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Transcriptional Activation and Repression by *Ultrabithorax* Proteins in Cultured Drosophila Cells

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Summary

Homeotic genes of Drosophila melanogaster such as *Ultrabithorax* (*Ubx*) and *Antennapedia* (*Antp*) have long been thought to select metameric identity during development by controlling the expression of various target genes. Here we describe a cotransfection assay in cultured D. melanogaster cells that is used to demonstrate that *Ubx* proteins (UBX) can repress an *Antp* promoter fusion and activate a *Ubx* promoter fusion, activities predicted from genetic studies. We show (a) that UBX proteins regulated the level of accurately initiated *Antp* P1 and *Ubx* transcripts, (b) that activation of the *Ubx* promoter required a downstream cluster of UBX binding sites, and (c) that binding site sequences were sufficient to confer regulation on a heterologous promoter, regardless of their orientation or precise position. We conclude that UBX proteins are transcriptional repressors and activators, and that their actions are mediated by binding to promoter region sequences. Each member of the UBX protein family has similar regulatory abilities, but the properties of synthetic mutant forms suggest that UBX proteins may have a modular design similar to other transcriptional regulators.

Introduction

A complex genetic program guides development in Drosophila melanogaster and serves to orient the major body axes of the animal, partition the animal along the anteroposterior axis into segments or metameric units, and distinguish the form and function of these units (reviewed in Akam, 1987; Scott and Carroll, 1987; Ingham, 1988). The developmental program is a spatial and temporal hierarchy in that genes that specify global features of the animal, such as its major body axes, are expressed first and in general are required for the appropriate expression of genes that specify more local features, such as segment boundaries and segment identities. Similarly, the appropriate expression of the homeotic genes that select and maintain segment identities depends upon the prior expression of certain segmentation genes that determine segment boundaries. There are myriad interactions between genes of one level and the next in the hierarchy, as well as examples of cross-regulatory and autoregulatory actions of genes of the same level. While the general organization of the hierarchy has begun to emerge from molecular genetic studies, current understanding of the biochemical functions of specific gene products remains largely inferential, relying primarily on sequence similarities between the encoded products and characterized proteins in other systems. Our goal is to determine the biochemical mechanisms by which homeotic genes such as *Ultrabithorax* (*Ubx*) of the bithorax complex and *Antennapedia* (*Antp*) of the Antennapedia complex specify metamere identities during development.

Ubx and *Antp* are involved in specifying metameric identities in the thorax and abdomen of the animal: *Ubx*$^-$ mutations transform primarily metameres in the posterior thorax and anterior abdomen to an anterior thoracic identity (Lewis, 1963, 1978; Minana and Garcia-Bellido, 1982), and *Antp*$^-$ mutations transform those in the anterior thorax to more anterior identities (Wakimoto and Kaufman, 1981). These genes are composed of long primary transcription units with large *cis*-acting control regions (see below), which direct developmental expression of their products in intricate spatial patterns that generally correspond to the anatomical regions and tissues affected in mutants (Akam, 1983; Levine et al., 1983; Beachy et al., 1985; White and Wilcox, 1985a; Wirz et al., 1986; Carroll et al., 1986). The ~77 kb *Ubx* transcription unit derives from a single promoter, P_{Ubx} (Hogness et al., 1985; Kornfeld et al., 1989). The temporal and spatial activity of this promoter during development depends on an ~40 kb upstream control region (the *bithoraxoid* region) and at least one other *cis*-regulatory region (the *anterobithorax/bithorax* region) located 30–50 kb downstream (Beachy et al., 1985; Cabrera et al., 1985; Hogness et al., 1985; White and Wilcox, 1985b; Lipshitz et al., 1987; Peifer et al., 1987; K. Irvine, S. Helfand, and D. S. H., unpublished data). The overlapping 103 kb and 36 kb *Antp* primary transcription units derive from two tandem promoters separated by ~67 kb ($P_{Antp\ P1}$ and $P_{Antp\ P2}$), with *cis*-acting control regions that are currently being studied (Laughon et al., 1986; Schneuwly et al., 1986; Stroeher et al., 1986; Boulet and Scott, 1988; Perkins et al., 1988). Differential splicing and polyadenylation of the primary transcripts give rise to sets of mRNAs that encode a family of at least five closely related *Ubx* proteins, UBX Ib, Ia, IIb, IIa, and IVa (Beachy et al., 1985; O'Connor et al., 1988; Kornfeld et al., 1989), and a family of *Antp* proteins (Bermingham and Scott, 1988) that execute functions required for metamere specialization.

It has long been hypothesized that the products of a single homeotic gene such as *Ubx* specify the dramatic differences between metameres by regulating the expression of other genes (Lewis, 1964; Garcia-Bellido, 1975). Subsequent molecular genetic studies have identified two genes as likely regulatory targets of the UBX proteins, which are the focus of this paper. One of these is the *Antp* gene, whose P1- and P2-derived products accumulate abnormally in *Ubx*$^-$ mutants (Hafen et al., 1983; Carroll et al., 1986; Wirz et al., 1986; Boulet and Scott, 1988; J. Bermingham, A. Martinez-Arias, and M. Scott, personal communication); indeed, it has been inferred that UBX proteins repress *Antp* expression because the accumulation

occurs in metameres where abundant *Ubx* expression is normally observed (Akam, 1983; Beachy et al., 1985; White and Wilcox, 1985a). Another likely target is the *Ubx* gene itself, at least in the visceral mesoderm of the embryo, where a high level of P_{Ubx} activity appears to be dependent on *Ubx* products (Bienz and Tremml, 1988).

How do *Ubx* products regulate the expression of their targets? UBX proteins are localized to the nucleus (White and Wilcox, 1984; Beachy et al., 1985), and each of these proteins contains the same 61 amino acid homeodomain (Beachy et al., 1985; O'Connor et al., 1988; Kornfeld et al., 1989). Homeodomain proteins (McGinnis et al., 1984; Scott and Weiner, 1984; for reviews, see Gehring and Hiromi, 1986; Scott et al., 1989) are also encoded by other homeotic genes (e.g., *Antennapedia*), certain segmentation genes (e.g., *fushi tarazu* and *paired*) and the *bicoid* gene required for anteroposterior polarity, and the homeodomain has sequence similarity to prokaryotic and eukaryotic regulators (Laughon and Scott, 1984; Shepherd et al., 1984; Herr et al., 1988) and can bind DNA in vitro (Desplan et al., 1985; Mihara and Kaiser, 1988; Müller et al., 1988). A purified UBX Ib protein produced in E. coli has sequence-specific DNA binding activity, and it interacts with clusters of binding sites located within several hundred base pairs downstream of the *Ubx* and *Antp* P1 transcription start sites and 6 kb upstream of *Antp* P1 (Beachy et al., 1988). Thus, the *Ubx-Antp* and *Ubx-Ubx* interactions observed in the animal might easily be mediated by binding of UBX proteins to regulatory sequences at its targets.

In this paper, we describe a cotransfection assay in cultured Drosophila cells designed to test for transcriptional regulatory functions of UBX and other proteins that govern Drosophila development. We show that in cultured cells UBX proteins can repress an *Antp* P1 promoter fusion and activate a *Ubx* promoter fusion, and experiments in the accompanying paper show that *Antp* proteins and a *fushi tarazu* protein activate these same targets (Winslow et al., 1989). The cotransfection assay is then used to investigate the role of UBX binding sites in regulation of transcription by UBX proteins, and to define which parts of the protein are necessary for regulation. Other developmental regulatory proteins are similarly amenable to study in this system (Jaynes and O'Farrell, 1988; Driever and Nüsslein-Volhard, 1989) and in a related system (Han et al., 1989), and it may be possible to reconstruct more complex aspects of the regulatory hierarchy in cultured cells.

Results

Repression of *Antennapedia* and Positive Autoregulation by UBX Proteins

The cotransfection assay is patterned after assays first used in vertebrate systems to study regulation by viral gene products and steroid hormone receptors. In our assay, a plasmid that directs expression of a developmental control protein (the effector plasmid) is transiently transfected into cultured D. melanogaster cells along with a second plasmid containing a candidate target promoter fused to a reporter gene (the reporter plasmid). Regulatory function is determined by the effect of the effector plasmid on reporter gene activity. The effect of UBX proteins on $P_{Antp\ P1}$ was selected as test case for the system because genetic studies implicated *Ubx* as a repressor of *Antp* (Hafen et al., 1983; Carroll et al., 1986), and because biochemical studies of a UBX protein revealed binding sites near the *Antp* P1 promoter (Beachy et al., 1988). The effect of UBX proteins on P_{Ubx} was also selected for study because UBX binding sites are also found near this promoter (Beachy et al., 1988), and because we suspected that UBX proteins might play a role in amplifying or maintaining their own expression during development through a positive autoregulatory action, a role since demonstrated in the embryonic visceral mesoderm by genetic studies (Bienz and Tremml, 1988).

