

Functional domains in the Deformed protein

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SUMMARY

A chimeric protein consisting of Deformed with a substituted Abdominal-B homeodomain (Dfd/Abd-B) is used to identify protein domains outside the homeodomain that are required for regulatory activity in vivo. A series of deletion proteins were generated based on regions showing amino acid composition similar to known regulatory domains. Each mutant protein can influence regulation of homeotic genes in a manner distinct from the intact protein. Activity was also tested using promoter elements from *empty spiracles* and *Distal-less*, two genes known to be directly regulated by *Abdominal-B*. Removal of the acidic region and the C-tail region convert the chimera from a strong

activator to a repressor of the *Distal-less* element, but had comparatively little effect on the activation of the *empty spiracles* element. Constructs without a third domain, the N domain, fail to show any regulatory activity. The N domain is the only domain of the Dfd/Abd-B protein which exhibits significant activation activity when fused to a heterologous DNA binding domain. Our results suggest transcriptional activity of the N domain can be modulated by the acidic and C-tail domains.

Key words: Abdominal-B, homeodomain, activation domain, *Drosophila*, Deformed

INTRODUCTION

One of the earliest events in *Drosophila* embryogenesis is the establishment of a metamer pattern of positional information along the anterior-posterior axis of the embryo. In response to this information, homeotic selector genes are activated in specific regions of the embryo and specify segmental identity (Lawrence, 1992). The homeotic genes are related to each other and to numerous other genes that control various developmental pathways in *Drosophila* through a conserved DNA sequence called the homeobox (McGinnis et al., 1984; Scott and Weiner, 1984). Homeobox-containing genes have been identified in other species and some exhibit a significant degree of homology to fly genes (reviewed by McGinnis and Krumlauf, 1992). Surprisingly, some vertebrate homeobox genes are capable of specifying the same developmental pathways in *Drosophila* embryos as their fly homologs (Malicki et al., 1990; McGinnis et al., 1990; Zhao et al., 1993; Bachiller et al., 1994; Halder et al., 1995). This observation suggests that the underlying mechanisms by which homeobox genes function have been conserved throughout evolution. Understanding these mechanisms would greatly enhance our comprehension of development at a molecular level in *Drosophila* and other species.

Various experimental approaches have demonstrated that homeodomain proteins act as transcription factors (Han et al., 1989; Krasnow et al., 1989; Samson et al., 1989; Winslow et al., 1989; Johnson and Krasnow, 1990). The protein domain encoded by the homeobox, referred to as the homeodomain, is a member of the helix-turn-helix class of sequence-specific DNA binding domains (Otting et al., 1988; Kissinger et al., 1990). Homeodomain swap experiments have shown that the

target specificity of a homeotic protein is determined by amino acids within and immediately surrounding the DNA binding domain (Kuziora and McGinnis, 1989; Gibson et al., 1990; Lin and McGinnis, 1992; Chan and Mann, 1993; Zeng et al., 1993). Some chimeric proteins exhibit regulatory activities distinct from the native proteins suggesting that protein domains outside the homeodomain can control transcriptional activity (Kuziora and McGinnis, 1990; John et al., 1995).

To identify functional domains in the Deformed protein, we have used a chimera that is composed of Deformed with a substituted Abdominal-B homeodomain. The chimera provides a useful tool for the analysis of functional domains since more segments are transformed than with the native Deformed protein, the types of transformations are diverse and easy to score and the regulation of several known Abdominal-B target genes can be tested. *Deformed* (*Dfd*) is normally required for the specification of the maxillary and mandibular segments (Chadwick and McGinnis, 1987; Merrill et al., 1987; Regulski et al., 1987). Ectopic expression of the Dfd protein in embryos leads to homeotic transformations of thoracic segments toward a maxillary identity without affecting the expression of other homeotic genes (Kuziora and McGinnis, 1988). *Abdominal-B* (*Abd-B*) is normally required for the specification of the 5th-10th abdominal segments (A5-A10). Ectopic expression of Abd-B proteins transform anterior segments toward an A8 identity and repress the expression of other homeotic genes (Lamka et al., 1992; Kuziora, 1993). The chimeric Dfd/Abd-B protein also transforms segments toward an A8 identity, but the expression of other homeotic genes is ectopically activated (Kuziora and McGinnis, 1990). Thus the chimera retains Abd-B target specificity, but exhibits regulatory activity distinct from the native Abd-B proteins.

What are the regions within the Dfd/Abd-B chimera that determine regulatory activity? To answer this question, we made several deletions within the Dfd/Abd-B gene and generated transgenic flies carrying each construct. The regulatory activity of each deletion mutant was assayed in embryos by examining alterations in the expression patterns of homeotic genes and reporter genes driven by promoter elements from *empty spiracles* and *Distal-less*, two genes known to be directly regulated by Abd-B. We have identified a region of the chimera which is essential for regulatory activity. This region functions as a strong activation domain in tissue culture cells when fused to a heterologous DNA binding domain, but is also present in a deletion mutant that represses the expression of *Distal-less* in embryos. Our results suggest other regions of the protein may modulate the ability of this domain to affect transcriptional activity. The data described here allow us to begin to assign functions to regions of a homeodomain protein that are critical for regulatory activity.

MATERIALS AND METHODS

Fly stocks

Drosophila were raised at 25°C on standard food. Microinjection of embryos for P element transformation was performed following standard procedures (Spradling and Rubin, 1982). Homozygous lethal insertions were balanced with *CyO* or *TM3, Sb* (Lindsley and Zimm, 1992). At least three lines were examined for each construct.

Injection constructs

Deletions were constructed as described by Eghtedarzadeh and Henikoff, (1986) or by amplifying desired regions by PCR and reassembling the construct with convenient restriction sites. All PCR products were confirmed by sequencing. Details of the construction of each mutant is available upon request.

Expression and purification of recombinant proteins

DNA fragments encoding each protein were amplified using PCR and cloned into pGEX2T (Smith and Johnson, 1988). Expression and purification of the GST-fusion proteins were performed as described by Ausubel et al. (1994).

Gel mobility shift assay

The gel mobility shift assay (Ausubel et al., 1994) used annealed synthetic oligonucleotides encoding a single Abd-B binding site (GATCATTATGCGCC and GATCGGCCATAAAAT). The binding reaction contained 20 mM Hepes (pH7.8), 10% glycerol, 1 mM DTT, 0.2 mM EDTA (pH8.0), 1 mg/ml BSA, 2 µg poly dI-dC, 180 mM KCl, 0.05% NP-40, 4×10⁴ cpm probe and various amounts of GST-fusion protein as indicated in Fig. 3. The binding reaction was performed at room temperature for 30 minutes and then fractionated using 4% polyacrylamide native gel electrophoresis.

Antibody staining

Embryos for the experiments shown in Fig. 2 were collected over 5 hours and heat shocked at 37°C for 1 hour, allowed to recover at 25°C for 4 hours, then fixed and stained as described by Patel et al. (1989). Embryos for the experiments shown in Fig. 9 were collected for 5 hours, heat shocked at 37°C for 1 hour, allowed to recover at 25°C for 5 hours, and then fixed for antibody staining.

Embryos for the experiments shown in Fig. 7 were heat shocked twice at 37°C for 30 minutes separated by a 1 hour recovery at 25°C. Embryos were fixed 1 hour after the second heat-shock. Monoclonal anti-β-galactosidase antibody (Promega) was used at 1:600 dilution.

β-galactosidase staining of embryos

Embryos to be stained for β-galactosidase activity (Fig. 6) were heat shocked at 37°C twice, beginning at the cellular blastoderm stage. Each heat-shock was administered for 30 minutes separated by a 1 hour recovery at 25°C. β-galactosidase activity was detected as described by Bellen et al. (1989).

