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

Gary M. Winslow,* Shigeo Hayashi,* Mark Krasnow,* David S. Hogness; and Matthew P. Scott*
Department of Molecular, Cellular, and Developmental Biology
University of Colorado
Boulder, Colorado 80309-0347
*Department of Biochemistry
Stanford University School of Medicine
Stanford, California 94305

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 Pl 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, 1997; 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 plasmids 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; Mahatley and Kaufman, 1989) while Ubx is a member of the Bithorax Complex (Duncan, 1967). 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-
tation 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 Blenck, 1987; Kornfeld et al., 1989), the two Antp promoters (Schneuwly et al., 1986; Strother et al., 1986; Laughon et al., 1988), the hsp70 promoter (Karch et al., 1986), 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., 1986; 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., 1990).

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 SC promoter (Bond and Davidson, 1986) was joined to the Antp cDNA G1100 (Laughon et al., 1986). actin SC 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 SC polyadenylation signals (Figure 1b). When the Antp protein—producing plasmid was introduced into S2 cells, Antp protein was immunologically 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 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 sequence 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 start of transcription. AU (pP & J) is the site of truncation of the intron preceding the homeodomain exons. Al (pP, AI) is the sites. Al (pP, AI) is the sites. FS90 and FS305 are solid bars above the constructions. FS90 and FS305 are solid bars above the constructions.

---
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 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).

(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 FM05 plasmid. Antp protein is expressed at high levels but is not localized to the nuclei (arrow).

(e) Protein produced from the hybrid Antp-Ubx plasmid (pP,AU).

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 T1, 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.
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 pPUBx-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 8–8). In three other repeats of the same experiment, the stimulation of deletion 8 by Antp protein ranged from 0.5– to 10-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 8; compare RO7+ and RO−, with deletion 5). 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 pPbx-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 T1, 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 Sienz, 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 pPubx-CAT plasmid was cleaved with Hinfl, end-labeled, and incubated with an Antp protein immunoprecipitate. The complexes were washed with 200 (a), 225 (b), 250 (c), and 275 (d) mM KCI 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 R 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 5' nontranslated region (+59 to +173) that contains a binding site for the Ubx protein Ubx-A; Beachy et al., 1986). 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 DNase I protection experiments (S. Hayashi et al., unpublished data). The protected nucleotides correspond to the "Ubx-B" region located at the Ubx promoter. The sequence of the oligonucleotide is: 5'-TAATAATCGTTCAAATCGTTAAAACCATAAAAATAATAATTGCAATAACAATAAACATA-GTAATAATG9' (+ 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 with 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, 1988). When the fragment D is eliminated from the pPubX-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-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.

Table 1. Activation of a Heterologous Promoter

<table>
<thead>
<tr>
<th>Insertion</th>
<th>Fold Change in Induction by Antp</th>
<th>Induction by ftz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal Levela</td>
<td>+c</td>
<td>+c</td>
</tr>
<tr>
<td>+c</td>
<td>-</td>
<td>+c</td>
</tr>
<tr>
<td>+c</td>
<td>-</td>
<td>+c</td>
</tr>
<tr>
<td>+c</td>
<td>-</td>
<td>+c</td>
</tr>
<tr>
<td>+c</td>
<td>-</td>
<td>+c</td>
</tr>
</tbody>
</table>

- Basal level CAT activity obtained without heat induction, using the F590 plasmid.
- 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 −c indicates orientation and number of oligonucleotide(s) in phsCasCAT relative to transcription initiation.

The sequence of the oligonucleotide is: 5'-TAATAATCGTTCAAATCGTTAAAACCATAAAAATAATAATTGCAATAACAATAAACATA-GTAATAATG9' (+ orientation), with the addition of a 5'GATC extension at each end.
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(dl-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 antibody (A) (1:2700, lane 3; 1:6100, 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 8; 1:6100, 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.

Figure 6. Deletion Analysis of the Ubx Upstream and Nontranslated Region
The arrow indicates 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 data show that fragment D is required for a significant portion of the activity obtained with the full-length P10-5′ nontranslated region (+115 to +808). The data in column 2 are the fold induction by Antp and ftz proteins. The data in column 3 are the fold induction by Antp and ftz proteins. Each experiment was repeated at least three times with similar results. nd: not determined.

