Cell-type-specific mechanisms of transcriptional repression by the homeotic gene products UBX and ABD-A in *Drosophila* embryos

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Communicated by M.Akam

The homeotic genes of *Drosophila melanogaster*, which are required for specification of segmental identities, encode proteins capable of regulating gene expression. We have chosen to study the organization and function of a regulatory target in an attempt to learn how homeotic gene products provide appropriate transcriptional controls. We identified 30 common binding sites for the proteins encoded by the *Ultrabithorax* (*Ubx*) and *abdominal-A* (*abd-A*) genes within a negatively regulated target, the P2 promoter of the *Antennapedia* (*Antp*) gene. By systematically mutagenizing binding sites and observing the resulting P2 expression pattern in embryos, we have found evidence for cell-type-specific interactions that are mediated by these sequences. In certain neuronal cells, UBX and ABD-A proteins appear to repress by competing for common binding sites with another homeodomain protein, which we propose to be ANTP acting to induce P2 transcription in an autoregulatory manner. In sets of cells that contribute to the tracheal system, UBX and ABD-A repress by counteracting the function of a factor acting at independent sites. The latter mechanism of repression requires only that multiple homeodomain binding sequences be present and is not dependent on any particular binding site.

Key words: *abdominal-A*/*Antennapedia*/*Drosophila*/transcription repression/*Ultrabithorax*

Introduction

The *Ultrabithorax* (*Ubx*) and *abdominal-A* (*abd-A*) genes of *Drosophila melanogaster* are responsible for conferring appropriate identities upon thoracic and abdominal segments. These genes have been classified as homeotic as their mutations result in transformations of segmental identity. For example, loss-of-function mutations in *Ubx* cause parasegments (PS) 5 and 6 to transform to an identity like that of PS 4 while PS 7–13 show partial transformation to a more anterior identity (Lewis, 1978). Likewise, mutations of *abd-A* result in complete transformation of PS 7–9 and partial transformation of PS 10–13 to more anterior identities (Lewis, 1978; Sánchez-Herrero, *et al.*, 1985; Busturia *et al.*, 1989).

Ubx and *abd-A* belong to a broader class of genes having sequence similarity across a 183 bp region termed the homeobox (reviewed by Scott *et al.*, 1989). Structural determinations of the homeodomains encoded by the *Drosophila Antennapedia* (*Antp*) and *engrailed* (*en*) genes

and the yeast *MATα2* gene showed that this domain forms a helix–turn–helix (Qian, *et al.*, 1989; Kissinger *et al.*, 1990; Wolberger *et al.*, 1991), similar to a structure found in certain bacterial transcription factors (Pabo and Sauer, 1984). *Drosophila* homeodomain proteins are known to function as transcription factors and this has been demonstrated by their ability to bind to DNA *in vitro* in a sequence-specific manner and to affect gene expression in a variety of *in vitro* and cultured cell assay systems (reviewed by Hayashi and Scott, 1990). Additionally, several demonstrations of the dependence of transcriptional activation on homeodomain binding sites have been made in embryos (Dearolf *et al.*, 1989; Driever *et al.*, 1989; Regulski *et al.*, 1991). The directness of homeodomain control in embryos has been established by making parallel changes in the *fushi tarazu* (*ftz*) homeodomain and its recognition sites within a *ftz* autoregulatory element (Schier and Gehring, 1992).

UBX and ABD-A proteins impose positive and negative controls on gene expression. *Ubx* function is required for high levels of *Ubx* and *decapentaplegic* expression in the visceral mesoderm and *abd-A* function is necessary for *wingless* expression, also in the visceral mesoderm (Bienz and Tremml, 1988; Immerglück, *et al.*, 1990; Reuter *et al.*, 1990). Conversely, *Ubx* and *abd-A* are required to prevent high levels of *Antp* expression in abdominal segments (Hafen *et al.*, 1984; Carroll *et al.*, 1986; Wirz *et al.*, 1986; Boulet and Scott, 1988; Birmingham *et al.*, 1990). Experimental evidence suggests that this control occurs at the level of transcription through the direct binding of UBX and ABD-A to DNA in a sequence-specific fashion. Within putative regulatory targets, UBX and ABD-A bind to sites having the core nucleotide sequence ATTA (Beachy *et al.*, 1988; Ekker *et al.*, 1991; this paper). Furthermore, UBX and ABD-A can activate or repress transcriptional activity in yeast, cell culture and *in vitro* experimental systems (Thali *et al.*, 1988; Krasnow *et al.*, 1989; Samson *et al.*, 1989; Johnson and Krasnow, 1990).

Very little is understood, however, about how homeodomain proteins function during development: how they act at appropriate targets, how positive and negative controls are differentiated and how they interact with other transcription factors to control gene expression. Some progress has been made toward identifying amino acids that determine a degree of homeodomain protein target specificity (Kuziora and McGinnis, 1989, 1991; Dessain *et al.*, 1992; Furukubo-Tokunaga *et al.*, 1992; Lin and McGinnis, 1992). Nevertheless, little information is available as to how functional target promoters are organized, primarily because of the difficulty in reconstructing the regulatory interactions that occur during development.

The complex nature of known homeotic gene regulatory targets presents problems for *in vitro* and cultured cell experimental systems. Although they have been used to

outline certain regulatory capacities of homeodomain proteins, these assays are incapable of providing the conditions necessary for the identification and examination of cell-specific mechanisms of transcriptional control. For instance, the *Ubx* promoter is activated by ANTP in cultured cells (Winslow *et al.*, 1989), although the *Ubx* expression pattern in embryos is unaffected by *Antp* mutations (Struhl and White, 1985; Bienz and Tremml, 1988). A cluster of homeodomain binding sites downstream of the *Ubx* promoter, which is required for *Ubx* positive autoregulation in the visceral mesoderm is not an effective mediator of autoactivation by UBX protein in cultured cells (Krasnow *et al.*, 1989; Müller *et al.*, 1989). These observations underscore the importance of studying transcriptional control in embryos.