Cuticle preparations

Embryos at the cellular blastoderm stage were given two 30-minute heat shocks at 37°C separated by a 1 hour recovery at 25°C and allowed to develop at 25°C for 36 hours. Cleared cuticles were prepared as described by Van der Meer, (1977).

RNA in situ hybridization

Beginning at the cellular blastoderm stage, embryos were heat shocked twice at 37°C for 30 minutes separated by a 1 hour recovery at 25°C. After recovery at 25°C for 3 hours, the embryos were fixed for RNA in situ hybridization (Jiang et al., 1991).

Cell culture, transient transfection and CAT assay

Sequences encoding each domain indicated in Fig. 8 were amplified using the PCR and cloned into a pRSV-LTR expression vector (Yang and Evans, 1992). QT6 cells were maintained in Dulbecco modified Eagle medium containing 8% fetal bovine serum, 2% chicken serum and antibiotics. Transient transfections using Lipofectin reagent (BRL) were performed as described by Yang and Evans (1992). A standard assay contains 20 ng of effector plasmid, 2 µg of a CAT reporter plasmid (Evans and Felsenfeld, 1991), 1 µg of pCH110, an internal control β-galactosidase expression vector (Hall et al., 1983).

Transfected cells were harvested 45-48 hours later and total cellular extract was prepared for CAT assays (Ausubel et al., 1994). A fraction of the extract was used to determine the β-galactosidase activity (Sambrook et al., 1989). The results were quantified using a Fuji phosphor-imaging system and normalized against the corresponding β-galactosidase activity.

RESULTS

The Dfd/Abd-B chimera can be divided into distinct regions that are enriched in certain amino acids. A region referred to as the N domain spans amino acids 38-259 and is characterized by a histidine-rich region flanked on both sides by glycine-rich sequences (Fig. 8C). Adjacent to this region is the acidic region spanning amino acids 260-340 containing a predominance of acidic amino acids. The IYPWM/homeodomain region, spanning amino acids 341-436, contains both the IYPWM motif (amino acids 343-347), a conserved sequence found in many but not all homeotic proteins, and the Abd-B homeodomain (amino acids 365-430). Finally, the C-tail domain, which spans amino acids 437-586, includes a poly-glutamine tract and a poly-asparagine tract. To ascertain the contribution of each domain to regulatory activity, we constructed five deletion mutants encoding different combinations of these regions (Fig. 1A). The constructs were cloned downstream of the hsp70 promoter and used to generate transgenic fly stocks by P element-mediated transformation (Spradling and Rubin, 1982).

Protein expression and DNA binding

Since nuclear localization signals in the Dfd or Abd-B proteins have not been identified, the subcellular localization of each mutant protein was confirmed by immunohistochemical staining with polyclonal anti-Dfd antibodies (kindly supplied

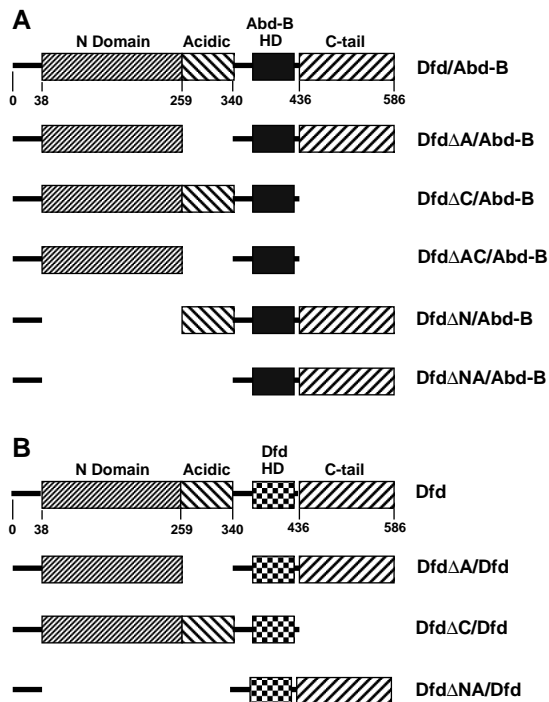


Fig. 1. The Dfd/Abd-B and Dfd deletion mutants. (A) Deletion proteins derived from the Dfd/Abd-B chimeric protein. (B) Deletion proteins derived from the intact Dfd protein. Each protein domain is labeled and indicated by different shading. Drawings are to scale and the numbering corresponds to the amino acids that define domain boundaries.

by Dr. William McGinnis). As shown in Fig. 2, each protein was detectable 4 hours after heat shock treatment and was properly localized to the nucleus. The Antennapedia homeodomain is sufficient to direct protein import into the nucleus (Gibson and Gehring, 1988), and consistent with this observation the IYPWM/homeodomain region contains sequences that resemble common nuclear localization signals (Powers and Forbes, 1994).

We next determined if deleting large regions of the protein adversely affected *in vitro* DNA-binding activity. The intact Dfd/Abd-B chimera and each deletion protein were expressed in *E. coli* as glutathione S-transferase (GST) fusion proteins (Smith and Johnson, 1988). Purified fusion proteins were assayed for their ability to retard the mobility of a double stranded oligonucleotide encoding an Abd-B homeodomain binding site (Ekker et al., 1991). The binding affinity of each mutant protein was comparable to the full length chimeric protein (Fig. 3). This result is consistent with studies demonstrating that the *in vitro* DNA binding activity of homeotic proteins

is dependent on an intact homeodomain (Hall and Johnson, 1987; Desplan et al., 1988; Ko et al., 1988; Hoey and Levine, 1988) and suggests that deletions outside the homeodomain do not introduce structural defects that interfere with DNA binding *in vitro*.

Regulation of homeotic gene expression

Following misexpression of the intact chimera many homeotic genes are expressed at high levels in ectopic positions (Kuziora and McGinnis, 1990; compare Fig. 4A-E with 4F-J). Although it is not known if this is an example of direct regulation by the chimeric protein, we used this observation as a convenient *in vivo* assay to gauge the regulatory activities of the deletion proteins.

Similar to the intact protein, misexpression of the DfdΔA/Abd-B protein did not affect expression of *Sex combs reduced* (*Scr*). Ectopic expression of *Antennapedia* (*Antp*), *Ultrabithorax* (*Ubx*), *abdominal -A* (*abd-A*) and *Abd-B* was also detected, although fewer segments were affected (Fig. 4K-O). Following misexpression of DfdΔC/Abd-B, expression patterns of homeotic genes were altered in a more complex manner. Levels of *Scr* transcripts were reduced (Fig. 4P) while expression of *Antp* was ectopically detected in anterior segments (Fig. 4Q). Levels of *Ubx* and *abd-A* transcripts were noticeably reduced within their normal domains of expression, but weak ectopic expression was also detected in anterior segments (Fig. 4R,S). *Abd-B* expression was slightly repressed, but no ectopic expression was detectable (Fig. 4T). The DfdΔAC/Abd-B protein exhibited regulatory activity that was strikingly different from the intact protein. Transcript levels of *Scr*, *Antp*, *Ubx*, and *abd-A* were all greatly reduced while levels of *Abd-B* were only slightly reduced (Fig. 4U-Y). Although the control of homeotic gene expression may be indirect, these results indicate that the protein domains under investigation in this study contribute to the regulatory activity of the chimeric protein.