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; Laugnon et al., 1986; Wilde and Akam, 1987; Weinzierl et al., 1987; 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 G100 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-
the cDNA construct (data not shown), and stimulates all on a polyacrylamide gel with the protein produced from the protein accumulates in nuclei (Figure 2d), comigrates produces apparently normal Anfp protein in cultured cells: those observed with the cDNA-encoded Anfp protein (Ta-
fication plasmid (Figure 1b; for details of the construction firmed that the correct splice donors and acceptors are cDNA-containing plasmid appear to function identically. cDNA promoter (Table 1). 1 is an Antp protein isoform encoded by the Antp cDNA YE12 (see text for details). 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. The PPaUbxb protein-expression plasmid (Krasnow et al., 1989). ND: not determined in the above experiment.

<table>
<thead>
<tr>
<th>Protein-Expression Plasmid</th>
<th>Reporter Plasmid</th>
<th>UbxCAT&lt;sup&gt;a&lt;/sup&gt;</th>
<th>hscCaspCAT75&lt;sup&gt;b&lt;/sup&gt;</th>
<th>P1CAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antp&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
<td>48.6 ± 5.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>36.5 ± 4.7</td>
<td>6.8 ± 1.5</td>
</tr>
<tr>
<td>Al</td>
<td>ND</td>
<td>42.6 ± 7.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>28.5 ± 4.0</td>
<td>ND</td>
</tr>
<tr>
<td>AU</td>
<td>ND</td>
<td>64.5 ± 11.3</td>
<td>32.0 ± 7.1</td>
<td>4.0 ± 2.3</td>
</tr>
<tr>
<td>YE12</td>
<td>ND</td>
<td>47.0 ± 11.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>FS305</td>
<td>ND</td>
<td>ND</td>
<td>1.9 ± 0.27</td>
<td></td>
</tr>
<tr>
<td>UbxB&lt;sup&gt;d&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>0.27 ± 0.07</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> The basal level CAT activity was that obtained using the pP<sub>re</sub> (for P1) or the FS90 (for Ubx) control plasmid. The use of pP<sub>re</sub> or FS90 gave essentially the same basal activity (see Figure 3b). The numbers indicate the fold activation over the basal level activity.

<sup>b</sup> The reporter plasmid is the truncated heat shock plasmid (see Figure 1b) containing three insertions of the oligonucleotide from the Ubx promoter (Table 1).

<sup>c</sup> 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).

<sup>d</sup> 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.

<sup>e</sup> The PPaUbxb protein-expression plasmid (Krasnow et al., 1989). ND: not determined in the above experiment.

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 MATA2 produces a homeo-
domain 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 (Kol et al., 1988; Müller et al., 1988a; Scheidereit et al., 1988; Ingraham et al., 1988; Bodner et al., 1988), which suggests that hom-
eodomains-containing proteins from Drosophila, yeast, and mammals, and presumably other homeodomain pro-
teins, 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.

**Discussion**

**Antp and ftz Act as Transcriptional Regulators**

We have shown that in cultured Drosophila cells the homeodomain proteins encoded by the Antp and Ubx promoters. Recent studies have demonstrated transcrip-
tional activation of other promoters by the ftz protein (Jaynes and O'Farrell, 1988; Fitzpatrick and Ingles, 1989; Han et al., 1989). Although the most relevant cis-acting se-
quence 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 pro-
moter and therefore has properties of an enhancer. While relatively uncommon, the positioning of enhancer ele-
ments in transcribed regions has been reported (Gillies et al., 1993; Hayashi et al., 1987). In addition, in vitro tran-
scription studies of the en (Soeller et al., 1988) and Ubx (Biggin and Tjian, 1988) promoters have identified identi-
ent regulatory sequences that lie in the 5' nontranslated region, although it has not been shown that these ele-
ments can function when placed upstream of the tran-
scription start site.

**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 ad-

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 Treml, 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 homeodomain sequences 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)n 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) (I. loey 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 PIT2/GH1-F 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 TAATATATCAT (Bodner and Karin, 1987; Nelson et al., 1988) and ATTTGCAT (Staudt et al., 1988; 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 Antp 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 vivo. 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 a2 homeodomain protein binds in vitro to operator sequences with an approximately 50-fold greater affinity in the presence of a ubiquitous cell protein (Kelleher 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#) is described in the accompanying paper (Krasnow et al., 1989). The Antp protein expression construct (pP#Antp) was constructed by blunt-end ligation of a 1.6 kb BalI–BglII fragment from the Antp cDNA G1100 (Laugthon et al., 1989) into the unique BamHI site of pP#. 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 BalXI 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 recircu- lated using T4 DNA ligase, and the sequence alterations were confirmed by nucleotide sequence analysis. The ftz protein expression plasmid (pP#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#.