Reporter plasmids were constructed by fusing a large *Antp* P1 promoter region fragment (−6.1 to +0.78 kb with respect to the transcription start site) or a *Ubx* promoter region fragment (−3.1 to +0.36 kb) to the bacterial chloramphenicol acetyl transferase (CAT) gene in the vector pC4CAT (Figure 1A). This vector contains the CAT coding sequences and the SV40 small t antigen splicing and polyadenylation signals (Thummel et al., 1988), and promoter activity can be determined from RNA analysis or CAT enzyme assays of extracts of transfected D. melanogaster cells (Di Nocera and Dawid, 1983; Benyajati and Dray, 1984; Thummel et al., 1988). Although the *Antp* P1 and *Ubx* promoter fragments used in these constructs contain only some of the *cis*-acting elements required for proper expression in the animal (K. Irvine and D. S. Hogness, unpublished data; Bienz et al., 1988; M. Scott, personal communication), they contain all of the sequences necessary for accurate in vitro transcription (Biggin and Tjian, 1988; K. Perkins and R. Tjian, personal communication) and all of the previously characterized UBX protein binding sites (Beachy et al., 1988).

To test for basal promoter activity, the *Antp* P1 ($pP_{Antp\ P1}CAT$) and *Ubx* ($pP_{Ubx}CAT$) reporter plasmids were each transfected by the standard calcium phosphate technique into a D. melanogaster cell line, Schneider's line 2 (S2), derived from late-stage embryos (Schneider, 1972). CAT enzyme activity was detectable in S2 cell extracts ~20 hr after transfection with either reporter plasmid, then increased linearly during the next 20–40 hr and remained relatively constant thereafter for at least 10 hr (Figure 2). Although reproducibility within a given experiment (i.e., a set of transfection assays with a given preparation of cultured cells) was good (± 15%), the activities of the *Antp* P1 and *Ubx* reporter genes varied considerably from one experiment to another. (Part of this variability may result from differences in transfection efficiency, i.e., the number of cells in the population that take up exogenous DNA, the amount of DNA each cell takes up, and the efficiency with which it is expressed.) The activity of the *Antp* P1 fusion was, however, always 10 to 100 times greater than that of the *Ubx* construct among many different experiments (Figure 2). Analysis of RNA from transfected cells confirmed that the *Antp* P1 and *Ubx* promoter constructs gave rise to transcripts initiated at the native start sites (Figures 4A and 4B). Six plasmids containing

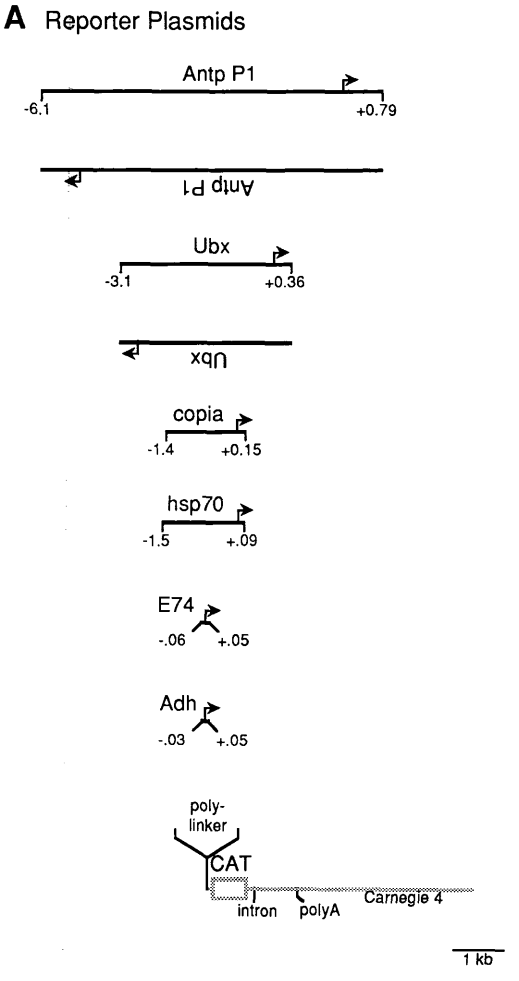

Figure 1. Plasmids Used in the Cotransfection Test for Regulation by UBX Proteins

In the standard cotransfection assay, a UBX effector plasmid is transiently transfected into cultured D. melanogaster Schneider line 2 (S2) cells along with a reporter plasmid, and the effect of UBX proteins on reporter gene expression is monitored by chloramphenicol acetyltransferase (CAT) activity assays or RNA analysis of extracts of the transfected cells. In these schematic representations, stippled lines and boxes represent vector sequences and solid lines and boxes represent inserted D. melanogaster sequences. The open boxes are protein coding sequences. Bent arrows show transcription initiation sites and direction, and the number below each promoter fragment endpoint is the nucleotide distance (in kb) from the initiation site.

(A) Reporter plasmids. Schematic representations are shown (from top to bottom) of the D. melanogaster promoter fragments in the reporter plasmids $pP_{Antp\ P1}CAT$, $pP_{Antp\ P1}CAT(-)$, pP_{UbxCAT}, $pP_{UbxCAT}(-)$, pC4copCAT, pC4hspCAT, pC4E74Δ4031CAT, and pD-33CAT; and the vector pC4CAT. The D. melanogaster promoter fragments represent the *Antennapedia* P1 promoter, the *Ultrabithorax* promoter, the promoter of the terminal repeat of the copia transposable element, the promoter of the *hsp70* heat shock gene, the promoter of the E74 ecdysone-inducible gene, and the alcohol dehydrogenase distal promoter. All reporter plasmids consist of the indicated promoter regions inserted into the polylinker of pC4CAT.

(B) The UBX effector plasmid, $pP_{ac}UBX$ Ib. The *act5C* exon 1 proximal promoter (of the putative cytoplasmic actin gene) drives expression of a Ubx Ib cDNA and allows production of UBX protein. The related UBX effector plasmid pUChsneo actUBX Ib, used in the experiments of Figure 3, contains the sequences shown and additional vector sequences necessary for P element transformation (H. D. Lipshitz and D. S. Hogness, unpublished data; Thummel et al., 1988).

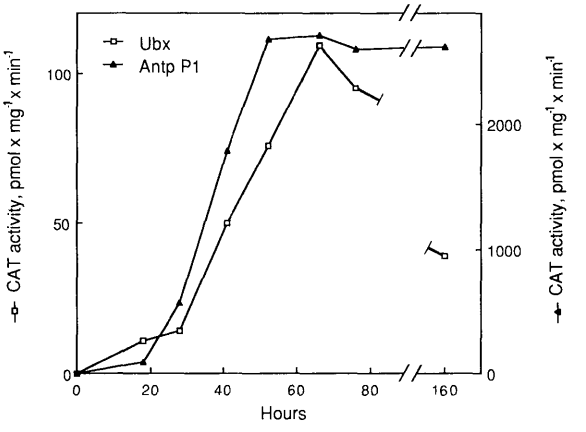

Figure 2. Time Course of Expression of Transfected *Antp* P1 and *Ubx* Reporter Genes

Parallel cultures of D. melanogaster S2 cells were transfected with 1 μg of either $pP_{Antp\ P1}CAT$ or pP_{UbxCAT} DNA by the calcium phosphate technique. At the times indicated after transfection, individual cultures were harvested and cell extracts were assayed for CAT activity as described in Experimental Procedures. CAT activity is given in pmol of chloramphenicol acetylated per mg extract protein per min.

other D. melanogaster promoters or the *Antp* P1 and *Ubx* promoters in the opposite orientation (diagrammed in Figure 1) were also tested in the S2 cells. CAT assays of extracts of transfected cells demonstrated that all of the promoters in their normal orientation were active, but the basal activity of the *Antp* P1 reporter was at least five times greater than that of the other promoter constructs tested (Figure 3 and data not shown).