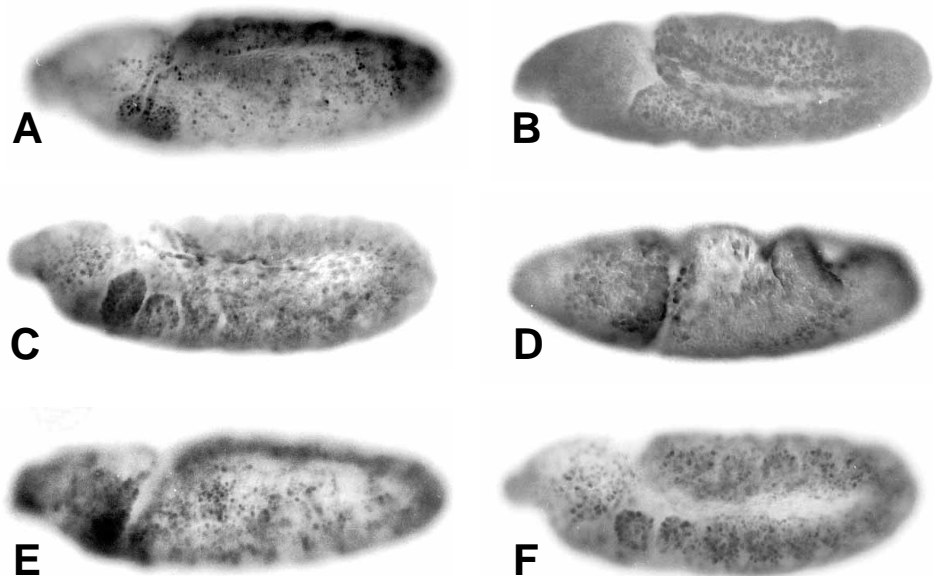


Fig. 2. Mutant proteins are localized to the nucleus. Four hours after heat shock, embryos containing each deletion construct were fixed and stained with a polyclonal anti-Dfd antibody. (A) Dfd/Abd-B, (B) DfdΔA/Abd-B, (C) DfdΔC/Abd-B, (D) DfdΔAC/Abd-B, (E) DfdΔN/Abd-B and (F) DfdΔNA/Abd-B.

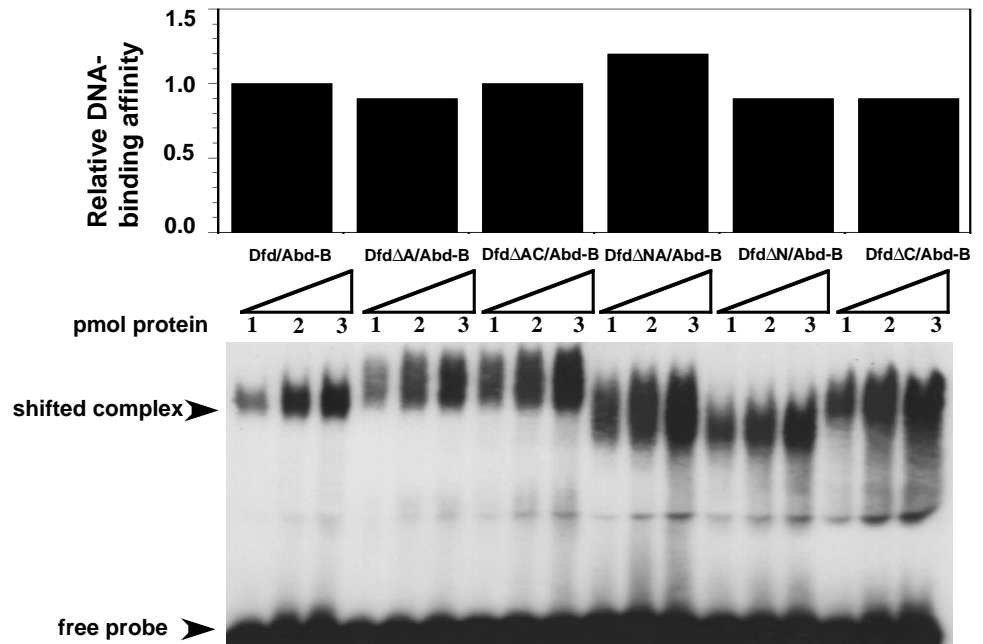


Fig. 3. Relative DNA binding affinity of the deletion proteins. Deletion proteins were expressed as GST-fusion proteins in *E. coli*. Purified fusion proteins were assayed in a gel shift experiment using a high affinity *Abd-B* binding site. The binding affinity of the intact chimera was determined at three concentrations of protein, the values were averaged and normalized to a value of 1. The affinity of each deletion protein is the average of three experiments plotted relative to the affinity of the intact chimera.

Altered homeotic expression patterns lead to ventral cuticular transformations

We next examined if altered homeotic expression patterns could be correlated with cuticular transformations in larvae. In wild-type larvae, the ventral region of the second and third thoracic segments (T2-T3) contain denticle belts composed of

fine hairs (Lohs-Schardin et al., 1979; Fig. 5A). The first thoracic segment (T1) contains two denticle belts, the main belt is a band of thick, hooked hairs while the 'beard' is a small patch of fine hairs centered within the ventral region of T1. Sensory organs such as ventral pits and Keilin's organs are also found in thoracic segments. Denticle belts in abdominal

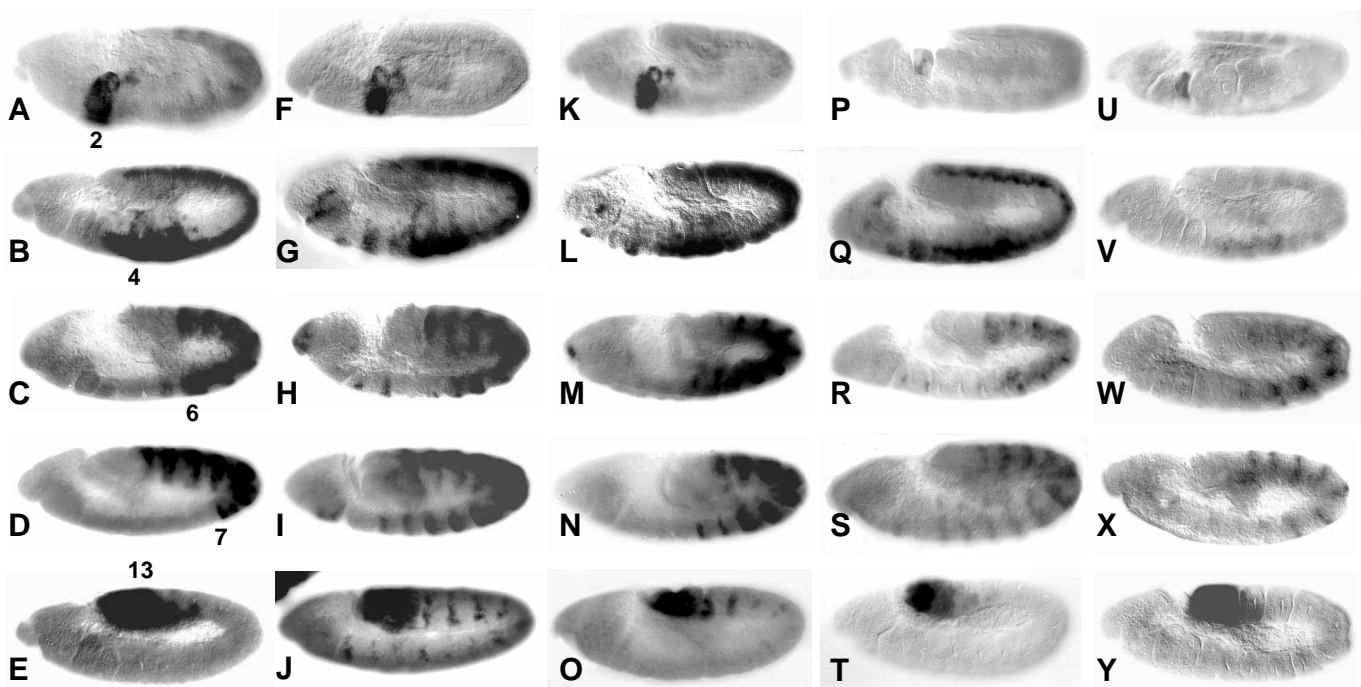


Fig. 4. Alteration in the expression patterns of homeotic genes. Embryos carrying the indicated constructs were heat shocked and allowed to recover for 3 hours, fixed and used for RNA in situ hybridization. The strains used are: *Dfd(1)yw* (A-E), embryos carrying the intact *Dfd/Abd-B* protein (F-J), the *DfdΔA/Abd-B* construct (K-O), the *DfdΔC/Abd-B* construct (P-T) and the *DfdΔAC/Abd-B* construct (U-Y). The probes used are *Scr* (A,F,K,P,U), *Antp* (B,G,L,Q,V), *Ubx* (C,H,M,R,W), *abd-A* (D,I,N,S,X) and *Abd-B* (E,J,O,T,Y). The numbers in A-E refer to the most anterior parasegment showing the highest level of expression in normal embryos.