The Antp “minigene” used in the homeodomain exchange experiment was constructed in the following manner: A Ball–BglII fragment from the Antp cDNA G1100 was linked at the BalI site in the nontranslated region with BalI linkers and inserted into plUC18, which had been digested with PvuII and linked with BglII, creating the plasmid pGgB. 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 fragment from phage A77 (Scott et al., 1983; Laugthon et al., 1988), 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 A77 (Scott et al., 1983; Laugthon et al., 1988), which contains exon H and upstream intron sequences, creating the plasmid pGgB.

Finally, a 3.6 kb BglII fragment from plgB containing the entire Antp minigene was inserted into the BamHI site of pP# creating the Antp minigene expression construct pP#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, 1986; -,tessier 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#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 C4-CAT (Thummel et al., 1988). In all cases, care was taken to avoid interruption of any possible open reading frame in the S' 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#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 pC4-CAT. The following fragments were inserted into the indicated polylinker sites of pP#CAT after blunting any cohesive ends using Klenow or T4 polymerase: PvuII–EcoRI (–2727 to –357) inserted into XbaI–Smal (deletion 1); NdeI–EcoRI (–961 to –357) inserted into XbaI–Smal (deletion 2); HillI–EcoRI (–625 to +357) inserted into XbaI–Smal (deletion 3); BstXI–EcoRI (+154 to +357) inserted into XbaI–Smal (deletion 4); XbaI–BstXI (–3151 to +154) inserted into Smal (deletion 5); PvuII–BstXI (–2727 to +154) inserted into XbaI–Smal (deletion 6); NdeI– BstXI (–2223 to +154) inserted into Smal (deletion 7); and XbaI–BstXI (+154 to +357) inserted into XbaI–Smal (deletion 8). Insertion of Antp–binding fragments from pP#CAT was performed by the ligation of the following restriction endonuclease fragments that bound Antp protein in vitro into deletion 8 at the Sall site in the pC4-CAT polymerase (pol- tion –626), 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– XbaI (+115 to +600) 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., 1986) with BamHI and BgIII ends was synthesized (for sequence of oligonucleotide see Table 1). The BamHI and BgIII cohesive ends were added to the oligonucleotide for convenience in subcloning. The oligonucleotide was subcloned, and its sequence was confirmed by nucleotide sequence analysis. The oligonucleotide was subsequently inserted into the unique BamHI site upstream of the truncated *hs70* promoter in the vector pbsCasPCAT (Figure 1), creating the plasmid pbsCasCAT75. The orientation of the oligonucleotide(s) was determined by restriction endonuclease digestion. To construct pbsCasCAT, a *SalI*-PstI fragment from the plasmid pG4702 (kindly provided by Dr. J. Lis), which contained a portion of the Drosophila *hs70* 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 Smal site of the plasmid pGEM7Zf (Promega), creating the plasmid pGSP. The sequence of the truncated heat shock promoter subclone was confirmed by nucleotide sequence analysis. A BamHI-*KpnI* fragment from pGSP containing the heat shock promoter element was inserted into the vector pCasPlac2 (Boulet and Scott, 1988), which had been cleaved with *KpnI* and *PstI*, creating the plasmid pCasPgpil. The coding sequence for lacZ in this plasmid was replaced with the coding sequence from the *CAT* gene by first digesting pCCAT with *SalI*, adding *PstI* linkers, digesting with *PstI*, and subcloning with a 1.6 kb fragment that contained the *CAT* gene into pGEMSZf (Promega). A *KpnI*-PstI fragment from this subclone, which contained the *CAT* gene and polyadenylation sequences, was then inserted into pCasPgpil, which had been digested with *KpnI* and *PstI*, creating the plasmid pCasPCAT.

**RNA Analysis**

Total RNA was isolated from S2 cells 48 hr after transfection with reporter and protein expression plasmids as described by Berringham 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 x 10⁶ cpm of [32P]-labeled antisense RNA probe, precipitated with ethanol, and resuspended in 20 µl of hybridization buffer. The sample was hybridized overnight at 46°C. Three microfilters of an ice-cold RNAase digestion mix (40 µg/ml of RNAase A, 2 µg/ml of RNAase T1) 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 0.6 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 auto-radiographed using Kodak X-AR film at -80°C with an exposing screen for 24 hr.