The S2 line does not express detectable levels of *Ubx* RNA (see below) or proteins (Beachy, 1986) and thus it provided a good background to test the effect of UBX proteins on reporter activity. Actin promoter vectors were used to produce UBX proteins in the cultured cells (Figure 1B). The *act5C* exon 1 proximal promoter in these vectors is active at most stages of D. melanogaster development (Fryberg et al., 1983) and in D. melanogaster K_c and S2 cultured cells (Bond-Matthews and Davidson, 1988; see Figure 4C below), and UBX proteins produced from a transiently transfected effector plasmid are readily detected by immunoblot analysis of transfected S2 cell extracts (E. R. Gavis and D. S. Hogness, unpublished data), and by indirect immunofluorescence of fixed and permeabilized S2 cells using anti-UBX antibodies (Winslow et al., 1989). Cotransfection of a UBX effector plasmid with the

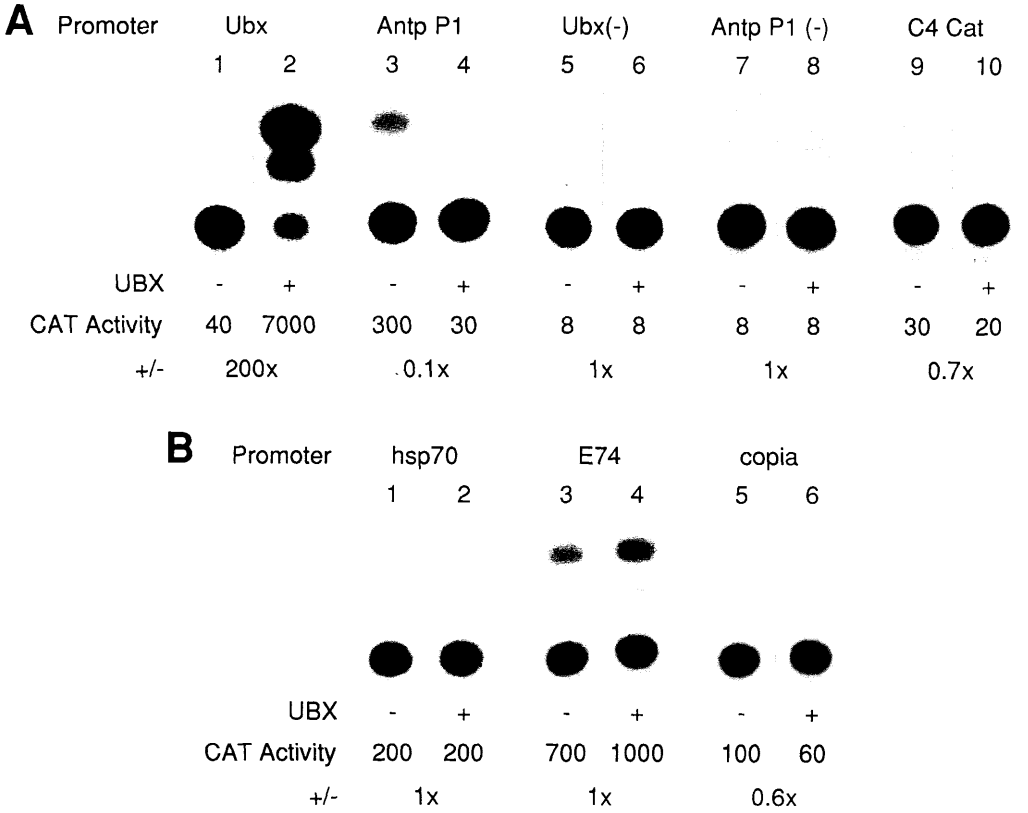

Figure 3. Effect of UBX Protein Expression on Reporter Gene Activity

S2 cells were cotransfected with a reporter plasmid containing the indicated D. melanogaster promoter-CAT fusion (described in Figure 1) and 9 μg of either a UBX effector plasmid (+ lanes; pUChsneo actUBX Ib) or a control plasmid that does not express UBX proteins (– lanes; pcopneo in [A] and pUChsneo act(Bam) in [B]). For the promoters in normal orientation, the amount of reporter plasmid used in these experiments (pC4hspCAT, 0.02 μg; $pP_{Antp\ P1}$CAT, $pP_{Antp\ P1}$CAT(–), and pC4copCAT, 0.1 μg; P_{Ubx}CAT, P_{Ubx}CAT(–), and pC4E74Δ4031CAT, 1 μg; pC4CAT, 10 μg) was within the range in which CAT expression was roughly proportional to the amount of transfecting reporter plasmid. Extracts of the transfected cells were assayed for CAT activity using ^{14}C-chloramphenicol and the reaction products were separated by thin-layer chromatography (TLC). An autoradiogram of the TLC plates from two separate experiments is shown in (A) and (B); the bottom spot in each lane is the unreacted chloramphenicol and the top two spots are the mono-acetylated products. The number below each lane gives the relative CAT activity of the extract in pmol of chloramphenicol acetylated per mg extract protein per min. For each reporter plasmid, qualitatively similar results were obtained in at least two experiments.

reporter plasmids resulted in a dramatic reduction in CAT production from the $_pP_{Antp\ P1}$CAT construct and a large increase from the $_pP_{Ubx}$CAT construct (Figure 3). The magnitude of the regulatory effects in different experiments ranged from a 9- to a 100-fold reduction in $P_{Antp\ P1}$CAT activity and from a 7- to a 200-fold increase from P_{Ubx}CAT, but in most experiments the effects on both were between 10- and 40-fold. The observed regulatory effects were mediated by UBX proteins, because similar effects were observed with three different UBX Ib effector plasmids, and effector plasmids with frameshift mutations in the UBX coding sequences did not significantly alter reporter gene activity (see below).

In the experiments shown in Figures 3A and 3B there was little effect of UBX protein expression on any of the other D. melanogaster promoter fusions, and similar results were obtained in other experiments with these as well as a D. melanogaster alcohol dehydrogenase distal promoter construct (pD-86CAT) and a yolk protein promoter construct (data not shown). These results demonstrate selectivity of UBX protein action in S2 cells: UBX proteins can repress or activate, or have little effect, on different promoter fusions.

RNA analysis demonstrated that UBX proteins modulated the levels of accurately initiated *Antp* P1 and *Ubx* fusion transcripts, and the effect on CAT RNA levels was sufficient to account for most or all of the observed changes in CAT enzyme activity in the extracts (Figures 4A and 4B). We could not detect transcripts from the endogenous *Ubx* and *Antp* P1 promoters, either in the absence or presence of UBX proteins (Figure 4A, lanes 5 and 6; Figure 4B, lanes 4 and 5), although our ability to detect UBX-induced transcripts may be somewhat compromised in these experiments because only about 10% of the cells take up exogenous DNA and express UBX proteins (Winslow et al., 1989).

UBX Proteins Are DNA Binding Transcriptional Regulators

If the regulatory activities of UBX proteins are mediated by binding to promoter region sequences, then removal of such functional binding sites should reduce or abolish