The P2 promoter of *Antp* provides an attractive opportunity to study how genes are regulated during development. Segmentation genes that control the promoter in the cellular blastoderm have been identified by genetic studies (Ingham and Martinez-Arias, 1986; Irish *et al.*, 1989; Riley *et al.*, 1991). The basal promoter has been dissected in an *in vitro* study (Perkins *et al.*, 1988). A comparison of sequences from the P2 promoter regions of *D.melanogaster* and *Drosophila virilis* has been carried out (Hooper *et al.*, 1993). Finally, the P2 promoter is repressed by *Ubx* and *abd-A* functions (Boulet and Scott, 1988; Bermingham *et al.*, 1990) and upstream regulatory regions responsible for particular aspects of the P2 expression pattern have been delimited (Boulet and Scott, 1988). We have been studying the P2 promoter with two goals in mind. First, we wish to determine its organization with respect to the number and distribution of functional homeodomain binding sites. Secondly, we wish to understand the mechanism of UBX and ABD-A repression by determining how they interact with factors that activate the P2 promoter in order to establish spatial and temporal limits of expression. Here, through a combination of DNA binding studies and *in vivo* functional tests, we show that UBX and ABD-A repress the P2 promoter by binding to common sites. Additionally, we present data to show for the first time that different mechanisms of repression by UBX and ABD-A proteins operate in different cell-types in the embryo.

Results

A 2.3 kb region of the *Antp* P2 promoter is repressed by *Ubx* and *abd-A* functions

Our experiments are based on an observation that a gene consisting of a DNA sequence extending 2.1 kb upstream and 200 bp downstream of the *Antp* P2 transcriptional start site linked to an *Escherichia coli lacZ* coding sequence (referred to here as 2.3P2-*lacZ*) is expressed in cells within the normal *Antp* expression domain (Boulet and Scott, 1988). To define more precisely the 2.3P2-*lacZ* expression pattern, we used an antibody that recognizes β -galactosidase and laser scanning confocal microscopy. β -galactosidase is first evident within a few ventrally located cells in PS 3, 4 and 5 of germ-band retracting embryos. At stages 15 and 16 (Campos-Ortega and Hartenstein, 1985), the pattern consists of three principal features (Figure 1A). Most conspicuous is the expression in a process extending from each side of the ventral nervous system (VNS) in PS 3, 4 and 5 (arrow). The cells that make up these processes originate as small clusters near the developing nerve cord and then extend laterally then dorsally. The second feature of the pattern is expression in a small group of cells located just posterior to the first (arrowhead). These cells do not elongate and remain within the nerve cord. Finally, expression is observed in a cluster of cells on either side of the nerve cord midline in each thoracic segment (outlined arrow). Staining marginally above background levels is occasionally observed within laterally extending processes in the abdominal VNS.

In embryos lacking *Ubx* and *abd-A* functions due to a chromosomal deletion (*Df Ubx¹⁰⁹*; Karch *et al.*, 1985) the expression pattern characteristic of PS 3-5 is reiterated in PS 6-PS 12 (Figure 1B). In embryos mutant for *Ubx* but wild-type for *abd-A*, 2.3P2-*lacZ* expression extends only through PS 6 (unpublished data). These results demonstrate that 2.3P2-*lacZ* is responsive to *Ubx* and *abd-A* control and is therefore a likely target for the direct action of their protein products.

2.3P2-*lacZ* is expressed by distinct cell-types that also express *Antp*

To determine whether 2.3P2-*lacZ* expression accurately portrays that of a subset of endogenous *Antp* gene expression,

Fig. 1. 2.3P2-*lacZ* expression in wild-type and *Ubx⁻ abd-A⁻* embryos. Ventral surfaces of embryos are shown with anterior to the left. Embryos were stained with an antibody specific to β -galactosidase, as described in Materials and methods. **A.** Stage 16 (Campos-Ortega and Hartenstein, 1985) wild-type embryo showing 2.3P2-*lacZ* expression in parasegments 3, 4 and 5. Three clusters of cells in each hemi-segment are labeled including a group extending laterally and dorsally from the VNS (arrow), a cluster at the base of the extended process (arrowhead) and a cluster bordering the midline of the VNS (outlined arrow). Expression is absent or barely detectable in abdominal segments. **B.** Similarly staged *Ubx⁻, abd-A⁻* (*Df Ubx¹⁰⁹*; Karch *et al.*, 1985) embryo showing reporter gene expression in PS 3-12. Each aspect of the PS 3-5 expression pattern is repeated in PS 6-12.

we double-labeled embryos with anti- β -galactosidase and anti-ANTP (Mab 8C11) antibodies. ANTP protein is detected in each of the β -galactosidase expressing clusters in late stage 12 embryos (Figure 2A). At later stages, ANTP

expression is evident within two of the three clusters in each hemi-segment. However, we no longer observe ANTP in those cells that form the lateral processes (unpublished data). We presume that high level expression of *Antp* ceases within