The radiolabeled RNA probe was generated using the pGEM7Zf riboprobe vector (Promega). A HindIII-*Mul* restriction fragment from the pUB*CAT* plasmid was subcloned into pGEM7Zf, the plasmid was linearized with *SalI*, and 0.5 µg was used in a transcription reaction containing 40 mM Tris-HCl (pH 7.5), 6 mM MgCl₂, 2 mM spermidine, [α-32P]CTP (800 cpm/mmol, 40 mCi/ml), 0.5 mM ATP, GTP, UTP, and 12.5 µM unlabelled CTP, 10 µl of RNAseIn and 1 µl of SP6 Polymerase (Promega). Reactions were for 1 hr at 40°C. One unit of RNAase-free DNAseA (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 RNA 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-CI [pH 7.5, 1 mM EDTA]). Fifty to seventy-five percent of the labeled nucleic acid 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 Goodman, 1986), supplemented with 10%-12% fetal calf serum (GIBCO), and 50 µU/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. (1986). 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, DiNocera and Dawid, 1983; Rio and Rubin, 1985). One microgram each of the protein-encoding and reporter plasmids was used in the transfections, with the exception of pICAT (0.3 µg) and pfsCasPCAT (0.5 µg). The cells were harvested 48-60 hr posttransfection, lysed with three cycles of freezing and thawing, heated to 60°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). RNA protection analysis was performed as described by Bermingham et al. (1988). The cells were transfected with the pUB*CAT* plasmid and the plasmid pcDNA. The sequence of the truncated heat shock promoter subclone, which contained the *CAT* gene and polyadenylation sequences, was then inserted into pCasPgpil, which had been digested with *KpnI* and *PstI*, creating the plasmid pCasPCAT.

**DNA Binding Assays**

A whole cell extract was prepared from a Drosophila S2 cell line (CA106) that produces Antp (G1100) protein from the inducible hs70 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, pUB*CAT* DNA was cleaved with HindIII and end-labeled with T4 DNA polymerase and [α-32P]dCTP and purified by electrophoresis on a 5% polyacrylamide gel. As a size marker, 10⁶ 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 76 bp double-stranded oligonucleotide described above, which contains an Antp binding site flanked by BamHI and BgIII cohesive ends, was subcloned into pGEM7Zf. The plasmid vector had been modified by treatment with the unique *SalI* site. The oligonucleotide was excised with BamHI and BgIII, labeled by replacement synthesis using T4 DNA polymerase and [α-32P]dCTP and purified by electrophoresis on a 5% polyacrylamide gel. A nuclear extract was prepared from an S2 cell line (RAIF2) that expresses the Antp protein (G1100) from the Drosophila melanogaster 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 precleared in 19 µl of buffer (22.5 mM HEpes-K [pH 7.5], 4.3 mM MgCl₂, 3 mM KCl, 1.1 mM DTT, 200 mM KCl, and 100 µg/ml of BSA [pH 7.5]). 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, and displayed on a 5% polyacrylamide gel. As a size marker, 10⁶ 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).

**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-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).

Acknowledgments

We would like to thank Drs. Deborah Andrew, Susan Dutcher, and John Tamkun, for critical comments and suggestions regarding the manuscript. Our thanks to Dr. Paskie for the SiZ cells, Beverly Bond, John Bermingham, John Lii, and Carl Thummel for plasmids, Danny Brower for the anti-Ant monoclonal antibody, Allen Laughon, Claude Desplan, and Patrick O'Farrell for helpful discussions; Alice Bernat for assistance with the graphics, and Cathy Inouye for help in preparation of the manuscript. S. H. is a Wesley Foundation fellow of the Life Science Research Foundation. S. H. was also supported by a Toyobo Biotechnology Foundation Postdoctoral Fellowship, and M. A. K. was a fellow of the Helen Hay Whitney Foundation and a Lucille P. Markey scholar in Biomedical Science. The research was supported by National Institute of Health grant #1563, March of Dimes grant #1-1129, a Searle scholar award to M. P. S., and by grants from The Lucille P. Markey Charitable Trust (to M. A. K.) and the National Institutes of Health (to D. S. H.).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 16 U.S.C. Section 1734 solely to indicate this fact.

Received December 12, 1986, revised March 13, 1987.

References