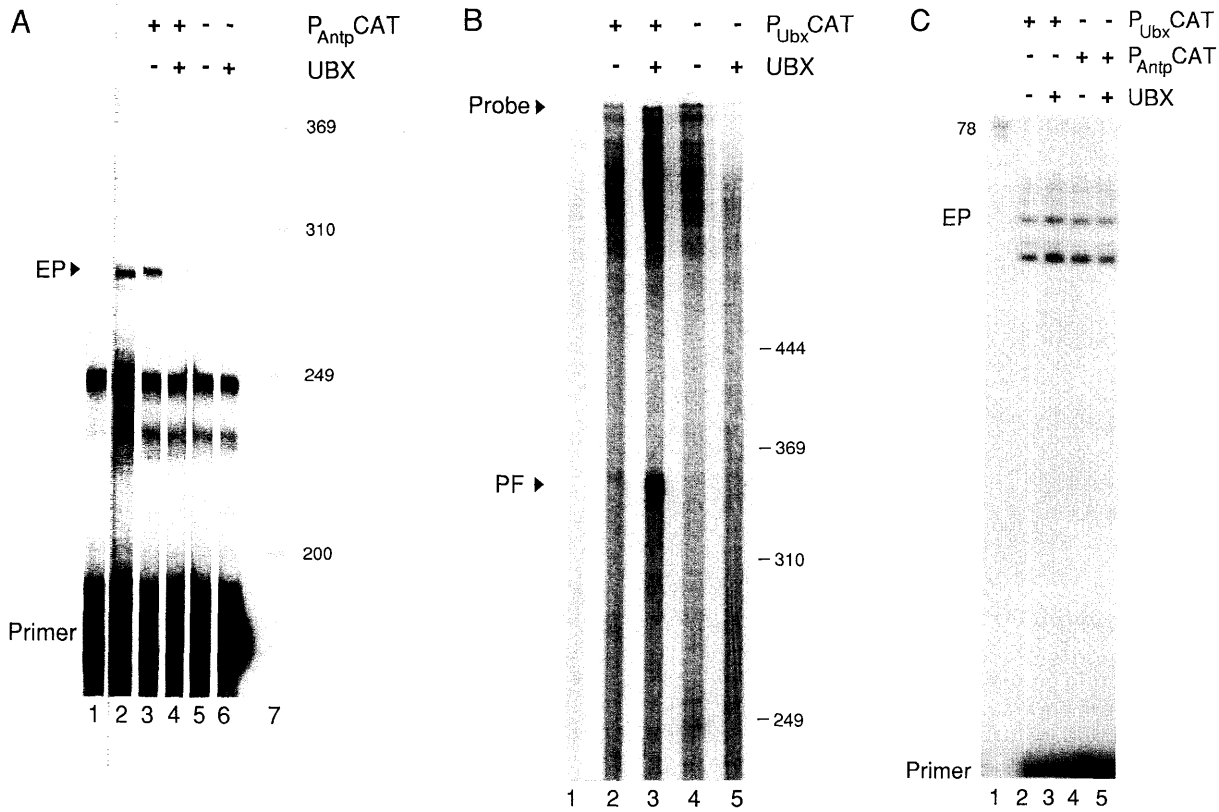

Figure 4. Effect of UBX Proteins on *Antp* P1 and *Ubx* Transcripts

(A) Primer extension analysis of *Antp* P1 transcripts. Primer extension reactions were carried out with a ^{32}P-labeled *Antp* P1 primer and either control RNAs (lane 1, 20 μg yeast tRNA; lane 2, poly A$^+$ RNA from 4–8 hr old D. melanogaster embryos) or 20 μg total RNA prepared from Schneider line 2 cells transfected with: p$P_{Antp\ P1}$CAT and pP_{ac}UBX Ib$^{S/B}$ (lane 3), p$P_{Antp\ P1}$CAT and pP_{ac}UBX Ib (lane 4), pP_{ac}UBX Ib$^{S/B}$ alone (lane 5), or pP_{ac}UBX Ib alone (lane 6). The control plasmid pP_{ac}UBX Ib$^{S/B}$ has a frameshift mutation in UBX Ib codon 8, which prevents UBX protein production (see Figure 7). BstNI-digested, ^{32}P-labeled SV40 DNA fragments were run as size standards in lane 7 and the sizes (in nucleotides) of four fragments are given at the right. The position of the 181 nucleotide primer is labeled, and the position of the 299 nucleotide authentic *Antp* P1 extension product (EP) is indicated by the arrowhead. The origin of the ~245 nucleotide species present in all the lanes is unknown but may result from self priming. No extension products were detected in lanes 4 through 6 even after much longer autoradiographic exposures, which would have revealed a signal of 3%–5% the intensity of that in lane 3, and the same result was obtained in a separate primer extension analysis. There was a 9-fold repression of $P_{Antp\ P1}$CAT by UBX protein in this experiment as determined by CAT activity assays.

(B) S1 nuclease protection analysis of *Ubx* transcripts. S1 nuclease protection reactions were carried out with a ^{32}P-labeled Ubx probe and either control RNA (lane 1, 20 μg total RNA from yeast) or 20 μg total RNA prepared from Schneider line 2 cells transfected with: pP_{Ubx}CAT and pP_{ac}UBX Ib$^{S/B}$ (lane 2); pP_{Ubx}CAT and pP_{ac}UBX Ib (lane 3); pP_{ac}UBX Ib$^{S/B}$ alone (lane 4); or pP_{ac}UBX Ib alone (lane 5). The positions and sizes of BstNI-digested SV40 DNA fragments are shown at the right, and the position of the residual intact 999 nucleotide probe is indicated at the left. Also indicated is the position of fragments run in an adjacent lane, which were protected by poly(A)$^+$ *Ubx* transcripts derived from 4–8 hr old embryos (protected fragments, PF; predicted size 359 nucleotides). No *Ubx* protected fragments were detected in lanes 2, 4, or 5, and the same result was obtained in a separate S1 analysis. Densitometric analysis of various autoradiographic exposures showed that there was at least a 10-fold stimulation of P_{Ubx}CAT transcripts by UBX protein, and there was a 34-fold stimulation as determined by CAT activity assays.

(C) Primer extension analysis of a control transcript. Primer extension analyses were performed as in (A) except that an *act5C* exon 1 proximal primer was used, and the RNA samples in lanes 2 through 5 were the same as those used in lanes 3 and 4 of (A) and lanes 2 and 3 of (B). HinfI-digested pBR322 DNA was run in lane 1 and the 78 nucleotide marker is shown. The *act5C* primer detects transcripts from both the endogenous *act5C* promoter and the *act5C* promoter on the transfected effector plasmids, and the position of the multiple extension products (EP; 60–70 nucleotides) are indicated. Reactions with the RNA samples used in lanes 5 and 6 of (A) and lanes 4 and 5 of (B) gave a similar *act5C* signal.

regulation. Purified UBX Ib protein produced in E. coli binds to two downstream regions of P_{Ubx}, a 48 bp promoter proximal region (+41 to +88 with respect to the transcription start site), and an 89 bp region located 130 bp further downstream (+218 to +306) (Beachy et al., 1988). The arrangement of these two clusters of UBX binding sites is diagrammed in Figure 5 for pP_{Ubx}CAT and several derivative plasmids in which promoter region sequences have been deleted or in which UBX binding sites have been replaced with essentially random sequences. All reporter plasmids that contain the distal downstream cluster of binding sites were activated by cotransfection with a UBX effector plasmid (Figures 5a, 5b, and 5e), whereas constructs in which this region was deleted or replaced showed little response to UBX proteins (Figures 5c and 5d). In contrast, the proximal binding site cluster was not required for UBX regulation, and replacement of these sequences actually increased the UBX stimulatory

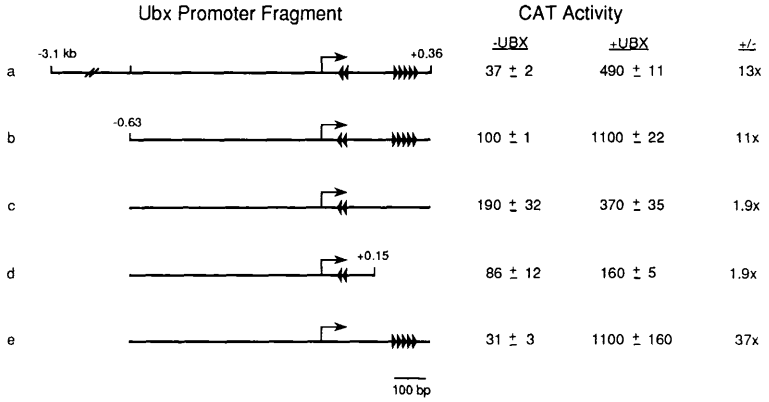

Figure 5. Effect of UBX Proteins on Deletion and Substitution Mutants of the *Ubx* Promoter

Standard cotransfection experiments were carried out with 9 μg of pP_{ac}UBX $Ib^{S/B}$ ("−UBX") or pP_{ac}UBX Ib ("+UBX") and 1 μg of the indicated P_{Ubx} reporter plasmid. a, pP_{Ubx}CAT. b, pP_{Ubx}CATΔ3. c, pP_{Ubx}CATΔ3subDE. d, pP_{Ubx}CATΔ35. e, pP_{Ubx}CATΔ3subB. The two groups of arrowheads indicate the two regions bound by purified UBX Ib protein (Beachy et al., 1988) and show the 5′ to 3′ orientation of the TAA binding site consensus repeats within a region. The numbers above the promoter diagrams are the distances (in kb) of the promoter fragment endpoints to the transcription start site. In the two substitution mutants, the promoter-distal (c) and promoter-proximal (e) UBX binding site clusters have been replaced with essentially random sequences of the same length, which are described in Experimental Procedures. Relative CAT activity is given in pmol of chloramphenicol acetylated per mg of extract protein per min and is the average and standard deviation of duplicate transfections. Similar results were obtained in three separate experiments.

effect (Figure 5e). Thus, UBX activates its own promoter in S2 cells through the distal downstream binding site cluster.

We tested whether UBX binding sites could function as regulatory elements in a novel context by inserting copies of a UBX binding site consensus sequence next to the alcohol dehydrogenase distal promoter, P_{Adh} (Heberlein et al., 1985). The consensus sequence used was a repeating TAA oligonucleotide, since a $(TAA)_x$ motif is found throughout the bound regions at the *Ubx* and *Antp* P1 promoters, and a synthetic 12 bp TAA repeat is sufficient for UBX protein binding in vitro (Beachy et al., 1988). Whereas cotransfection with the UBX effector plasmid had only a modest effect on CAT production from the P_{Adh} base construct (pD-33CAT), there was more than 100-fold stimulation of derivative plasmids containing a 72 or a 144 bp region of UBX binding sites (Figure 6A). Similar effects of UBX proteins were observed with constructs in which the binding sites were inserted in the opposite orientation at the same position. UBX proteins stimulated to a smaller extent (5- to 11-fold) reporters containing a 36 bp region of UBX binding sites inserted at the same position, or inserted 52 bp further upstream of the Adh transcription start site in the plasmid pD-86CAT. Primer extension analysis of RNA from the transfected cells demonstrated that the stimulation in CAT production was paralleled by a dramatic increase in accurately initiated Adh-CAT fusion transcripts (Figure 6B). Thus, UBX binding sites are sufficient to confer UBX regulation on a heterologous promoter, and they can function upstream of the transcription start site in either orientation and in at least two positions.