Fig. 2. 2.3P2-*lacZ* is co-expressed with *Antp* in neuronal and tracheal cells. **A.** Single optical section focused on the ventral surface at PS 4 of a late stage 12 embryo labeled with antibodies recognizing cytoplasmic β -galactosidase (red) and nuclear ANTP protein (green). Five cells appear yellow from the co-localization of β -galactosidase and ANTP. These cells correspond to those cells that will form the lateral process and those that will appear at the base of the lateral process in later stages. The 2.3P2-*lacZ* expressing cluster near the VNS midline is outside the focal plane of this optical section. **B.** Single optical section showing a portion of the thoracic ventral nerve cord at PS 4 of a stage 16 embryo stained with antibodies to β -galactosidase (red) and HRP (green). The arrow indicates the proximal tip of the lateral process which is not labeled by anti-HRP antibody. In contrast, the two β -galactosidase-expressing cells near the base of the lateral process (arrowheads) are outlined in green and yellow by virtue of their simultaneous staining with anti-HRP, which recognizes an antigen on the surfaces of neuronal cells (Jan and Jan, 1982). The outlined arrow indicates anti-HRP staining of the intersegmental nerve. **C.** Stage 16 embryo stained with anti- β -galactosidase antibody (red) and the 2A12 monoclonal antibody (green) which identifies the tracheal system (N.Patel and C.Goodman, unpublished results). This single optical section is focused on the laterally extending process of PS 4. The co-localization of these antigens along with the position of these cells relative to the VNS identify them as part of the ventral ganglionic tracheae. Scale bars represent 5 μ M.

these cells, while the persistence of the β -galactosidase signal is due to greater protein stability. We conclude that 2.3P2-*lacZ* marks cells in which the endogenous P2 promoter is active.

The use of an anti-horseradish peroxidase (HRP) antibody, which stains *Drosophila* neurons (Jan and Jan, 1982), has allowed us to identify a distinction between these cell clusters with respect to neuronal character. The non-elongating

cluster of about three or four cells at the lateral edge of the VNS, as well as the cell cluster located closer to the midline, can be classified as neuronal-based on their expression of the HRP epitope (Figure 2B). The third group of cells, which form the laterally and dorsally extending processes, are not labeled with the anti-HRP antibody. The monoclonal antibody 2A12, which recognizes the tracheal system (N.Patel and C.Goodman, unpublished results), stains the

Fig. 3. ABD-A and UBX binding to wild-type and mutant DNA. Increasing amounts of partially purified ABD-A (A) and UBX (B) proteins were incubated with radiolabeled 290 bp DNA fragments from between 1155 bp and 865 bp upstream of the *Antp* P2 transcription start site. This complex was then subjected to DNase I cleavage as described in Materials and methods. A. Lanes 3–6 show the DNase cleavage pattern generated by ABD-A protein compared with the control lane 7 having no protein. Six regions of protection are evident and are labeled with respect to the position of core ATTA sequences 5' of the P2 transcription initiation site. Lanes 1 and 2 represent G and G + A Maxam–Gilbert sequencing reactions, respectively. B. Lanes 3 and 4 exhibit the UBX protection pattern compared with a no protein control in lane 2. The same six sites bound by ABD-A protein are bound by UBX. Lanes 5–7 show the effect of base substitution mutations on the ability of UBX to bind the DNA. Mutations are as follows. Sites 990, 982 and 912, core ATTA sequences changed to ACTA; site 1076, ATTA changed to CCTA; site 925, GCAATAAA changed to GCACCAAA; sites 1096, 967, no changes.

same processes as anti- β -galactosidase demonstrating them to be the ventral ganglionic tracheae (Figure 2C).

UBX and ABD-A proteins bind many common sites within the 2.3P2 promoter

Partially purified UBX Ib and ABD-A proteins (see Materials and methods) were used to identify binding sites within the 2.3 kb P2 fragment. Immunoprecipitation of ABD-A protein-DNA complexes indicated that binding sites are widely distributed (data not shown). *In vitro* DNase I protection assays identified ~30 binding sites for each of the proteins throughout the 2.3P2 promoter. Figure 3A shows the protection pattern generated by ABD-A protein bound to a DNA fragment extending from 1155 bp to 865 bp upstream of the P2 transcription start site. Lanes 2–4 of Figure 3B shows the protection pattern produced by UBX protein within the same 290 bp fragment. The two proteins protect the same six sites with small differences in the way the DNase I cleavage pattern is affected. This indicates that although the manner of contacting the DNA may differ slightly, each protein is capable of recognizing the same DNA sequences. Although sites 1096–1076 and 990–982 appear as single sites, a mutagenesis experiment reveals that sites 1076 and 1096 can be considered as independent (see below). Within this set of bound sequences, small differences in relative affinity for each protein exists. For example, site 925 is more fully protected than site 912, suggesting at least a 2-fold difference in affinity. Comparison of other sites indicates that some differences may be as great as 5-fold (data not shown). In general, these differences in relative affinities for individual sites are approximately equal for UBX and ABD-A proteins.

Most sequences protected by UBX and ABD-A can be aligned with respect to a common ATTA core (Figure 4). Those without the ATTA core are generally A-T rich. The size of the protected regions range from 7–90 bp (site 1606). Although UBX protein has been shown to bind large tracts within the *Ubx* and *Antp* P1 promoters (Beachy *et al.*, 1988), the sequences within our data set lack the TTA and CGATTA tandem repeats common to those targets. As a single homeodomain protein protects a site of ~12 bp (Florence *et al.*, 1991) the larger areas of protection are likely to represent occupation by more than one protein molecule. Finally, of 30 protected sites, UBX and ABD-A proteins differ in their recognition of only a single site. The sequence GCCGCCATTATCTATG located 116 bp upstream of the start site is bound only by UBX protein in our assays.