segments are composed of short hairs that are thick at the base and hooked at the end.

Ectopic expression of the intact *Dfd*/*Abd-B* chimera transforms thoracic segments toward abdominal segmental identity, as determined by the abdominal type denticles in the T2-T3 belts (Fig. 5B). The head fails to involute and mixed patches of thoracic and abdominal denticles appear in cephalic segments occasionally forming complete belts. The ectopic expression of *Antp*, *Ubx*, *abd-A* and *Abd-B* described above are consistent with these posterior transformations. Ectopic expression of the *Dfd* Δ /*Abd-B* protein produced a weaker thoracic to abdominal transformation, but 70% of cuticles ($n=79$) exhibited a duplicated T1-like beard in labial segments (Fig. 5C). The duplicated T1 was also observed following misexpression of the intact chimera, but at a much lower frequency (17%, $n=57$).

Transformations generated by *Dfd* Δ C/*Abd-B* or *Dfd* Δ AC/*Abd-B* were similar (Fig. 5D,E). In each case, the T2 and T3 denticle belts were unaffected but the T1 beard was reduced in size and often missing. Thoracic-like denticles appeared in head segments, but a T1-like beard was not observed. *Dfd* Δ C/*Abd-B* and *Dfd* Δ AC/*Abd-B* also produced a ventral transformation of abdominal segments towards thoracic identity as judged by the appearance of ventral pits in abdominal segments (Fig. 5G,H). An average of 3.7 abdominal segments contained at least one ventral pit ($n=30$) following misexpression of the *Dfd* Δ AC/*Abd-B* protein, while an average of 1.2 ($n=30$) abdominal segments were transformed in a similar manner by the *Dfd* Δ C/*Abd-B* protein. Additionally, misexpression of *Dfd* Δ AC/*Abd-B* leads to a reduction in size of individual abdominal denticle hairs (compare Fig. 5F with 5H). The reduced levels of *Ubx* and *abd-A* transcripts can account for these abdominal to thoracic transformations (Hayes et al., 1984; Lamka et al., 1992; Kuziora, 1993). Lower levels of *Scr*

expression and the ectopic activation of *Antp* are consistent with suppression of T1 and the transformation of head segments toward thoracic identity (Gibson and Gehring, 1988; Zhao et al., 1993).

Ectopic expression of either the *Dfd* Δ N/*Abd-B* or *Dfd* Δ NA/*Abd-B* proteins was not lethal to embryos, and larval cuticles appeared normal in all aspects (data not shown). Additionally, multiple heat-shock treatments given to embryos carrying either construct alone or in combination with a deficiency for the bithorax complex (BX-C) which deletes *Ubx*, *abd-A* and *Abd-B*, failed to generate any consistent visible

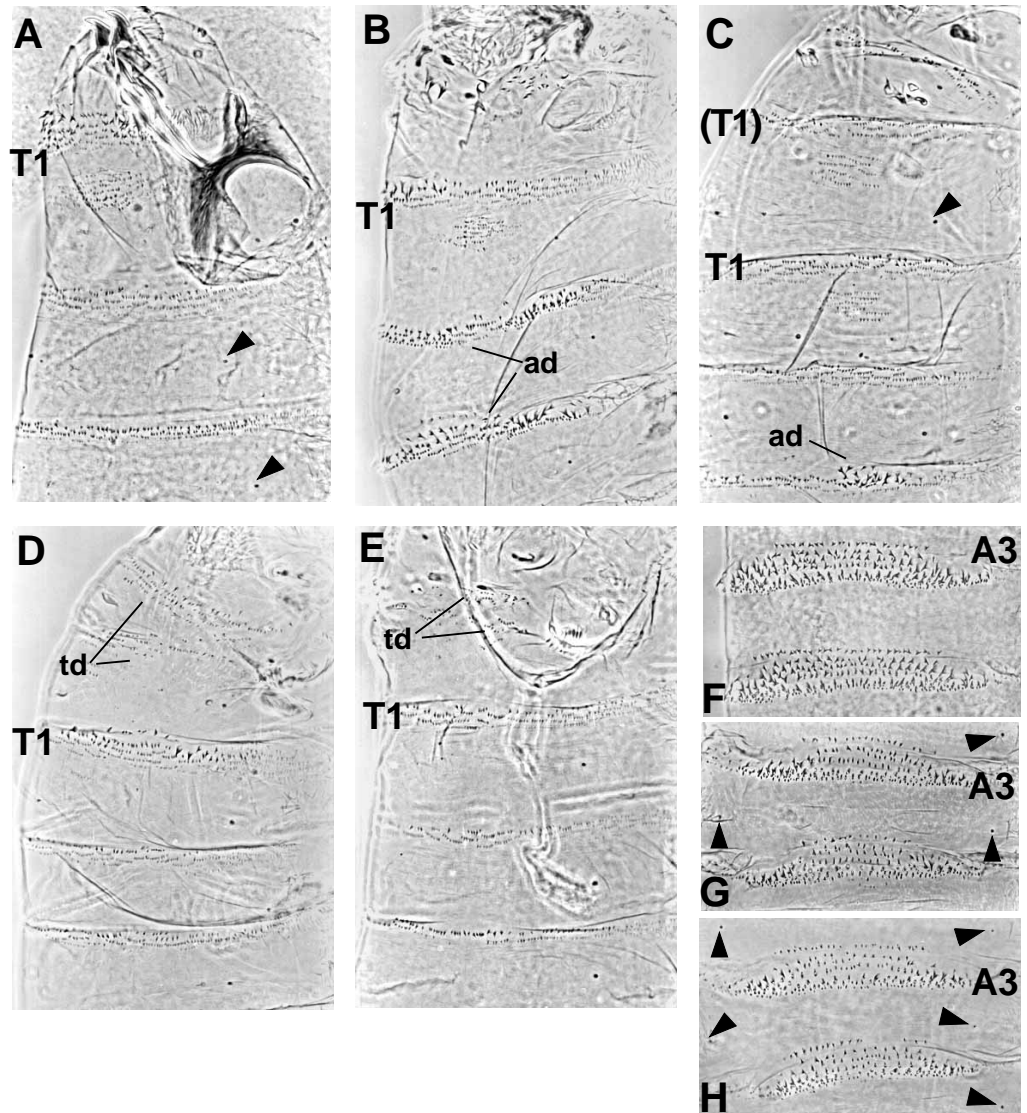


Fig. 5. Ventral cuticular transformations. Embryos were heat shocked at the cellular blastoderm stage and allowed to develop into first instar larvae. Cuticles were cleared and viewed with phase contrast optics. Cuticles are oriented with the anterior up. (A) A control *ry*⁵⁰⁶ larva. Ventral pits are indicated in thoracic segments by arrowheads. (B) A cuticle from strain A48 carrying the *Dfd*/*Abd-B* construct. (C) A cuticle from strain Z5 carrying the *Dfd* Δ /*Abd-B* construct (arrowhead, ventral pit). (D) A cuticle from strain C16 carrying the *Dfd* Δ C/*Abd-B* construct. (E) A cuticle from strain pCAS-9 carrying the *Dfd* Δ AC/*Abd-B* construct. (F) Close up of the abdominal region of a control *ry*⁵⁰⁶ cuticle. (G) The abdominal region from strain C16 carrying the *Dfd* Δ C/*Abd-B* construct, note the ectopic ventral pits indicated by arrowheads. (H) The abdominal region from strain pCAS-9 carrying the *Dfd* Δ AC/*Abd-B* construct. Ectopic ventral pits are indicated by arrowheads. T1, the first thoracic segment; ad, ectopic abdominal-type denticles; (T1), ectopic first thoracic segment; td, duplicated thoracic denticles; A3, the third abdominal segment.