Regulatory Activity of Other UBX Proteins

Alternative splicing of *Ubx* transcripts yields a set of mRNAs that encode a family of at least five ~40 kd proteins with different temporal and tissue distributions during development (O'Connor et al., 1988; Kornfeld et al., 1989; J. Lopez and D. S. Hogness, unpublished data). Figure 7A shows that the members of this family (UBX Ib, Ia, IIa, IIb, and IVa) have the same N- and C-terminal sequences flanking a differential region consisting of different combinations of three optional elements: b (9 amino acids), I (17 amino acids), and II (17 amino acids). Each UBX protein contains the same homeodomain located in the C-constant region, next to the differential region. An analysis of the functional organization of UBX proteins was initiated by constructing effector plasmids that express the five naturally occurring UBX proteins as well as four synthetic mutant derivatives, diagrammed in Figure 7B. Each of the effector plasmids produced a similar amount of UBX protein of the expected M_r as determined by immunoblot analysis of transfected cell extracts, except that: (a) the short peptides encoded by the N-terminal frameshift constructs (UBX $Ib^{S/B}$ and UBX $Ib^{S/H}$) lack the relevant epitope and were not detected, and (b) the b-form constructs also produced some proteins of slightly lower M_r due to aberrant RNA splicing (E. R. Gavis, K. K., and D. S. H., unpublished data; see legend to Figure 7).

In cotransfection experiments, all of the effector plasmids that express wild-type members of the UBX family behaved similarly. Each repressed the *Antp* P1 promoter fusion and stimulated the *Ubx* promoter fusion, although the UBX IIb and UBX IVa constructs were somewhat more potent than the others in activating the *Ubx* reporter (Figure 7A). When the amount of effector plasmid transfected was varied from 1 ng to 20 μg to determine the apparent dependence of the regulatory responses on UBX protein concentration, no substantial differences between forms were detected: 50% repression of *Antp* P1 required 0.04 ± 0.01 μg of effector, and 2-fold stimulation of the *Ubx* reporter required 0.8 ± 0.4 μg.

In contrast to the wild-type proteins, mutant forms with frameshift mutations in the homeodomain (UBX Ib^{X-}) or in the N-terminal coding sequences (UBX $Ib^{S/B}$ and UBX $Ib^{S/H}$) lacked both regulatory activities (Figure 7B). The most interesting derivative tested, UBX $Ib^{\Delta N}$, was a synthetic deletion in which sequences that encode almost the entire N-constant region of the protein were removed. This deletion left largely intact repression of the *Antp* P1 promoter fusion but abolished activation of the *Ubx* promoter fusion. Indeed, the UBX $Ib^{\Delta N}$ construct caused a modest

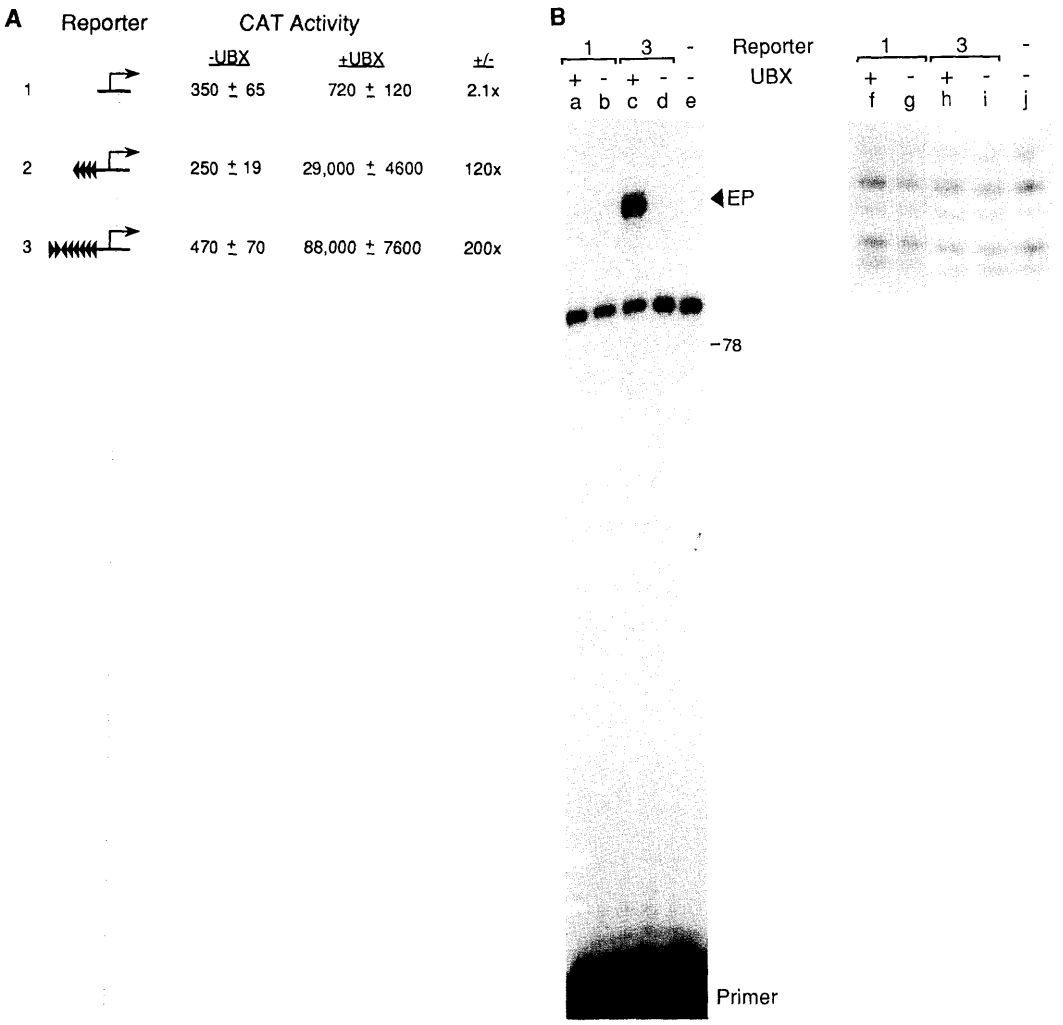

Figure 6. UBX Binding Sites Confer UBX Regulation on the Adh Promoter

Cultures of S2 cells were transfected with either pP_{ac}UBX $Ib^{S/B}$ ("UBX") or pP_{ac}UBX Ib ("+UBX") and either the P_{Adh} reporter plasmid pD-33CAT (reporter 1) or a derivative plasmid containing UBX binding sites, $p(U-)_2$D-33 (reporter 2) or $p(U+)(U-)_3$D-33 (reporter 3). After 48 hr, cultures were harvested and samples were analyzed for CAT activity (A). RNA was prepared from the remainder of the cells and analyzed by primer extension analysis as described in Figure 4A; a CAT gene primer was used to detect Adh-CAT fusion transcripts ([B], lanes a–e) and an *act5C* primer was used to detect actin transcripts as control ([B], lanes f–j). Lanes e and j of (B) show the results using RNA from cells transfected with 20 μg of pUC18 DNA alone. Similar results were obtained in three separate experiments.

(A) The Adh promoter fragment (−33 to +53 with respect to the transcription start site) of the reporter plasmids is shown, and the position, size, and orientation of the inserted UBX binding sites are indicated by the arrowheads. Each pair of arrowheads represents one copy of the oligonucleotide $5'CTAG(AAT)_{12}$ and shows its 5′ to 3′ orientation. CAT activity is given as the average and standard deviation of samples of duplicate transfections in pmol of chloramphenicol acetylated per mg of extract protein per min.

(B) Left: The positions of the 25 nucleotide CAT gene primer and the 97 nucleotide Adh-CAT extension products (EP) are indicated. pBR322 HinfI fragments were run in an adjacent lane and the position of the 78 nucleotide fragment is marked. The transcription start site was confirmed by comparison of the extension products to sequencing ladders (Sanger et al., 1977) generated with the same primer and pD-33CAT DNA. Extension reactions with RNA from cells transfected with reporter 2 and pP_{ac}UBX Ib gave the same extension product but of ~50% of the intensity of that with reporter 3. The origin of the ~80 nucleotide band present in all lanes is unknown.

(B) Right: The 60–70 nucleotide *act5C* exon 1 proximal promoter extension products are shown.