Mutations in ATTA sequences disrupt UBX and ABD-A binding

We used mismatched primers and the PCR (described in Materials and methods) to introduce specific base pair changes within UBX and ABD-A binding sites. Crystallographic (Kissinger *et al.*, 1990), NMR (Otting *et al.*, 1988) as well as ethylation and methylation interference studies (Affolter *et al.*, 1990; Percival-Smith *et al.*, 1990) of DNA-homeodomain complexes have revealed protein contacts with all nucleotides of the ATTA core. Furthermore, mutagenesis of nucleotides within the core dramatically reduce the affinity of UBX and FTZ homeodomains for their respective optimal binding sequences *in vitro* (Ekker *et al.*, 1991; Florence *et al.*, 1991). To minimize disruption of binding by factors that potentially

overlap homeodomain binding sites, we limited our mutagenesis to one and two base substitutions within the binding sites, changing ATTA to either ACTA or CCTA.

The effects of these mutations on the binding of UBX protein are demonstrated in lanes 5–7 of Figure 3B. Two base pair mutations at sites 1076 and 925 fully eliminate binding. Single base pair mutations at sites 990, 982, 958 and 912 greatly reduce binding. Occasionally, novel protection patterns are generated in the mutagenized DNA. For example, binding at site 958 is disrupted by the mutation but a new binding site is revealed at a sequence that overlaps the original binding site at the 3' end. Notably, disruption of binding by the mutation at site 1076 does not interfere with the ability of UBX protein to protect the intact 1096 site nor is binding at site 967 inhibited by nearby mutations. Thus, we have no evidence of cooperative binding at closely aligned sites. Additionally, this indicates that protected regions scored as single binding sites may actually comprise multiple sites for interaction with homeodomain protein.

Mutations in homeodomain binding sites reveal cell-type-specific mechanisms of transcriptional control

We tested the effects of mutations within homeodomain binding sites on the expression pattern of 2.3P2-*lacZ* by combining different sets of mutations and placing these altered reporter gene constructs into flies via P-element-mediated transformation (Rubin and Spradling, 1982). Figure 5 presents the combinations tested in schematic form along with a summary of the resulting expression patterns. Our strategy was to target first those sites that were judged to have the greatest affinity for UBX and ABD-A proteins *in vitro* followed by mutagenesis of weaker sites. The total

Site:	Protected Sequence:
2052	GGAAGTGCATTAGGGA
1997	CACATTAAACATCCATTAA
1957/1952	GAAATAAATTAATTA
1921/1918	AAATATTAATAAGATGG
1781	GATTAATAATCAACATACGACAATC...
1715	AAATGCATTAAT
1605	...TTAGATTTATGGCTTCACGCAATTAGTCTTAAATATATGCAAAAG...
1524	GGTATGGCTTCATTAACGGC
1242/1231	GGTAAATTTTGTGATTATGTT
1099/1096/1076	ATTAATATATATTCATGGCAGCAATATCGG
990/ 982	TAATTAATAAT
958	CCACAATCATTAATTT
912	GGCATAAATTAATAACACA
852/ 849	TGAATGTAAATTAATTTGCAATAT
837	GAATATTAAC
744	AGGATAAATTGTTATTATAAATTT
719	AAAATTAATTC
687	GAATATGTTA
636/ 625	GTTCATTAATAATAATAATTT
555/ 542	TAATATATAAGCAATTAAGGTA
452/ 445	ACAGATTATGGATTAG
422	TCATATTAATGGACGTTATG
372	CTAACATTAACTTGT
359	ATAATTA
303/ 297	TGAATATAAATTAATTTG
1258	TAAACATAAA
967	TGGAATA
925	TGAAAGCAATAAAGAAA
391	GCATTTAAAGTAAATTT
UBX only: 116	GCCGCCATTATCTATG

Fig. 4. Compilation of sequences protected by UBX and ABD-A proteins. Sequences protected from DNase I cleavage by UBX and ABD-A proteins are aligned with respect to a common ATTA sequence. Additional ATTA sequences that appear apart from the aligned core are underlined. Five protected sequences do not contain the ATTA core and are represented at the bottom. One sequence, site 116, is bound only by UBX protein in our assays. Each protected site is designated with respect to the nucleotide position of each ATTA sequence within it 5' of the P2 transcription start site (Hooper *et al.*, 1992). Those sites having no ATTA core are designated with reference to the 3' extent of protection.

Fig. 5. Schematic representation of mutant binding site combinations and summary of their effects on 2.3P2-*lacZ* expression. Each ATTA sequence within a site protected by UBX and Abd-A proteins and each protected sequence having no ATTA core is represented as a box. The numbers above the boxes are nucleotide position designations relative to the P2 transcription start site and correspond to the sequences compiled in Figure 4. Open boxes represent unmutated sequences, filled-in boxes represent sites with base pair substitution mutations. Each promoter combination is labeled to indicate the number of mutant sites it contains. For example, pt11 contains mutations within 11 positions. Neuro and tracheal categories refer to cell-type-specific expression of the 2.3P2 promoter in neuronal and tracheal cells (see text). + and - denote presence and absence of reporter gene expression, respectively. 2.3P2; *Antp*⁻ refers to the unmutated promoter in homozygous *Antp*⁻ embryos. 2-6 independent transposant lines were analyzed for each construct with the exception of pt16a and pt16c, for which single lines were examined. The pattern of expression was consistent between all lines representing individual constructs with only small differences in the level of expression being evident.

number of mutations placed within the promoter is greater than the number of sites recorded by the DNase protection assays as some protected sites have multiple ATTA sequences within them. Mutations within 11 binding sites with the highest relative affinities for UBX and ABD-A proteins do not alter the pattern of 2.3P2-*lacZ* expression. High levels of β -galactosidase are observed in neuronal and tracheal cells of PS 3-5 with little or no expression in abdominal segments (pt11, Figure 6A). By comparison, introduction of mutations at 15 positions in addition to those of pt11 result in a significant change in the expression pattern. Although PS 3-5 expression in these lines is normal, high levels of expression arise in ventral ganglionic tracheal cells from PS 6-PS 12 (pt26, Figure 6B). These cells are equivalent to a subset of those expressing 2.3P2-*lacZ* in embryos mutant for *Ubx* and *abd-A* functions (Figure 1B, arrow). These results argue strongly that repression is the consequence of a direct interaction of UBX and ABD-A proteins with binding sites in the 2.3P2 promoter.