transformation, suggesting these proteins are non-functional *in vivo*. Together with the RNA *in situ* hybridization data described above, these results are consistent with the N domain being required for activity while the acidic and C-tail regions play a role in modulating regulatory activity.

Dorsal-lateral cuticular transformations and regulation of the *empty spiracles* target gene

Ectopic expression of the Dfd/Abd-B chimera produces a transformation of the dorsal-lateral epidermis in abdominal and thoracic segments toward an A8 identity as determined by the appearance of ectopic Filzkörper and perispiracular hairs (Kuziora and McGinnis, 1990; Fig. 6B), cuticular structures which normally develop only in A8 (Jürgens, 1987; Fig. 6A). Other deletion proteins that contain the N domain also produced ectopic Filzkörper and perispiracular hairs, however the response was somewhat weaker (Fig. 6C). For example, Filzkörper appeared in segments anterior to A4 in 55% of the embryos with the intact chimera ($n=57$), but in only 16% of Dfd Δ A/Abd-B larvae ($n=79$), 20% of Dfd Δ C/Abd-B larvae ($n=30$) and 27% of Dfd Δ AC/Abd-B larvae

($n=30$). Constructs lacking the N domain failed to produce any ectopic Filzkörper.

The development of Filzkörper requires the *empty spiracles* (*ems*) gene, which is directly regulated by Abd-B proteins through a *cis*-regulatory element known as the *ems*-ARFE (Jones and McGinnis, 1993). In transgenic flies, the *ems*-ARFE directs expression of a *lacZ* reporter gene in a pattern identical to *ems* in two dorsal-lateral clusters of cells in A8 (Jones and McGinnis, 1993; Fig. 6D). Misexpression of Abd-B proteins or the Dfd/Abd-B chimera activates ectopic expression of the *ems*-ARFE in anterior segments (Jones and McGinnis, 1993; Fig. 6E). We used the *ems*-ARFE to test the ability of the deletion proteins to directly regulate a known Abd-B target gene. Following misexpression of the Dfd Δ A/Abd-B protein, ectopic β -galactosidase activity was detected in embryos (Fig. 6F). Identical results were obtained with the Dfd Δ C/Abd-B and Dfd Δ AC/Abd-B proteins (data not shown). In contrast, no ectopic expression of *lacZ* was detected following misexpression of Dfd Δ N/Abd-B or Dfd Δ NA/Abd-B (data not shown). These results suggest that while the acidic and C-tail regions are not essential, the N

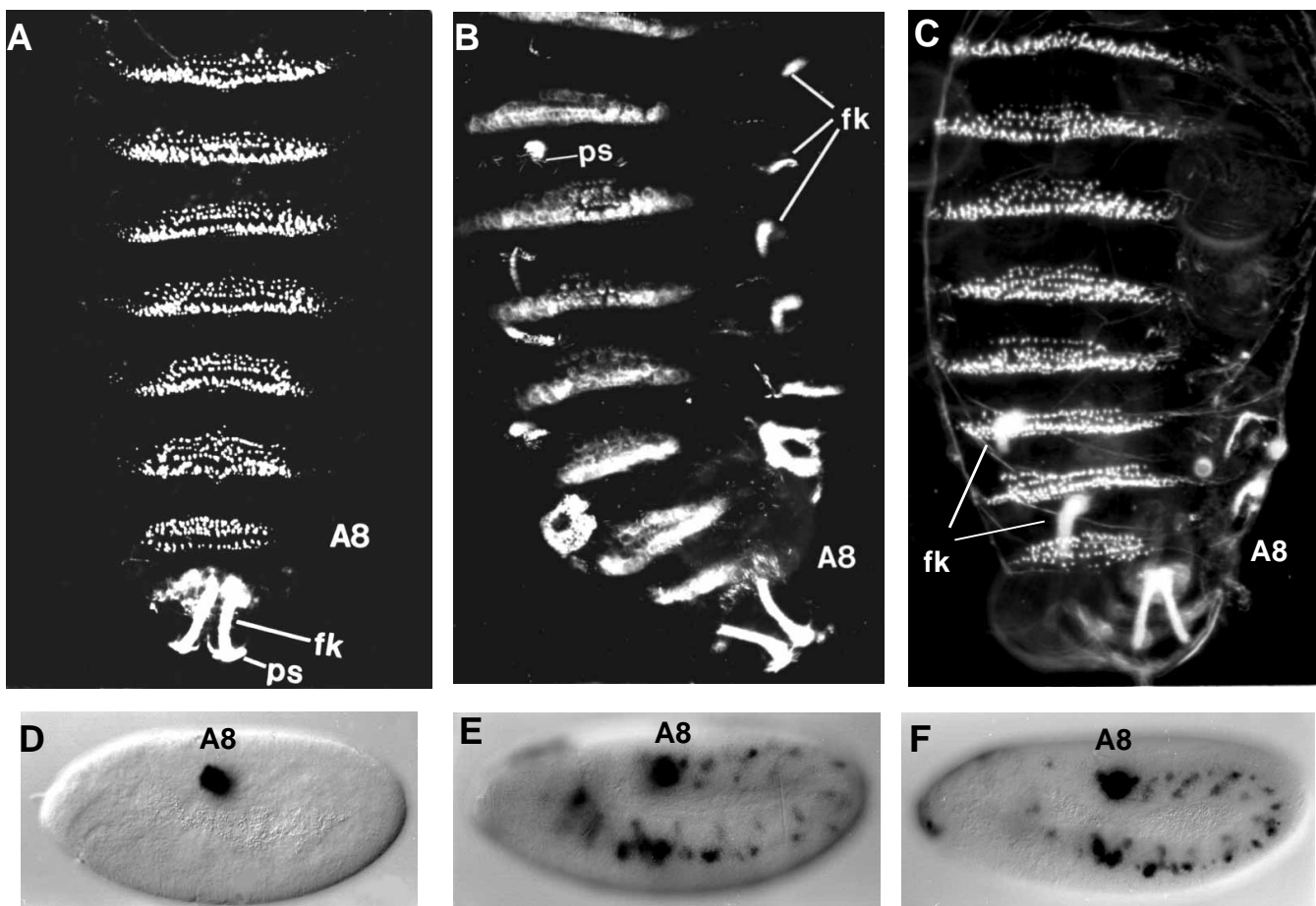


Fig. 6. Posterior cuticular transformations and activation of the *ems*-ARFE. For the cuticles in A-C, embryos were heat shocked at the cellular blastoderm stage and allowed to develop into first instar larvae. Cuticles were cleared and viewed with dark-field microscopy. Posterior is down and the plane of focus is dorsal. (A) A control larva from the *ry*⁵⁰⁶ strain. (B) A larva carrying the Dfd/Abd-B intact chimera. (C) A larva carrying the Dfd Δ A/Abd-B protein. fk, Filzkörper; ps, perispiracular hairs; A8, eighth abdominal segment. The embryos in D-F, each carry the *ems*-ARFE and the deletion construct indicated below. Embryos were heat shocked, allowed to recover for 1 hour then fixed and stained for β -galactosidase activity. Embryos are oriented so that anterior is to the left and dorsal is up. (D) An embryo carrying only the *ems*-ARFE; (E) the Dfd/Abd-B intact chimera; (F) the Dfd Δ A/Abd-B construct.