2- to 4-fold repression of the *Ubx* reporter (Figure 7B and data not shown).

Discussion

Using a cell culture cotransfection assay, we have provided direct evidence that UBX proteins are transcriptional regulators with the ability to repress and activate target genes. By suppressing the high basal activity of an *Antp* P1 promoter fusion while activating a *Ubx* promoter fusion, UBX proteins switch these promoters from an anterior thoracic state (*Antp* ON, *Ubx* OFF or low) to a posterior thoracic state (*Ubx* ON, *Antp* OFF or low) in the cultured cells. In this reciprocal effect on the transcription of two genes that direct alternative developmental pathways, UBX proteins are reminiscent of key control mole-

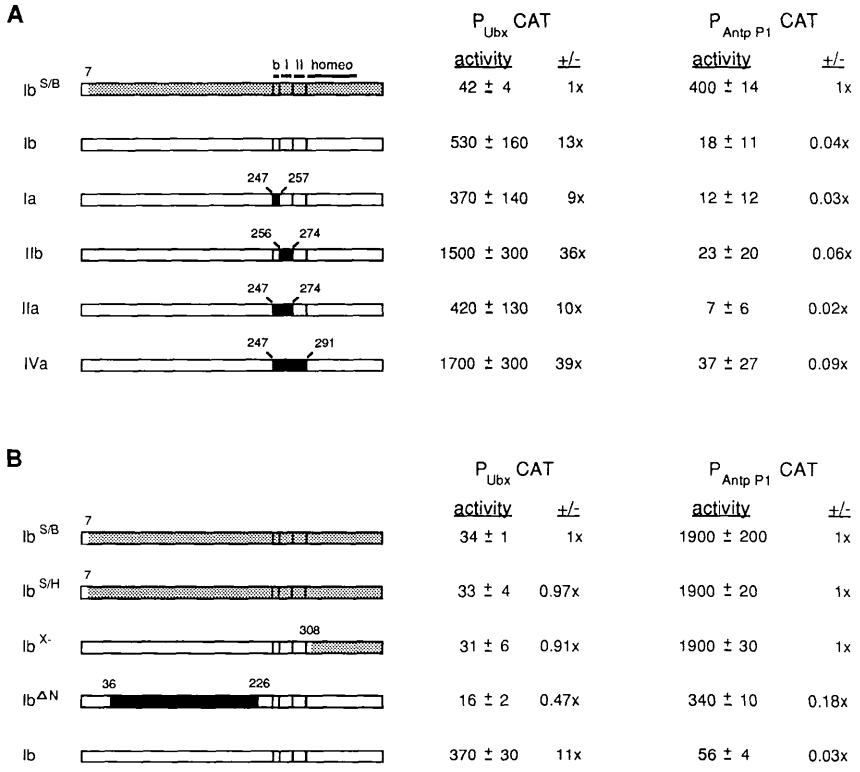

Figure 7. Regulatory Effects of UBX Proteins and Mutant Derivatives

Standard cotransfections were performed with 1 μg of $pP_{Ubx}CAT$ or $pP_{Antp\ P1}CAT$ and 9 μg of an effector plasmid containing the UBX coding sequences indicated. UBX Ib, Ia, IIb, IIa, and IVa are wild-type members of the UBX protein family, and their synthetic mutant derivatives are indicated by superscripts. Labeled in (A) are the rectangles representing the UBX Ib open reading frame (389 codons), the optional elements b, I, and II, which distinguish the native UBX forms, and the homeobox. Filled regions are sequences not present in a construct; stippled regions are present but not translated in frame. The numbers above the diagrams are the codons adjacent to the deletions and the frameshift mutations. Constructs that express b-forms also produce some aberrant, lower molecular weight UBX proteins due to RNA splicing that occurs between the a-form 5′ splice site and cryptic 3′ splice sites in the transcripts (E. R. Gavis, K. Kornfeld, and D. S. Hogness, unpublished data). CAT activity is given in pmol of chloramphenicol acetylated per mg of extract protein per min and is the average and standard deviation of triplicate transfections in (A) and duplicate transfections in (B); values for pP_{ac}UBX $Ib^{S/B}$ were used as the negative reference in the +/− column. Similar results were obtained in at least three separate experiments.

cules in much simpler systems, such as the cI protein of bacteriophage λ, which governs the lysis/lysogeny decision via an analogous genetic switch (Ptashne, 1986). The Drosophila developmental regulatory hierarchy may be in large part a cascade of transcriptional switches, since many other components of the hierarchy have structural (homeodomain) and biochemical (DNA binding activity) similarity to UBX proteins (Gehring and Hiromi, 1986; Scott et al., 1989), and mutations in many genes of the hierarchy alter the distribution or abundance of transcripts of other loci (Akam, 1987; Scott and Carroll, 1987; Ingham, 1988). Indeed, recent studies have demonstrated regulatory capabilities in the cell culture system of several other homeodomain proteins, including *Antp, fushi tarazu, bicoid,* and *paired* proteins (Winslow et al., 1989; Jaynes and O'Farrell, 1988; Driever and Nüsslein-Volhard, 1989; Han et al., 1989), and homeotic gene products can activate synthetic target genes in mammalian cells (Thali et al., 1988).

Although it is appealing to view *Ubx* as a transcriptional switch, there are complexities in its developmental regulatory actions that indicate that this view may be too simplistic. One example of this complexity is the difference in *Ubx* functions in various tissues. While down regulation of *Antp* expression by *Ubx* products has been observed in all tissues examined, positive autoregulation so far appears restricted to the visceral mesoderm (Hafen et al., 1983; Boulet and Scott, 1988; Bienz and Tremml, 1988; J. Botas, K. Irvine, R. Mann, and D. S. Hogness, unpublished data). This implies that maintenance of *Ubx* expression during development need not involve autoregulation in all tissues. Further, it suggests that there are tissue-specific regulatory effects of UBX proteins, a suggestion that curiously parallels the recent observation that the different UBX proteins are differentially distributed between tissues (J. Lopez and D. S. Hogness, unpublished data). It is unlikely, however, that the tissue-specific distribution of the proteins provides a sufficient explanation for the tissue differences in regulation since all UBX proteins tested in the cell culture assay could repress the *Antp* P1 and stimulate the *Ubx* promoters (Figure 7A). Rather, we favor models in which tissue-specific factors, or factors that in-

teract differentially with the UBX protein forms, alter their regulatory actions.

The cell culture approach allows parts of the regulatory hierarchy to be removed from some of the complexities of their developmental context and studied in isolation, and both the regulators and the target promoters can be easily manipulated in this system. This system should therefore facilitate studies of the biochemical mechanisms that govern development in Drosophila, and we used the system to probe the mechanism of regulation by UBX proteins. High-level activation of the *Ubx* reporter required a downstream cluster of UBX protein binding sites (Figure 5), and activation could be transferred to a heterologous promoter by the insertion of UBX binding sites upstream of the transcription start site (Figure 6). Since activation was dependent on UBX protein binding sites, and since it occurred at the level of accurately initiated transcripts but did not require specific sequences within the transcripts, we conclude that UBX proteins regulate transcription initiation of target genes, and that this action is direct and mediated by binding to promoter region sequences. Furthermore, UBX proteins appear to have characteristics of enhancer-activating proteins since activation of a heterologous promoter was not dependent on the precise position or the orientation of the inserted UBX binding sites.

Surprisingly, only one of the two identified clusters of UBX binding sites at the *Ubx* promoter, the distal downstream cluster, was required for high-level activation (Figure 5). Removal of the promoter-proximal cluster paradoxically increased activation. It may act as an inactive sink for UBX proteins, or it may somehow interfere with the function of the distal cluster, perhaps by directly interacting with protein bound there or by competing for a factor required for activation. Whatever the mechanism, these results imply that there are at least two different classes of UBX binding site clusters: activating clusters, which increase the activity of a nearby promoter in response to UBX proteins, and accessory clusters, which apparently have little effect on their own but can influence the response of other elements to UBX proteins. Also, since UBX proteins repress the *Antp* P1 promoter fusion, and since the repression involves, in part, downstream regions containing UBX binding site clusters (E. Parker and M. A. Krasnow, unpublished data), it is very likely that there is a third class that reduces the activity of a linked promoter in response to UBX proteins. It will be important to clarify the functional differences between these classes and to determine which characteristics of the clusters, such as their sequence, size, or promoter context, distinguish their roles in regulation by UBX proteins.