To determine whether a single, high affinity binding site is sufficient to mediate repression in abdominal non-neuronal cells, we restored site 1076 to its wild-type sequence. Lines transformed with this promoter show no difference in expression from pt26 lines with high levels of β -galactosidase in tracheal cells of PS 6-12 and normal expression in PS 3-5 (pt25, Figure 5; unpublished data).

Because we observed derepression in abdominal tracheal cells when only a subset of homeodomain binding sites were mutated, we wondered if the repression function might operate through a particular site or set of sites. To test this, we constructed promoters with subsets of the pt26 mutant site combinations, all of which maintain the original pt11

mutations. (Figure 5). Each of these combinations (pt16a, pt16b, pt16c and pt21) direct reporter gene expression in a pattern identical to that of pt26: expression in tracheal cells of PS 6-12 with the normal PS 3-5 pattern maintained (Figure 6C; unpublished data). Because the pt16 promoters share no combination of mutated binding sites other than those of pt11, which is correctly regulated, these results argue that derepression is not a function of any particular binding site mutation. What appears to be critical, instead is the total number of disrupted binding sites. As mutation of about one half of the total number of binding sites results in derepression, these results demonstrate that multiple binding sites are required for this aspect of 2.3P2 repression.

Finally, lines with mutations in all binding sites, with the exception of two low affinity non-ATTA sites (pt39), exhibit a second major difference from the original 2.3P2-*lacZ* pattern. In addition to derepression of tracheal cell expression from PS 6-PS 12, neuronal cell expression in PS 3-5 is lost or reduced (Figure 6D). This indicates expression of 2.3P2-*lacZ* in these cells is dependent on a factor that contacts homeodomain binding sites.

***Antp* is required for 2.3P2-*lacZ* expression in thoracic neuronal cells**

The loss of expression of the pt39 mutant reporter gene in neuronal cells of PS 3-5 suggested to us that the activating factor was likely to be a homeodomain protein. Because some homeotic genes have been demonstrated to positively regulate expression from their own promoters (Bienz and Tremml, 1988; Bergson and McGinnis, 1990) and the ANT-P homeodomain recognizes sequences similar to those bound by UBX and ABD-A *in vitro* (Müller *et al.*, 1988), we reasoned that this aspect of 2.3P2-*lacZ* expression may be

Fig. 6. Expression patterns of 2.3P2-*lacZ* reporter genes containing mutations in homeodomain binding sites. Each embryo is stained with anti- β -galactosidase antibody and oriented with the ventral surface facing out and anterior to the left. **A.** pt11 embryo having mutations in 11 positions. Expression is limited to PS 3–5. **B.** pt26 embryo with mutations in 26 positions. Expression in PS 3–5 is normal. However, expression appears in ventral ganglionic tracheal cells of PS 6–12 (optical sectioning does not include PS 12 due to interference by yolk autofluorescence). **C.** High magnification view of pt16a embryo showing thoracic neuronal (outlined arrow and arrowhead) and tracheal (arrow), and abdominal tracheal cell expression. Tracheal expression appears posteriorly through PS 12. Scale bar represents 25 μ M. **D.** pt39 embryo having mutations in 39 positions. High magnification view showing absence or decreased levels of expression in neuronal cells of PS 3–5 (outlined arrow, arrowhead). Abdominal tracheal expression remains through PS 12.

due to ANTP protein. Although *Antp* function was determined not to be essential for the expression of a 10 kb P2 reporter gene (Boulet and Scott, 1988), the lower sensitivity of the assays used may not have revealed autoregulation within a subset of the expressing cells. Therefore, the unaltered 2.3P2-*lacZ* was crossed into an *Antp*^{w10} background, which is an apparent null allele of *Antp* (Wakimoto and Kaufman, 1981). In homozygous *Antp*⁻ embryos, normal levels of expression in tracheal cells of PS 3–5 are present. However, expression in all neuronal cells is absent (Figure 7). This pattern is similar to that of pt39 embryos in PS 3–5, suggesting that ANTP protein acts through sequences identified as UBX and ABD-A binding sites to autoregulate the P2 promoter in a cell-type-specific manner. Furthermore, it suggests that UBX and ABD-A proteins can compete with ANTP protein for common binding sites to prevent high level expression from the P2 promoter.

Discussion

UBX, ABD-A and ANTP proteins control expression of the P2 promoter

The *Antp* P2 promoter is subject to *Ubx* and *abd-A* regulatory control as defined by its derepression in embryos mutant for those functions (Boulet and Scott, 1988; Bermingham *et al.*,

1990). We have identified 30 sites bound by UBX and ABD-A proteins within 2.3 kb of DNA encompassing the P2 transcription start site, consistent with this promoter being a direct regulatory target for these proteins. With a single exception, each of these sites is bound by both proteins. The most common feature of these binding sites is an ATTA motif, which is also characteristic of sequences bound by other homeodomain proteins (Beachy *et al.*, 1988; Desplan *et al.*, 1988; Hoey and Levine, 1988; Müller *et al.*, 1988; Driever and Nüsslein-Volhard, 1989; Pick *et al.*, 1990; Regulski *et al.*, 1991). A mutagenesis experiment has demonstrated that some protected regions may actually comprise multiple, independent binding sites (Figure 3B). Thus, the 2.3P2 promoter is potentially bound by many molecules of homeodomain protein at one time.