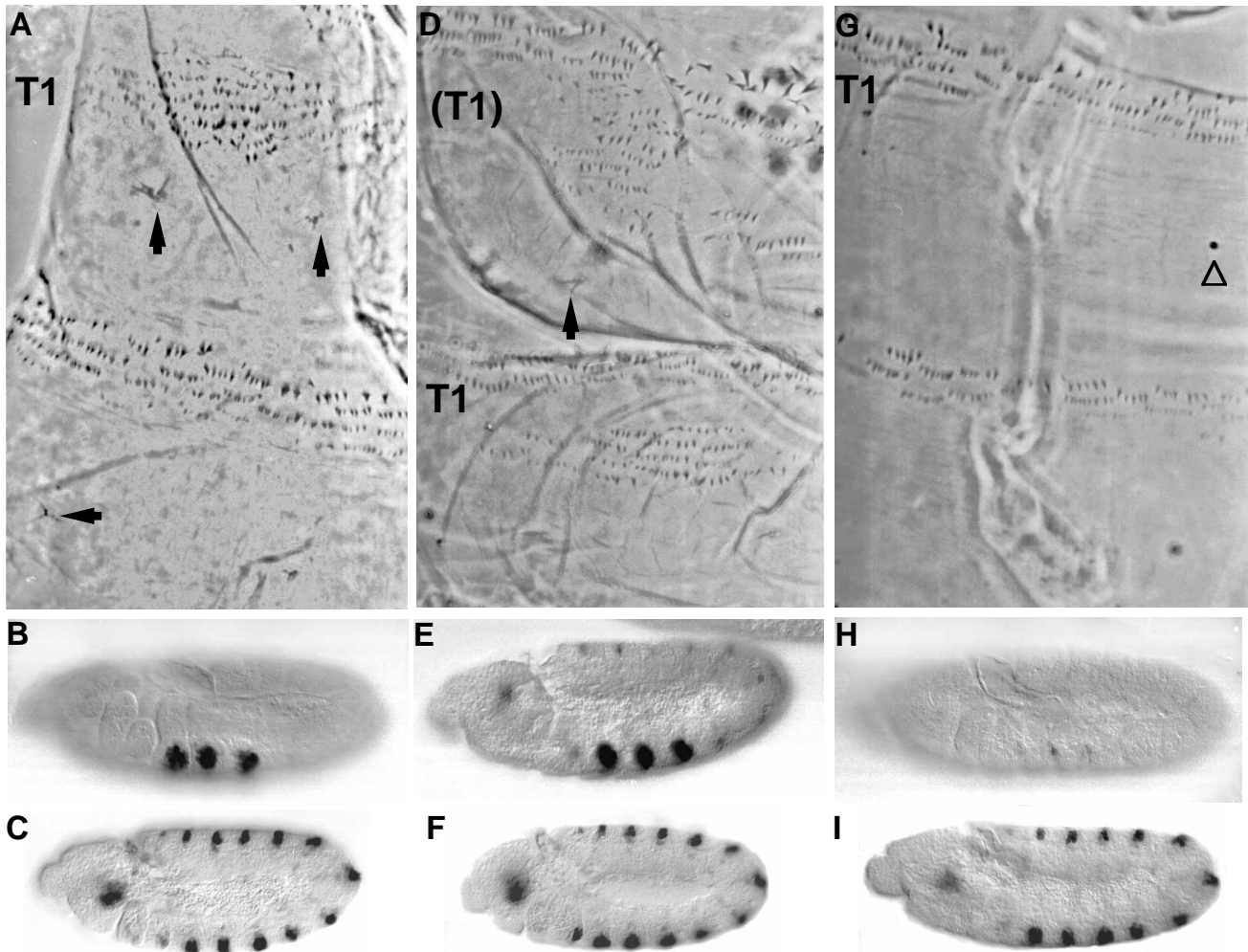


Fig. 7. Regulation of the *Dll NRE-BX*. Cuticles were prepared from embryos heat shocked at the cellular blastoderm stage and allowed to develop into first instar larvae. Embryos were heat shocked and fixed 1 hour later. Expression of *lacZ* was detected immunologically. (A) View of the second and third thoracic segments of a wild-type larva. Filled arrow indicate Keilin's organs. (B) An embryo carrying the *Dll NRE-BX 304*. Note that *lacZ* is expressed in thoracic segments but is repressed in abdominal segments. (C) An embryo carrying the *Dll NRE-BX 305* showing derepression of *lacZ* expression in abdominal segments. (D) The second and third thoracic segments of a larva carrying the *DfdΔA/Abd-B* construct showing ectopic Keilin's organs (filled arrow) in the (T1) duplicated first thoracic segment. (E) An embryo carrying *Dll NRE-BX 304* and the *DfdΔA/Abd-B* construct. Note the ectopic activation of *lacZ* expression in abdominal and cephalic segments. (F) Embryo carrying the *Dll NRE-BX 305* and the intact *Dfd/Abd-B* construct. (G) View of the first and second thoracic segment of a *DfdΔAC/Abd-B* larva. Note that Keilin's organs fail to develop, while ventral pits are unaffected (open arrowhead). (H) An embryo carrying *Dll NRE-BX 304* and the *DfdΔAC/Abd-B* construct. Note that *lacZ* expression is repressed. (I) Embryo carrying the *Dll NRE-BX 305* and the *DfdΔAC/Abd-B* construct.

domain is required for the activation of *ems* and the development of Filzkörper.

Direct regulation of a *Distal-less* regulatory element

The observation that homeotic gene expression is repressed following misexpression of the *DfdΔAC/Abd-B* protein is in sharp contrast to its ability to activate the *ems*-ARFE. Since it is unknown if homeotic gene expression is directly regulated by the mutant proteins, we examined the regulation of a second known *Abd-B* target gene, *Distal-less* (*Dll*). *Dll* expression is required in thoracic segments for development of the Keilin's organs (Cohen et al., 1989; Fig. 7A) but is repressed in abdominal segments by the products of the BX-C (Vachon et al., 1992). Repression is mediated by a *cis*-regulatory element called the *NRE-BX* which contains two homeodomain binding

sites. Linkage of the *NRE-BX* to a *lacZ* reporter gene is sufficient to direct β -galactosidase expression in a pattern identical to *Dll* expression in thoracic segments, and deletion of the homeodomain binding sites results in derepression of *lacZ* in the abdomen.