What parts of the UBX protein are required for its different regulatory functions? The differential region that distinguishes the UBX proteins is not essential for repression of the *Antp* P1 reporter or stimulation of the *Ubx* reporter since proteins lacking part or all of this region retained these capabilities (Figure 7A). In contrast, a synthetic deletion (UBX $Ib^{\Delta N}$) that removes most of the N-constant region of the protein defines a domain necessary for activation, since this deletion abolishes activation of *Ubx* but not repression of *Antp* P1 (Figure 7B). Additionally, we infer that the DNA binding homeodomain is required for both activities, because a frameshift mutation in the homeodomain coding sequence eliminates both, and both are affected by promoter region deletions that remove UBX binding sites. Thus, UBX proteins may have a modular design like that demonstrated for several yeast (Brent and Ptashne, 1985; Hope and Struhl, 1986) and vertebrate transcriptional regulators (Giguere et al., 1986; Courey and Tjian, 1988), with domains required for activation distinct from those required for DNA binding and repression. This idea is supported by experiments presented in the accompanying paper (Winslow et al., 1989), in which a hybrid protein containing the N-terminal sequences of an *Antp* protein fused to the UBX homeodomain and C-constant region activates both the *Antp* P1 and the *Ubx* reporters.

An important mechanistic question raised by our studies is how a single UBX protein can both activate and repress target genes. This may be a more general question for DNA binding transcriptional regulators, since the glucocorticoid receptor (Adler et al., 1988; Akerblom et al., 1988; Sakai et al., 1988), and probably a yeast silencer binding protein (Shore and Nasmyth, 1987; Buchman et al., 1988) and a bovine papilloma virus regulatory protein (Thierry and Yaniv, 1987) have opposite effects on the expression of different target genes. One possibility is that different regulatory capabilities are conferred by post-translational modifications, such as the multiple phosphorylations of the UBX proteins that occur in the cultured cells and in the developing animal (E. R. Gavis and D. S. Hogness, unpublished data). Even so, there must still be sequences and perhaps associated proteins at the *Ubx* and the *Antp* P1 promoters that discriminate between the modified species, or that alter the activity of, or respond differently to, a particular form. One such promoter-specific alteration could be the masking of a UBX activation domain at $P_{Antp\ P1}$ to reveal a cryptic repressor function; this would also explain why the disruption of a putative activation domain leaves a protein that represses both $P_{Antp\ P1}$ and, to a lesser degree, P_{Ubx} (UBX $Ib^{\Delta N}$; Figure 7B). By this model, the N-terminal part of *Antp* protein would harbor an activation domain insusceptible to such masking, since the *Antp-Ubx* fusion protein described above activates both the P_{Ubx} and $P_{Antp\ P1}$ reporters (Winslow et al., 1989).

In addition to the use of the cell culture system in the characterization of interactions between a regulator such as UBX and its targets, it may also be possible to adapt the system for the study of more sophisticated aspects of the developmental regulatory hierarchy, such as the combinatorial control of target gene expression by multiple regulators. Such studies will critically depend on the generality and fidelity of the cell culture system, i.e., the ability of other factors to function in the cell culture system in ways that reflect their native actions. There are now several additional examples of hierarchy interactions deduced from genetic studies that have been modeled in the cell culture system, including the activation of the *Ubx* promoter fusion by the *fushi tarazu* protein (Winslow et al., 1989) and the activation of a *hunchback* promoter fusion

by a *bicoid* protein (Driever and Nüsslein-Volhard, 1989). However, there are instances in which predicted interactions are not observed in the cell culture system, and there are also cases of unexpected interactions. For example, in more recent experiments with an effector plasmid (pP_{ac}UBX Ib) different from that used in Figure 3, we found that UBX proteins activated an E74 promoter-fusion and a heat-shock promoter fusion (E. E. Saffman and M. A. Krasnow, unpublished data). Equally surprising, Winslow et al. (1989) found that an *Antp* protein activates the *Ubx* promoter construct. Some of these may be anomalous effects due to abnormally high levels of the effector protein in transfected cells or the absence of key regulatory sequences in the reporter genes. Alternatively, particular combinations of gene products may be necessary for proper regulation, and coregulators may be missing from the cultured cells. If so, cotransfecting effector plasmids that express candidate coregulators along with the original effector plasmid may restore the natural situation, and in this manner it may be possible to reconstruct larger parts of the regulatory hierarchy in cultured cells.

Experimental Procedures

Plasmids

The effector vector pP_{ac} was constructed by inserting a 3.8 kb EcoRI–SalI fragment of the P-element vector pUChsneo act(Bam) (Thummel et al., 1988) between the EcoRI and SalI sites of pUC18. The pUChsneo act(Bam) fragment is composed of a 2.7 kb *act5C* exon 1 proximal promoter fragment (−2.6 kb to +0.09 kb with respect to the transcription start site) and a 1.1 kb fragment containing the three *act5C* polyadenylation signals. pP_{ac} has a unique BamHI site separating the *act5C* promoter and polyadenylation sequences, which was the site used to insert protein coding sequences. Transcripts produced from effector plasmids with intron-containing inserts are spliced in Schneider line 2 cells (Winslow et al., 1989), but introns are not necessary for expression, as most cDNA inserts also yield the expected RNAs and proteins.

pP_{ac}UBX Ib contains the 2.8 kb end-filled EcoRI fragment of the *Ubx* cDNA pφ3605 (D. Peattie and D. S. Hogness, unpublished data) in the sense orientation in the end-filled BamHI site of pP_{ac}. The cDNA sequences are derived from pφ3712 and extend from +353 to +3129 in the Ubx Ib transcript (Kornfeld et al., 1989). The related UBX effector construct, pUChsneo actUBX Ib was constructed by H. Lipshitz. It contains the same *Ubx* and *act5C* sequences as pP_{ac}UBX Ib but differs from it by the presence of vector sequences required for P element transformation (Thummel et al., 1988). The UBX Ia, IIb, IIa, and IVa effector plasmids are derivatives of pP_{ac}UBX Ib in which the StuI–XhoI fragment containing the differential region of the Ubx Ib protein coding sequences was substituted with an analogous fragment derived from cloned cDNAs encoding the other UBX proteins. The Ia fragment was derived from pφ3602, IIa from EC13, and IVa from EC1 (Kornfeld et al., 1989). The IIb fragment was from cDNA E6-16T#5, which was a gift from Michael O'Connor (O'Connor et al., 1988). pP_{ac}UBX $Ib^{S/B}$ and pP_{ac}UBX $Ib^{S/H}$ were constructed by inserting a 10 bp BamHI (5′CGGGATCCCG) or HindIII (5′GCAAGCTTGC) linker, respectively, into pP_{ac}UBX Ib at the unique StuI site in the eighth codon of the 389 codon UBX Ib open reading frame. pP_{ac}UBX Ib^{X-} was generated by cleaving pP_{ac}UBX Ib at the unique XhoI site, end filling, and religating to generate a frameshift mutation in codon 309 (the 16th codon in the *Ubx* homeobox). pP_{ac}UBX $Ib^{\Delta N}$ is an in-frame deletion of the coding sequences between two NotI sites and removes codons 37 through 225. The *Ubx* sequence coordinates used in this paper are those of Kornfeld et al. (1989).

Reporter plasmids were constructed by inserting flush-ended, promoter-containing fragments into the SmaI site of the vector pC4CAT (Thummel et al., 1988). The 3.5 kb genomic EcoRI fragment, −3154 to +358 with respect to the *Ubx* transcription start site, derived from pφ3102 (R. Saint and D. S. Hogness, unpublished data), was end-filled and used to construct pP_{Ubx}CAT and the related plasmid with the insert in the opposite orientation, pP_{Ubx}CAT(−). The 7 kb genomic HindIII fragment, ∼−6.1 kb to +793 with respect to the *Antp* P1 transcription start site (Laughon et al., 1986), derived from p599 (obtained from Matthew Scott and described in Beachy et al., 1988), was end-filled and used to construct $pP_{Antp\ P1}$CAT and $pP_{Antp\ P1}$CAT(−). The reporter plasmids that contain D. melanogaster promoters from the copia transposable element (pC4copCAT), the heat shock gene *hsp70* (pC4hspCAT), and the ecdysone-inducible gene E74 (pcC4E74δ4031-CAT) were constructed by Carl Thummel (Thummel et al., 1988). The E74 sequences extend from −65 to +55 with respect to the E74 transcription start site (C. Thummel and D. S. Hogness, unpublished data). The alcohol dehydrogenase gene reporter plasmids pD-33CAT and pD-86CAT were provided by Bruce England and contain sequences extending from −33 or −86 to +53 with respect to the Adh distal promoter (Heberlein et al., 1985). The upstream Adh sequences present in pD-86CAT but not pD-33CAT contain a binding site for the transcription factor Adf-1 (Heberlein and Tjian, 1988). The sequences at the junctions of the vector polylinker and the Adh promoter are: 5′TCTAGA-GGATCTCG**GTATTT** (pD-33CAT) and 5′TCTAGAGGATCTC**CCGCT**(pD-86CAT), where the underlined sequences are unique XbaI restriction sites and the Adh sequences are in bold print.