Two published reports provide the opportunity for a comparison of UBX binding specificities. Beachy *et al.* (1988) identified several large UBX binding sites in the vicinities of the *Antp* P1 and *Ubx* promoters. The most notable feature of these binding sites is the presence of the tandemly repeated trinucleotide TTA or the hexanucleotide CGATTA. Although the *in vivo* role of these sequences has not been established, TTA and CGATTA repeats do confer transcriptional activation upon a heterologous promoter by UBX protein in a cell culture system (Krasnow *et al.*, 1989). One of our protected sequences is also large, covering 90

Fig. 7. 2.3P2-*lacZ* expression in *Antp*⁻ embryos. High magnification view of 2.3P2-*lacZ* expression in an *Antp*⁻ (*Antp*^{w10}; Wakimoto and Kaufman, 1982) embryo showing expression limited to tracheal cells of PS 3-5 (arrow). No 2.3P2-*lacZ* expression is evident in neuronal cells (outlined arrow, arrowhead).

bp. However, TTA or CGATTA tandem repeats are not a feature of our set of binding sites. Ekker *et al.* (1991) isolated oligonucleotide sequences from a random pool that were selectively bound by UBX protein. The optimal binding sequence derived in this way was 5'-CCATTAA-3'. This particular sequence appears only once within our set (site 1997). However, their study demonstrated that positions outside the core make considerably smaller contributions to affinity than those within, confirming that many sequence variants may be avidly bound.

We have demonstrated that the sequences bound by UBX and ABD-A *in vitro* are important for the correct regulation of the *Antp* P2 promoter by introducing mutations designed to disrupt binding by homeodomain proteins. First, the mutations result in ectopic expression in a set of ventral ganglionic tracheal cells within the *Ubx* and *abd-A* functional domains. This expression mirrors an aspect of the 2.3P2-*lacZ* pattern seen in embryos lacking *Ubx* and *abd-A* functions suggesting that reporter gene activity in abdominal segments is normally repressed through a direct interaction of UBX and ABD-A proteins with the P2 promoter. Secondly, when nearly all of the *in vitro* binding sites are mutated, 2.3P2 expression is lost from a set of cells in PS 3-5. We have shown that expression in precisely the same cells is dependent on *Antp* function suggesting the binding of ANTP protein to these sequences enhances P2 activity in an autoregulatory fashion. The large number of homeodomain binding sites within the P2 promoter makes a more rigorous demonstration of direct interactions as carried out for *ftz* autoregulation by Schier and Gehring (1992) difficult. However, the coincidence of ANTP, UBX and ABD-A expression domains (Beachy *et al.*, 1985; White and Wilcox, 1985; Carroll *et al.*, 1986; Wirz *et al.*, 1986; Karch *et al.*, 1990; Macias *et al.*, 1990) with positively (by ANTP) or negatively (by UBX and ABD-A) regulated cells strongly argues that these proteins function as sequence-specific transcription factors in the embryo.

Repression by UBX and ABD-A occurs through two different mechanisms

2.3P2-*lacZ* has the *cis*-acting regulatory elements sufficient to drive *Antp* P2-like expression within two different cell-types. Our mutagenesis experiments have revealed that

Fig. 8. Model of cell-specific regulatory mechanisms for the *Antp* P2 promoter. A. In neuronal cells of PS 3-5, ANTP protein positively autoregulates the P2 promoter by binding to specific DNA sequences, represented by an open box. In neuronal cells of PS 6-12, UBX and ABD-A proteins bind to these same sequences, preventing ANTP autoregulation. When the binding sites are mutated (black box), no binding occurs and the promoter remains inactive in neuronal cells of both thoracic and abdominal segments. B. In tracheal cells of PS 3-5, expression results from the activity of a factor binding to sequences distinct from homeodomain binding sites. In tracheal cells of PS 6-12, UBX and ABD-A prevent this activation by binding to multiple sites within the promoter. When some of the homeodomain binding sites are mutated, UBX and ABD-A fail to bind the DNA sufficiently to prevent activation in PS 6-12. A possible consequence of such binding is shown as the looping of DNA in the promoter region.

distinct regulatory mechanisms are responsible for activation and repression of the promoter within these cells. As we discussed above, both ANTP protein and intact homeodomain binding sites are required to drive expression of the P2 promoter in neuronal cells of the thoracic VNS. Accordingly, we propose the model represented in Figure 8A in which, following low level activation by an as yet unknown factor, ANTP acts through ATTA sequences within the P2 promoter to increase and maintain its transcriptional activity in PS 3-5. In PS 6-12, UBX and ABD-A proteins would displace ANTP, preventing its autoregulatory function. Repression resulting from competition between activating and non-activating homeodomain proteins for common binding sites has previously been proposed on the basis of cell culture and *in vitro* experiments (Jaynes and O'Farrell, 1988; Han *et al.*, 1989; Ohkuma *et al.*, 1990). Our results argue that such a mechanism indeed constitutes a molecular basis of repression of gene activity in embryos.