Strains carrying the intact protein, *DfdΔA/Abd-B* or *DfdΔAC/Abd-B* were crossed with flies carrying the intact *Dll NRE-BX 304*, or flies carrying *Dll NRE-BX 305*, in which the homeodomain binding sites were deleted. Heat shock treatment did not disrupt expression of *lacZ* in thoracic segments of flies carrying the *Dll NRE-BX 304* alone (Fig. 7B), nor did it affect derepression in flies carrying *Dll NRE-BX 305* alone (Fig. 7C). Misexpression of the *DfdΔA/Abd-B* protein is capable of activating ectopic *lacZ* expression from *Dll NRE-BX 304* in cephalic and abdominal segments (Fig. 7E). Ectopic

expression of *Dll* in cephalic segments is probably responsible for the occasional appearance of ectopic Keilin's organs (Fig. 7D). Misexpression of the Dfd/Abd-B protein (Fig. 7F) or the Dfd Δ A/Abd-B protein had no effect on the expression of *Dll NRE-BX 305*. The protein Dfd Δ AC/Abd-B repressed *lacZ* expression from the *Dll NRE-BX 304* (Fig. 7H), but not from the mutated *Dll NRE-BX 305* construct (Fig. 7I). Consistent with this observation, Keilin's organs are often suppressed following misexpression the Dfd Δ AC/Abd-B protein (Fig. 7G). These data indicate that the Dfd Δ AC/Abd-B protein has dual regulatory activities which enable it to mediate repression of the *Dll NRE-BX* but activate expression of the *ems-ARFE*.

The N domain is a transcription activation domain

The demonstration that the N domain is required for activation of the *ems-ARFE* reporter gene suggests that it may function as an activation domain. To test this hypothesis, we separately fused the N domain, the acidic region, the Abd-B homeodomain or the C-tail region to the GAL4 DNA binding domain and performed transient cotransfections in a quail fibroblast cell line QT6 (Moscovici et al., 1977). Transcriptional activity of each fusion protein was determined by measuring activity from a chloramphenicol acetyltransferase (CAT) reporter construct containing GAL4 binding sites (Evans and Felsenfeld, 1991). A protein composed of the potent acidic activation domain of VP16 fused to the GAL4 DNA binding domain activated transcription about 3500 fold (Fig. 8A). We observed similarly high levels of activation with the N-domain fusion protein. In contrast, neither the acidic region, the Abd-B homeodomain, nor the C-tail region were capable of activating expression of the CAT reporter gene significantly above background levels.

To further define the sequences required for activation within the N domain, we tested several subregions for activity when fused to the GAL4 DNA binding domain. The N domain consists of a histidine enriched sequence flanked on both sides by tracts of glycine-rich sequences indicated as G1 or G2 in Fig. 8B. Either glycine-rich region by itself was incapable of activating transcription in QT6 cells, while the histidine-rich region alone exhibited only about 10% of the activity seen with the intact N domain (Fig. 8B). Constructs composed of either G1 or G2 fused to the histidine rich region exhibited only 40-50% of the activity shown by the intact domain. We conclude that the complete N-domain region of 220 amino acids is required for full activity in QT6 cells.

Deletions in the native Dfd protein

Our results suggest that the N, acidic and C-tail domains contribute to transcriptional regulation

by the Dfd/Abd-B chimeric protein. We next asked if these domains were important for activity of the native Deformed protein by constructing a similar set of deletion proteins (Fig. 1B). Misexpression of the intact Dfd protein transformed thoracic segments toward a maxillary identity, as determined by the development of ectopic cirri and mouth hooks (Kuziora and McGinnis, 1988; Fig. 9C). Autoactivation of the endogenous *Dfd* expression is required for these transformations thus establishing *Dfd* as a regulatory target gene of the Dfd protein

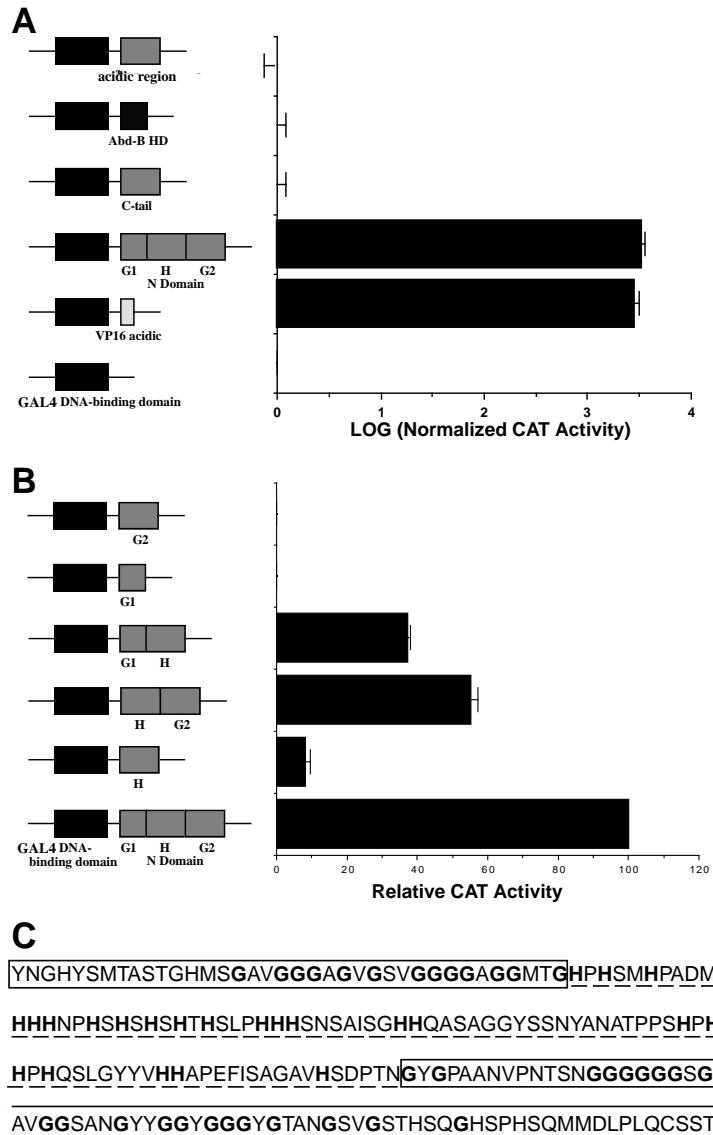


Fig. 8. The N domain functions as an activation domain in QT6 cells. (A) cDNA fragments encoding each region were fused in frame to the GAL4 DNA binding domain and cotransfected with a reporter gene. Each construct is diagrammed to the left, adjacent to the plot of its CAT activity. The activation level of each construct is normalized against the activity of the GAL4 DNA binding domain alone and the logarithm of the relative CAT activity is plotted. (B) Various subregions of the N domain were fused to the GAL4 DNA binding domain and their ability to activate CAT expression was compared to the levels induced by the intact N domain. G1, glycine enriched sequence from amino acids 39-75; H, the histidine enriched central core, amino acids 76-179; G2, glycine enriched sequence from amino acids 180-259. (C) Amino acid sequence of the N domain. G1 and G2 are boxed, the histidine rich region is indicated with a dashed line.

