be present in homeodomain protein extracts used for DNA binding experiments and may or may not be present in S2 cells. Another (nonexclusive) possibility is that many or all homeodomain proteins may bind to similar sequences, but the transcriptional outcome is dependent on the particular protein that is bound. For example, we observe opposite effects of the *Antp* and *Ubx* proteins on the P1 promoter. Finally, as has been suggested (Rowe and Akam, 1988), different homeotic gene products may in many cases perform similar functions in different cells or different segments, but each gene is uniquely regulated, temporally and spatially, so as to produce different morphological structures. All of these mechanisms may be used in concert to control pattern formation during development.

Experimental Procedures

Plasmid Constructions

Protein Producing Plasmids

The actin protein expression vector (pP_{ac}) is described in the accompanying paper (Krasnow et al., 1989). The *Antp* protein expression construct (pP_{ac}Antp) was constructed by blunt-end ligation of a 1.6 kb BalI–BglII fragment from the *Antp* cDNA G1100 (Laughon et al., 1986) into the unique BamHI site of pP_{ac}. In a parallel construction, sequences from the *Antp* cDNA YE12 (Bermingham and Scott, 1988) were inserted into the actin vector. Frameshift mutations were created in the open reading frame of the *Antp* cDNA by KpnI or BstXI restriction endonuclease digestion (Figure 1); cohesive ends were then made blunt by using E. coli DNA polymerase I (Klenow fragment), or T4 DNA polymerase, and deoxyribonucleotides. The plasmids were recircularized using T4 DNA ligase, and the sequence alterations were confirmed by nucleotide sequence analysis. The *ftz* protein expression plasmid (pP_{ac}ftz) was constructed by blunt-ended insertion of a HindIII–EcoRI cDNA fragment from pF392 (Struhl, 1985) containing the entire *ftz* open reading frame, into the BamHI site of pP_{ac}.

The *Antp* "minigene" used in the homeodomain exchange experiments was constructed in the following manner: A BalI–BglII fragment from the Antp cDNA G1100 was linkered at the BalI site in the nontranslated region with BglII linkers and inserted into pUC18, which had been digested with PvuII and linkered with BglII, creating the plasmid pGBB. The *Antp* cDNA was replaced with intron sequence by performing a tripartite ligation of a 3.2 kb SphI–BamHI fragment of the plasmid pGBB, a 2.2 kb SphI–EcoRI from phage A77 (Scott et al., 1983; Laughon et al., 1986), which contains part of exon E, all of exons F and G, and part of the following intron, and a 0.5 kb EcoRI–BamHI fragment from phage 2015 (Scott et al., 1983; Laughon et al., 1986), which contains exon H and upstream intron sequences, creating the plasmid pGBI. Finally, a 3.6 kb BglII fragment from pGBI containing the entire *Antp* minigene was inserted into the BamHI site of pP_{ac}, creating the *Antp* minigene expression construct pP_{ac}AI.

To construct the intron-containing *Antp–Ubx* hybrid gene, the *Antp* minigene plasmid was digested with EcoRI, the cohesive ends were made blunt with Klenow fragment, and BamHI linkers were added. The plasmid was next digested with BamHI (thereby deleting a 0.5 kb fragment from exon H and the upstream intron) and recircularized. A 3.2 kb BamHI fragment from pDM3144 (Scott and Weiner, 1984; Bender et al., 1983) containing the *Ubx* homeodomain exon, upstream intron sequence, and downstream nontranslated sequences was inserted into the BamHI site of the deletion derivative of the *Antp* minigene, creating the hybrid homeobox plasmid pP_{ac}AU.

Reporter Plasmids

For descriptions of the CAT reporter vector and the constructs containing promoter elements from the P1, *Ubx*, *hsp70*, and *copia* genes, see the accompanying paper by Krasnow et al. (1989). To construct the P2–CAT reporter construct, a 10 kb fragment from a pC4neo-lacZ containing the P2 *Antp* promoter (Boulet and Scott, 1988) was inserted into C4CAT (Thummel et al., 1988). In all cases, care was taken to avoid interruption of any possible open reading frame in the 5′ noncoding region. All plasmids were purified by two cycles of CsCl density gradient centrifugation.