In contrast to what we observe in thoracic neuronal cells, expression of 2.3P2-*lacZ* in thoracic ventral ganglionic tracheal cells is maintained despite mutations of homeodomain binding sites. In addition, the reporter gene becomes derepressed in comparable cells of abdominal segments. We conclude that first, expression of 2.3P2-*lacZ* in tracheal cells is due to a factor not dependent on homeodomain binding sites for its function and secondly, UBX and ABD-A act directly through sequences defined as *in vitro* binding sites to repress the promoter in these cells. By binding to a site, UBX and ABD-A may prevent the binding of a positively acting factor recognizing an overlapping site, as proposed for steroid hormone receptors (Akerblom *et al.*, 1988; Glass *et al.*, 1988; Sakai *et al.*, 1988; Drouin *et al.*, 1989). Mechanistically, this would be very similar to repression by competition with ANTP protein for common binding sites. If true, one of the following two predictions should be confirmed. If the positive activator works through a single or limited number of sites, PS 6–12 tracheal cell derepression should be observed only with certain combinations of homeodomain binding site mutations. Conversely, if the factor is able to act through many sites, we should recognize a common sequence element closely associated with many of our homeodomain binding sites. Our results support neither of these predictions. Instead, any combination of mutant sites above a certain number (lying between 11 and 16) results in derepression, none of which exhibit recognizable repeated sequences other than the core homeodomain binding sequence. Thus, derepression is a function of the total number of homeodomain binding site mutations and not of mutation of any particular sequence or combination of sequences. This suggests that occlusion of an overlapping or nearby binding site is not the mechanism of repression as suggested for regulation of *even-skipped* stripe 2 (Small *et al.*, 1991). Instead, it appears that UBX and ABD-A may prevent activation of a factor binding some distance away from homeodomain binding sites. The *engrailed* (EN) and *even-skipped* (EVE) proteins have been demonstrated to have the ability to repress transcription through a mechanism different from competition for binding sites. In cultured cells, EN can act through homeodomain binding sites to prevent activation by mammalian and yeast transcription factors bound to separate sites (Jaynes and O'Farrell, 1991). Purified EVE has been shown to work through homeodomain binding sites to repress the *Ubx* promoter *in vitro*, the activation of which is independent of those homeodomain binding sequences (Biggin and Tjian, 1989). We have shown here that this manner of repression by homeodomain proteins also serves to regulate gene activity during development.

The requirement for multiple intact binding sites to mediate UBX and ABD-A repression of the 2.3P2 promoter in abdominal tracheal cells implies that many molecules of these proteins are assembled on the transcriptionally inactive P2 promoter. We find this suggestive of a mechanism in which repression is imposed by a physical block between the activator and the transcription complex, perhaps by affecting chromatin structure (Figure 8B). DNA loops dependent on UBX protein and homeodomain binding sites have been visualized by electron microscopy (P. Beachy and J. Varkey, personal communication). Additionally, the yeast MAT α 2 homeodomain protein has been proposed to repress gene expression by influencing the position of nucleosomes (Roth

et al., 1990, 1992). This particular model provides the basis for an important prediction: repression is independent of the activating molecule. Whereas mechanisms that operate by protein contacts between repressor and activator would require the ability of UBX and ABD-A to make specific interactions with each factor they counteract, our model states such specificity is not necessary, thereby allowing a general mechanism to be applied to a variety of promoter contexts.

Materials and methods

Protein expression and purification

To produce full-length, non-fusion ABD-A protein, the T7 polymerase expression system (Studier and Moffatt, 1986) was utilized. The *abd-A* cDNA E20 was altered using oligomers to introduce a *NdeI* site at the putative translation initiation codon located at +1801 of the cDNA sequence. A *NdeI*–*EcoRI* fragment from this altered cDNA was inserted into the plasmid pET-3a and the resulting construction was used to transform the bacterial strain BL21(DE3), which contains a single copy of the T7 polymerase gene under the control of the IPTG inducible *lac UV5* promoter. Induction results in expression of a 42 kDa ABD-A protein.

Early-log phase cultures were induced with 0.4 mM IPTG and were treated, after 1 h following induction, with 400 μ g/ml rifampicin A. Cells were harvested after 4 h and stored at -80°C . Analysis of soluble and insoluble fractions by Western blotting revealed no ABD-A protein in soluble form. Therefore, the following protocol was developed in order to recover ABD-A protein from the insoluble fraction. Cells from 1 l of induced culture were thawed with 10 ml lysis buffer (50 mM Tris–HCl pH 7.9, 200 mM NaCl, 2 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 1 μ M pepstatin A and 0.1 mM leupeptin), followed by addition of solid lysozyme to 0.4 mg/ml. After 45 min incubation on ice, the solution was passed through five freeze–thaw cycles using dry-ice/ethanol and a water bath at 37°C . Nucleic acids were digested by addition of MgCl_2 to 10 mM, DNase I to 10 μ g/ml and RNase A to 10 μ g/ml with incubation on ice for 30 min. Insoluble material was recovered by centrifugation at 10 000 g for 15 min. The insoluble pellet was resuspended using 2 M GuHCl wash buffer (25 mM HEPES pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT and 2 M guanadine hydrochloride) and a Dounce homogenizer to remove trapped soluble proteins. The insoluble portion was again recovered with centrifugation at 10 000 g. Solubilization of the protein was achieved by addition of 6 M GuHCl buffer (wash buffer with GuHCl to 6 M) with stirring on ice and/or Dounce homogenization. The resulting solution was fractionated by applying 200 μ l samples to a Superose 6 column (FPLC, Pharmacia) that had been equilibrated with 6 M GuHCl buffer. Fractions containing ABD-A protein were identified using a monoclonal anti-ABD-A antibody. ABD-A containing fractions from repeated column runs were pooled and dialyzed for 2 h at 4°C against renaturation buffer (10 mM HEPES pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, 0.5 mM PMSF, 1 μ M pepstatin A, 0.1 mM leupeptin) containing 2 M GuHCl. The dialyzed solution was adjusted to 0.1% Triton X-100, then added dropwise to renaturation buffer containing 0.1% Triton X-100 to achieve a dilution of 50-fold. The protein was concentrated using a stirred cell (Filtron) and Centriprep-30 concentrators (Amicon). The protein was dialyzed against renaturation buffer, the glycerol concentration was adjusted to 50% and aliquots were stored at -80°C .