The plasmids containing UBX binding sites inserted near the alcohol dehydrogenase distal promoter, including $p(U-)_2$D-33 and $p(U+)(U-)_3$D-33, are derivatives of either pD-33CAT or pD-86CAT in which the oligonucleotides 5′CTAG$(AAT)_{12}$ and 5′CTAG$(TTA)_{12}$ were annealed to each other and inserted into the XbaI site upstream of the Adh promoter. The number and orientation of inserted oligonucleotides in each construct was determined by DNA sequence analysis (Sanger et al., 1977).

The Δ3 and Δ35 deletion mutants of pP_{Ubx}CAT were a gift from Shigeo Hayashi and Matthew Soctt. They were constructed by inserting end-filled HindIII–EcoRI and flush-ended HindIII–BstXI genomic fragments containing the *Ubx* promoter between the end-filled XbaI and the SmaI sites of pC4CAT (Winslow et al., 1989). The Δ3subB and Δ3subDE mutants were constructed like Δ3 except that the HindIII–EcoRI fragments were derived from mutant sequences constructed by Mark Biggin (pUbx FP B sub and pUbx FP DE sub) in which the UBX binding sites are replaced by randomly generated sequences. Translation initiation codons in the random sequences, which might interfere with translation of the CAT open reading frame, were then altered by site-directed mutagenesis. The resultant plasmids have the following changes from pP_{Ubx}CATΔ3:

subB:(+43 to +85 with respect to the *Ubx* transcription start site)
5′GGGTACCTTCGAGCAACCACCGCTTCGACGCTCAGAGGTACCC

subDE:(+221 to +307) 5′GGAATTTACCCTTGCTACGAGTCAGG-
GCAAACGAGTACGCAACAGTAGCGGAATTACGATCGACG-
CTTTCACACCAGACGCTCCTGT

All plasmids used in transfection experiments were grown in E. coli DH1 or DH5 α cells, and purified by an alkaline-lysis procedure followed by CsCl density gradient sedimentation in the presence of ethidium bromide (Maniatis et al., 1982).

Cell Culture and Transfections

Schneider line 2 (S2) cells (Schneider, 1972) were grown as described by Di Nocera and Dawid (1983) except that the growth medium contained 12% fetal bovine serum (heat inactivated at 56°C for 30min). Cells were passaged every 3 days and maintained at a density of 1 × 10^6 to 8 × 10^6 cells/ml in 25 or 75 cm^2 T flasks (Corning). For transfections, a late log culture (∼6 × 10^6 cells/ml) was diluted with growth medium to 8 × 10^5 cells/ml and then 5 ml aliquots were plated onto 6 cm diameter tissue culture plates. After 22–26 hr at 25°C, the cells were transfected with 20 μg of supercoiled plasmid DNA per plate by the calcium phosphate technique described by Di Nocera and Dawid (1983) except that 0.4 ml of 0.25 M $CaCl_2$ was used and precipitates were formed for 30–35 min at 25°C in 16 ml (17 × 100 mm) polypropylene culture tubes. The cells were incubated with the precipitates at 25°C for 58–62 hr. In the standard cotransfection assay, 9 μg of expression plasmid was combined with 1 μg of reporter plasmid and 10 μg of pUC18 DNA. pUC18 was included in all transfections to bring the total amount of DNA to 20 μg, because in preliminary experiments with

1 μg of pP_{Ubx}CAT reporter this amount of pUC18 carrier gave maximal CAT expression.

CAT Activity Assays

Cell extract preparation and CAT activity assays were performed essentially as described by Thummel et al. (1988). Transfected cells were harvested by pipetting, then pelleted and washed once with phosphate-buffered saline (137 mM NaCl, 27 mM KCl, 65 mM Na_2HPO_4, 15 mM KH_2PO_4 [pH 6.8]). After harvesting the cells, all manipulations were carried out at 4°C except where noted. The washed cell pellets were resuspended in 0.1 ml of 0.25 M Tris-HCl (pH 7.8) and lysed by freezing in liquid nitrogen and thawing followed by sonication for 60 sec at ~90 watts in a cup horn (Branson Sonifier Cell Disrupter, Model W185). Cell lysates were heated to 65°C for 5 min and cell debris and precipitated protein were removed by a 10 min centrifugation in a microcentrifuge. The concentration of protein in the supernatant (cell extract) was determined by the method of Bradford (1976) with bovine serum albumin as standard and was typically 2–4 mg/ml. Extracts were assayed for CAT activity immediately or stored for up to 2 weeks at −20°C with little loss of activity. Ten to fifty micrograms of extract protein was assayed using 0.2 μCi ^{14}C-chloramphenicol (~54 mCi/mmol) for 30 min according to Gorman et al. (1982). Because the assay is nonlinear above ~50% conversion, assays of active extracts were performed with less protein or for shorter periods. CAT activities are given as the pmol of ^{14}C-chloramphenicol substrate converted to mono-acetylated forms per mg of extract protein per minute, as determined by TLC analysis and liquid scintillation counting of the substrate and product spots cut out of the TLC plate. All values were corrected for the background activity of extracts transfected with 20 μg pUC18 alone (usually <5 pmol × mg^{-1} × min^{-1}).

RNA Isolation and Analysis

For RNA analysis, transfections were scaled up 3-fold and performed in duplicate, and 48 μg of effector plasmid was used along with 12 μg of reporter plasmid or pUC18 to give a total of 60 μg of plasmid DNA per transfection. After addition of the DNA precipitates, cells were left at 25°C for 48–54 hr, and then extracts were prepared from one-sixth of the cells and analyzed for CAT activity. Total cellular RNA was prepared from the remainder of the cells as described by Burtis (1985). Cells were resuspended in a solution of 6 M guanidine hydrochloride/0.1 M sodium acetate (pH 5.2), dounced several times, and debris removed by centrifugation. The homogenate was layered onto 2 ml of 5 M CsCl/50 mM EDTA (pH 7.5) and sedimented at 42,000 rpm for 18–21 hr in a Beckman SW50.1 rotor. The RNA pellet was resuspended in 10 mM EDTA (pH 7.0), ethanol-precipitated twice, and resuspended in 0.3 M sodium acetate (pH 5.2). The RNA was then extracted three times with phenol:chloroform (1:1) and once with chloroform, ethanol-precipitated, and resuspended in 0.1 ml H_20 and stored at −20°C. The typical yield was ~0.2 mg RNA.

Primer extension and S1 nuclease protection experiments were performed essentially as described (Lipshitz et al., 1987). Twenty micrograms of RNA was mixed with primer or probe, heated to 80°C, and annealed under oil in 10 μl of reannealing buffer (0.4 M NaCl/40 mM PIPES-Cl/1 mM EDTA/pH ~6.4) at 37°–65°C (depending on the primer or probe). For primer extension reactions, 50 μl of PE buffer (75 mM Tris-HCl [pH 8.1], 70 mM KCl, 7 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM dNTPs) and 10 U AMV reverse transcriptase were added, and the mixtures were incubated at 42°C for 60 min. The extension products were concentrated by ethanol precipitation and analyzed on denaturing 4% or 5% acrylamide gels containing 7.8 M urea. The ^{32}P *Antp* P1 primer (181 nucleotides) and the ^{32}P *Ubx* S1 probe (999 nucleotides) were uniformly labeled, single-stranded M13 extension products generated from the recombinant phages M13*Antp* P1(PE1) and M13dm5105 according to Lipshitz et al. (1987) using [^{32}P]dATP and [^{32}P]dCTP, respectively. M13Antp P1(PE1) has an *Antp* genomic BamHI–NruI fragment (+115 to +263 with respect to the P1 transcription start site) inserted between the BamHI and SmaI sites of M13mp19. The 181 nucleotide primer contains 17 nucleotides of the M13 sequencing primer, 19 nucleotides of M13 polylinker sequence, and 145 nucleotides of *Antp* sequence terminating at +119. M13dm5105 has a *Ubx* genomic HindIII–EcoRI fragment (−629 to +358 with respect to the transcription start site) inserted between the HindIII and EcoRI sites of M13mp19. The *act5C* primer (5′GGCTGATGGAGCGGCTTTGTGTCGG) and the CAT gene primer (5′TCCATTTTAGCTTCCTTAGCTCCTG) were synthetic oligonucleotides ^{32}P-labeled at their 5′ ends using T4 polynucleotide kinase. The *act5C* primer (Heberlein and Tjian, 1988) is complementary to nucleotides +43 to +67 of *act5C* exon 1 proximal promoter transcripts. The CAT primer is complementary to nucleotides +73 to +97 of Adh-CAT fusion transcripts generated from pD-33CAT and derivative plasmids.

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