Deletion and Insertion Reporter Gene Plasmids

Deletions of the *Ubx* upstream region were made by excision of restriction fragments from the plasmid pP_{Ubx}–CAT, which contained the entire upstream regulatory region used in this study, followed by reinsertion of the *Ubx* DNA into the polylinker of the vector pC4CAT. The following fragments were inserted into the indicated polylinker sites of pC4CAT after blunting any cohesive ends using Klenow or T4 polymerase: PvuII–EcoRI (−2727 to +357) inserted into XbaI–SmaI (deletion 1); NdeI–EcoRI (−961 to +357) inserted into XbaI–SmaI (deletion 2); HIII–EcoRI (−625 to +357) inserted into XbaI–SmaI (deletion 3); BstXI–EcoRI (+154 to +357) inserted into XbaI–SmaI (deletion 4); XbaI–BstXI (−3151 to +154) inserted into SmaI (deletion 5); PvuII–BstXI (−2727 to +154) inserted into XbaI–SmaI (deletion 6); NdeI–BstXI (−2223 to +154) inserted into SmaI (deletion 7); and HIII–BstXI (−625 to +154) inserted into XbaI–SmaI (deletion 8). Insertion of *Antp*-binding fragments from pP_{Ubx}–CAT was performed by the ligation of the following restriction endonuclease fragments that bound *Antp* protein in vitro into deletion 8 at the SalI site in the pC4CAT polylinker (position −625; the fragments contained some polylinker sequences at each end): EcoRI–PvuII (−3151 to −2727; site A); PvuII–EagI (−2727 to −2407; site B); and TaqI–EcoRI (+175 to +357; site D). A BamHI–XhoII (+115 to +606) fragment from the *Antp* P1 promoter was similarly inserted into deletion 8. The orientation of the inserted fragments was determined by restriction endonuclease digestion. A double-stranded

oligonucleotide (75 bp) with a sequence from the *Ubx* 5′ nontranslated region (+225 to +292; Beachy et al., 1988) with BamHI and BglII ends was synthesized (for sequence of oligonucleotide see Table 1). The BamHI and BglII cohesive ends were added to the oligonucleotide for convenience in subcloning. The oligonucleotide was subcloned, and its sequence was verified by nucleotide sequence analysis. The oligonucleotide was subsequently inserted into the unique BamHI site upstream of the truncated *hsp70* promoter in the vector phsCaspCAT (Figure 1), creating the plasmid phsCaspCAT75. The orientation of the oligonucleotide(s) was determined by restriction endonuclease digestion.

To construct phsCaspCAT, a SalI–PstI fragment from the plasmid p4370Z (kindly provided by Dr. J. Lis), which contained a portion of the Drosophila *hsp70* promoter from an artificial SalI site at −40 to the PstI site at +90 (Karch et al., 1981), was blunt-ended with Klenow polymerase and inserted into the SmaI site of the plasmid pGEM7Zf (Promega), creating the plasmid pGSP. The sequence of the truncated heat shock promoter was confirmed by nucleotide sequence analysis. A BamHI–KpnI fragment from pGSP containing the heat shock promoter element was inserted into the vector pCasplacZ (Boulet and Scott, 1988), which had been cleaved with KpnI and PstI, creating the plasmid phsCaspβgal. The coding sequence for lacZ in this plasmid was replaced with the coding sequence from the CAT gene by first digesting pC4CAT with SacI, adding PstI linkers, digesting with PstI, and subcloning with 1.6 kb fragment that contained the CAT gene into pGEM5Zf (Promega). A KpnI–PstI fragment from this subclone, which contained the CAT gene and polyadenylation sequences, was then inserted into phsCaspβgal, which had been digested with KpnI and PstI, creating the plasmid phsCaspCAT.