For preparation of UBX protein, a system expressing the UBX Ib isoform as described by Beachy *et al.* (1988) was used. The initial steps of the purification were essentially as described in that paper. However, following a 50% ammonium sulfate precipitation, the protein was resuspended in oligo buffer (50 mM Tris–HCl pH 7.4, 1 mM Na_2EDTA , 1 mM DTT, 5% glycerol) with 100 mM NaCl and applied directly to a DNA affinity column consisting of the sequence (TAA) $_{12}$. UBX protein was eluted by developing the column with a linear gradient (0.1–1.0 M NaCl) in oligo buffer. Fractions containing UBX protein were identified using the FP3.38 monoclonal antibody (White and Wilcox, 1984) and Western blotting and stored at -80°C .

DNase I protection assays

Binding conditions for UBX protein were as described by Beachy *et al.* (1988). For ABD-A protein, a standard reaction in 50 μ l consisted of 10 mM HEPES pH 7.5, 100 mM NaCl, 10 mM MgCl_2 , 1 mM DTT, 20 μ g/ml poly d(I–C), ~ 30 fmol ^{32}P -labeled DNA fragment and ABD-A as indicated in Figure 3 legend. After a 20 min incubation at 24°C , 50 μ l of diluent (10 mM CaCl_2 and 20 mM MgCl_2) were added followed by 2 ng DNase I (Pharmacia). After 1 min, reactions were terminated by addition of 0.3

ml stop buffer (300 mM NaCl, 20 mM Na₂EDTA, 50 mM Tris-HCl pH 8.0, 1% SDS and 33 µg/ml *E.coli* tRNA). After extraction with phenol-chloroform (1:1), nucleic acids were recovered by precipitation, electrophoresed in 6% polyacrylamide gels containing 8 M urea and detected by autoradiography. Sequencing reactions were as described by Maxam and Gilbert (1980).

Site-directed mutagenesis

A two-step PCR amplification process was used to introduce base substitution mutations within homeodomain binding sites. First, in two separate reactions, sense and anti-sense primers covering the binding site and having complimentary one or two nucleotide changes from the wild-type sequence were used, along with appropriate flanking primers, to synthesize fragments extending in both directions from the binding site. By using restriction sites, the 2.3 kb P2 fragment was treated as four separate cassettes ranging from ~400–900 bp in length. 50 µl PCR reactions contained 1 µM concentration of each primer, 0.2 mM concentration of each deoxynucleotide triphosphate, 20 mM Tris-HCl pH 8.8, 10 mM KCl, 5 mM MgSO₄, 100 µg/ml BSA, 0.1% Triton X-100, 1.5 U of Vent DNA polymerase (New England Biolabs) and template DNA. 25 cycles of PCR were used to achieve amplification. Products from the first set of reactions were purified on agarose gels, combined and used with the original flanking primers to synthesize a full-length fragment. These products were also gel-purified and used either as templates for mutagenesis of additional binding sites or were cloned, using restriction sites into the 2.3P2 promoter. The entire pt26 and pt39 promoters were sequenced as were portions of pt16a, pt16b, pt16c and pt21 to confirm the homeodomain binding site mutations and to identify errors resulting from the multiple rounds of PCR. Two errors that did arise were restored to the original sequence.

Immunocytochemical staining of embryos

Embryos from each transformant line were processed and stained with antibodies as described previously by Boulet and Scott (1988). Primary antibodies used were polyclonal anti-β-galactosidase (Cappel) at 1:1000, monoclonal anti-β-galactosidase (Promega) at 1:800, anti-ANTP (Mab 8C11, provided by D.Brower) at 1:500, anti-HRP (Jackson Immunoresearch) at 1:500 and Mab 2A12 (provided by N.Patel) at 1:10. Secondary antibodies used were fluorescein-conjugated goat anti-mouse IgG (Jackson Immunoresearch) at 1:500, biotinylated horse anti-mouse IgG (Vector) at 1:2000 and biotinylated goat anti-rabbit IgG (Vector), also at 1:2000. A Texas Red-Avidin conjugate (Vector) was used in combination with biotinylated secondary antibodies. After dehydration in ethanol, embryos were mounted in a 1:2 solution of benzyl alcohol and benzyl benzoate. For microscopy, a Zeiss Axioplan equipped with a Bio-Rad MRC600 Lasersharp Confocal system was used.

Acknowledgements

We wish to thank A.Boulet, C.Thummel and D.Stillman for their helpful criticisms of the text and members of the Sakonju laboratory for discussions. We are also indebted to J.Hooper and M.Scott for providing the P2 promoter DNA sequence prior to publication, P.Beachy and J.Varkey for communicating unpublished results, B.Johnson and M.Krasnow for the UBX lb expression plasmid and protein purification protocols, D.Brower for the gift of Mab 8C11 and N.Patel for Mab 2A12. This work was supported by a predoctoral training grant to B.A. from the NIH Cancer Training Grant CA09602 and by the Howard Hughes Medical Institute.

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Received on September 11, 1992; revised on December 8, 1992