RNA Analysis

Total RNA was isolated from S2 cells 48 hr after transfection with reporter and protein-expression plasmids as described by Bermingham et al. (1988). RNA protection analysis was performed essentially as described by Melton et al. (1984). For analysis of the *Ubx* transcripts, 25–50 μg of total RNA was mixed with 1 × 10^4 cpm of ^{32}P-labeled antisense RNA probe, precipitated with ethanol, and resuspended in 20 μl of hybridization buffer (80% formamide, 40 mM PIPES [pH 6.4], 0.4 M NaCl, 1 mM EDTA), heated to 85°C for 5 min, and incubated overnight at 46°C. Three hundred microliters of an ice-cold RNAase digestion mix (40 μg/ml of RNAase A, 2 μg/ml of RNAase T_1) was added and incubated at 30°C for 30 min. The samples were treated with 25 μl of 2 mg/ml Proteinase K and 10 μl of 20% SDS at 30°C for 15 min, extracted with phenol/chloroform and precipitated twice with 0.5 vol of 7.8 M ammonium acetate and 2.5 vol of ethanol. Samples were analyzed in a 5% polyacrylamide gel containing 7.8 M urea and autoradiographed using Kodak X-AR film at −80°C with an enhancing screen for 24 hr.

The radiolabeled RNA probe was generated using the pGEM7Zf riboprobe vector (Promega). A HindIII–MluI restriction fragment from the pP_{Ubx}–CAT plasmid was subcloned into pGEM7Zf, the plasmid was linearized with SacII, and 0.5 μg was used in a transcription reaction containing 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, [α-^{32}P]CTP (800 ci/mmol, 40 mCi/ml), 0.5 mM ATP, GTP, UTP, and 12.5 μM unlabeled CTP, 10 μ of RNAsin and 1 μ of SP6 Polymerase (Promega). Reactions were for 1 hr at 40°C. One unit of RNAase-free DNAase I (Promega) was added, and the sample was incubated at 37°C for 15 min. Reaction products were extracted with phenol/chloroform, 5–10 μg of tRNA was added as carrier, and nucleic acids were precipitated with 0.5 vol of ammonium acetate and 2.5 vol of ethanol, dried, and resuspended in TE (10 mM Tris-Cl [pH 7.5], 1 mM EDTA). Fifty to seventy-five percent of the labeled nucleotide was incorporated into acid-precipitable nucleic acid.

Drosophila Cell Culture, Cotransfections, and CAT Assays

Drosophila S2 cells were a gift from Dr. P. Beachy. Cells were maintained in modified Schneider's media (Vaughn and Goodwin, 1986), supplemented with 10%–12% fetal calf serum (GIBCO), and 50 U/ml of penicillin, and 50 μg/ml of streptomycin and maintained at 25°C in air.

Transfections were essentially as described by Krasnow et al. (1989). The protein-encoding plasmids and the reporter plasmids were mixed with carrier DNA (to 20 μg total) and transfected using the calcium phosphate precipitation technique (Gorman et al., 1982; Di Nocera and Dawid, 1983; Rio and Rubin, 1985). One microgram each of the protein-encoding and reporter plasmids was used in the tranfections, with the exception of P1CAT (0.3 μg) and phsCaspCAT (0.5 μg). The cells were harvested 48–60 hr posttransfection, lysed with three cycles of freezing and thawing, heated to 65°C for 5 min, and centrifuged in an Eppendorf microcentrifuge for 10 min. The protein concentration of the cell extract was determined by the method of Bradford. Five to twenty micrograms of protein was used in each CAT assay according to the method of Gorman et al. (1982). Incubations were for 30 min at 37°C. For quantitation, radioactive spots were excised from the thin-layer chromatography plate and counted in a scintillation counter. As necessary, assays were performed for shorter or longer durations to obtain results in the linear range of the assay. The CAT assay is linear from at least 5%–60% conversion of chloramphenicol to acetyl-chloramphenicol. Alternatively, a direct diffusion assay was used to assay CAT activity (Neumann et al., 1987) as modified by Eastman (1987), using ^{3}H-acetyl coenzyme A (Amersham). In this method, the reaction was performed at 25°C in a Beckman scintillation counter. A series of reaction timepoints were generated, and the data from timepoints in the linear range of the assay were used to calculate the relative levels of CAT activity. In these experiments, nonradioactive acetyl coenzyme A was added to 125 μM.

DNA Binding Assays

A whole cell extract was prepared from a Drosophila S2 cell line (CA106) that produces *Antp* (G1100) protein from the inducible *hsp70* promoter (Hayashi et al., unpublished data). *Antp* protein was immunoprecipitated from about 1 mg of extract using polyclonal anti-*Antp* antisera and protein–A Sepharose and washed extensively with the binding buffer (see below). For the DNA binding experiments, pP_{Ubx}–CAT DNA was cleaved with HinfI and end-labeled with T4 DNA polymerase and α-^{32}P deoxyribonucleotides, and the probe (5 × 10^4 cpm; 2.5 ng) was incubated with about 1/20th of the total *Antp* protein immunoprecipitate in 20 μl of binding buffer (10 mM HEPES, 5 mM MgCl, 1 mM DTT, 200 mM KCl, and 100 μg/ml of BSA [pH 7.6]). After 30 min on ice, the immunoprecipitate was washed three times with various concentrations of KCl (see Figure 5) in the binding buffer, phenol extracted, ethanol precipitated, and displayed on a 5% polyacrylamide gel. As a size marker, 10^4 cpm of the input probe was used. Control experiments using an S2 extract from the original (nontransfected) cell line did not give any detectable binding (data not shown).

Gel Mobility Shift Assay

The 75 bp double-stranded oligonucleotide described above, which contains an *Antp* binding site flanked by BamHI and BglII cohesive ends, was subcloned into pGEM7Zf. The plasmid vector had been modified by the addition of a BglII linker at the unique SmaI site. The oligonucleotide was excised with BamHI and BglII, labeled by replacement synthesis using T4 DNA polymerase and [α-^{32}P]dCTP, and purified by elution after polyacrylamide gel electrophoresis. A nuclear extract was prepared from an S2 cell line (RAIF2) that expresses the *Antp* protein (G1100) from the Drosophila metallothionein promoter upon induction with copper sulfate (Bunch et al., 1988). The details of the construction of the cell line and the preparation of protein extracts will be reported elsewhere (Hayashi et al., unpublished data). Cell extract, 200 ng, was preincubated in 19 μl of buffer (22.5 mM HEPES-K [pH 7.6], 4.3 mM $MgCl_2$, 3 mM KCl, 1.1 mM DTT, 7% glycerol, 50 μg/ml of poly(dI-dC), and 625 ng/ml of sonicated salmon sperm DNA) on ice for 5 min. One microgram of labeled probe (2 × 10^4 cpm, about 1 ng) was added, and the incubation was continued for 10 min. The mixture was electrophoresed at 10 V/cm through a TBE 4% polyacrylamide gel. The gel was dried and exposed on Kodak X-RP film with an intensifying screen. A monoclonal anti-*Antp* antibody (4C3) was kindly provided by D. Brower. Mouse ascites fluid containing the antibody was diluted and added to the preincubation reaction as described in the figure legend. The control ascites fluid containing IgG2b,k from myeloma MOPC 141 was obtained from Sigma (M-7644). Competitor oligonucleotide was added to the reaction with the probe DNA.

Immunocytochemistry

Cells were transfected, as described above, except that the cells were plated on glass coverslips. Twenty-four to forty-eight hours posttransfection, the cells were fixed in 3.7% formaldehyde in phosphate-buffered saline for 20 min, permeabilized with a 0.5% NP-40 for 5 min,

stained with polyclonal affinity-purified anti-*Antp* antisera followed by horseradish peroxidase–conjugated, goat anti–rabbit secondary antibody (Jackson Immunoresearch Laboratories), developed, and observed by differential interference contrast microscopy using a Zeiss IM-35 microscope. The affinity-purified polyclonal antisera was prepared from a full-length *Antp–β-gal* fusion protein that was synthesized in E. coli, purified by gel filtration chromatography, and used for immunization, as described by Carroll and Laughon (1987).

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