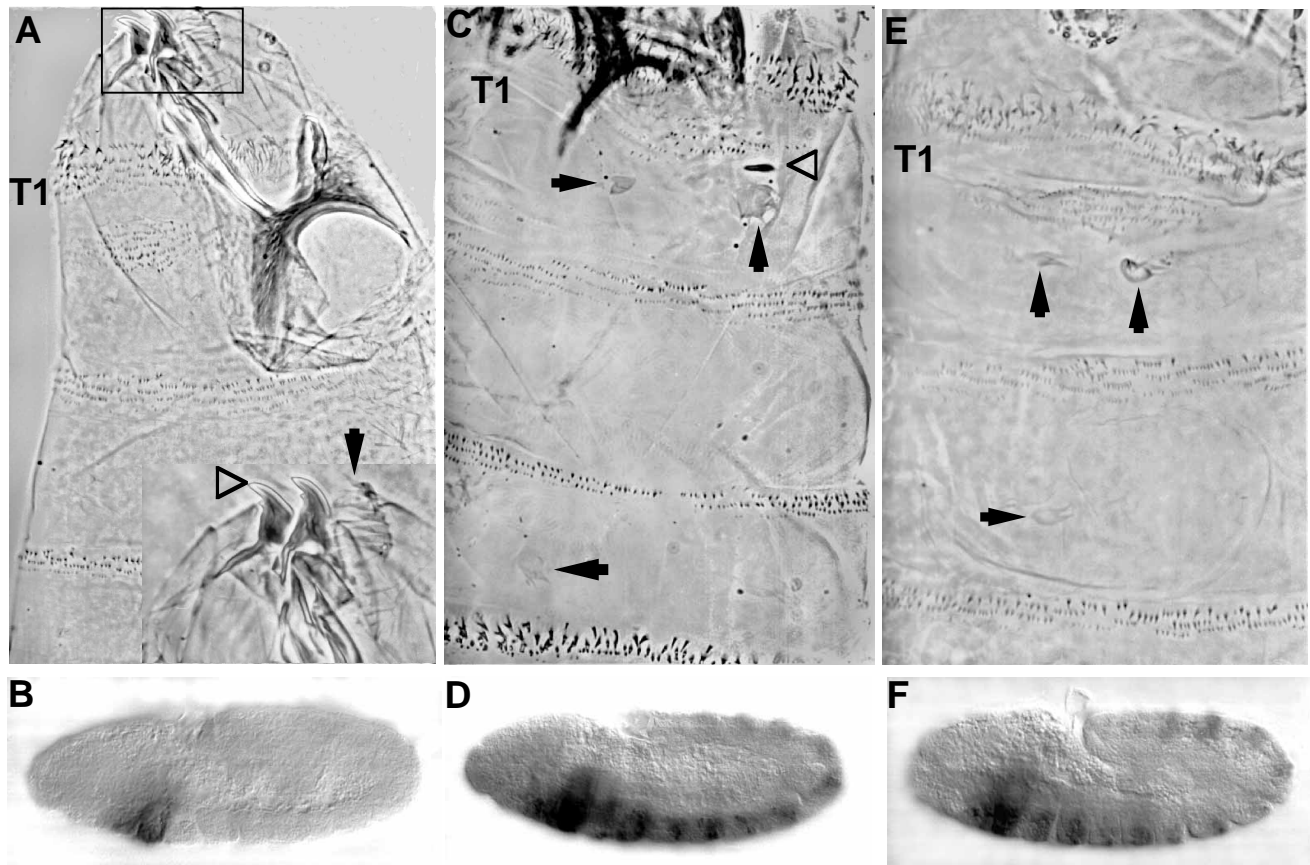


Fig. 9. Regulatory activity of the Dfd deletion proteins. Cuticles were prepared as described for Fig. 8. Embryos were heat shocked at the cellular blastoderm stage and allowed to recover 5 hours. Embryos were then fixed and prepared for antibody staining. (A) Cuticle of a *ry*⁵⁰⁶ embryo. The inset is a close-up of the boxed area showing mouth hooks (open arrowhead) and cirri (arrow). (B) Expression of *Dfd* in *ry*⁵⁰⁶ embryos is normal. (C) Thoracic segments of a larva carrying the intact *Dfd* construct. Note the ectopic cirri (arrows) and a rudimentary mouth hook (open arrowhead). (D) Ectopic expression of the intact *Dfd* protein activates autoregulation of *Dfd* in posterior segments. (E) Thoracic segments of a larva carrying the *Dfd*ΔC/*Dfd* construct. Note the ectopic cirri (arrows). (F) Ectopic expression of the *Dfd*ΔC/*Dfd* construct activates autoregulation of *Dfd* in posterior segments.

(Fig. 9D). Consistent with the results obtained with the chimeric protein, *Dfd*ΔNA/*Dfd* is an inactive protein unable to generate any transformation in larval cuticles suggesting the N domain is required for activity. Deletion of the C-tail (Fig. 9E) or the acidic region (data not shown) did not affect the ectopic formation of cirri, but ectopic mouth hooks were rarely observed. Antibody staining demonstrates that both proteins can ectopically activate the *Dfd* autoregulatory circuit (Fig. 9F). These results suggest the acidic or C-tail regions are individually dispensable, but the N domain is required for the ectopic specification of cirri and autoactivation of the endogenous *Dfd* gene.

DISCUSSION

The regulation of transcription involves a number of protein-DNA and protein-protein interactions (Tjian and Maniatis, 1994). To begin to understand these interactions with relevance to homeodomain proteins, we have performed a deletion analysis of a *Dfd*/Abd-B chimeric protein to identify and char-

acterize domains that contribute to the activation or repression of target genes.

The N domain is essential for regulatory activity

The N domain stimulates high levels of transcription in QT6 cells when fused to a heterologous DNA binding domain indicating it functions as an activation domain. Activation domains mediate protein-protein interactions with the basal transcriptional apparatus (Gill et al., 1994), and the observation that the N domain is functional in quail cells suggests it may interact with some evolutionary conserved component of the transcriptional machinery.

Our deletion analysis suggests that the N domain may be an essential part of the protein required for any regulatory activity in embryos. The N domain makes up a major portion of the *Dfd*ΔAC/Abd-B protein, which activates expression from the *ems*-ARFE, but represses reporter gene transcription from the *Dll* *NRE-BX*. Constructs lacking the N domain fail to produce any visible cuticular transformations, and do not alter the expression of any target gene tested. Despite this lack of biological activity, these proteins are properly localized to the nucleus and retain the ability to bind DNA, at least in vitro.

These observations suggest that interactions between the N domain and other transcription factors may be essential for both activation and repression.

The Dfd Δ AC/Abd-B protein is functionally similar to proteins encoded by *Abd-B*

Abd-B encodes two protein isoforms that carry out genetically defined functions during embryogenesis (Casanova et al., 1986). Similar to the Dfd/Abd-B and Dfd Δ AC/Abd-B proteins, each Abd-B protein isoform activates the *ems*-ARFE (Jones and McGinnis, 1993) and can generate ectopic Filzkörper in larval cuticles (Lamka et al., 1992; Kuziora, 1993). It should be noted that both *ems* and *Abd-B* are required for the specification of ectopic Filzkörper (Jones and McGinnis, 1993), yet we were unable to detect ectopic expression of the endogenous *Abd-B* gene in heat shocked embryos carrying the Dfd Δ AC/Abd-B construct. Additionally we have observed rudimentary ectopic Filzkörper in heat shocked BX-C deficient embryos carrying the Dfd Δ AC/Abd-B construct (data not shown). These observations suggest that the Dfd Δ AC/Abd-B protein can functionally substitute for *Abd-B* activities required to specify Filzkörper.

Both Abd-B proteins (Ali and Bienz, 1991) and Dfd Δ AC/Abd-B contain activation domains but exhibit a dual regulatory function in the embryos by acting as transcriptional repressors of the *Dll NRE-BX* and activators of the *ems*-ARFE. Several mechanisms could be used to explain the dual regulatory nature of the Dfd Δ AC/Abd-B protein. Cofactors may modulate its transcriptional activity via protein-protein interactions as shown for the product of the tumor suppressor gene *WT1* and its association with p53 (Maheswaran et al., 1993). Alternatively, the switch may involve target promoter context which determines the number and location of homeodomain binding sites, as well as binding sites for necessary cell-specific cofactors required for transcriptional regulation (Appel and Sakonju, 1993; Lehming et al., 1994).

The role of the acidic and C-tail regions

Despite the failure of the acidic region and C-tail to stimulate CAT reporter gene expression in transfection assays it could be argued that some cell- or species-specific cofactor is required for activity. This argument is not, however, supported by the observation that proteins lacking the N domain exhibit no detectable activity in embryos. It remains a possibility that these regions are required for gene regulation in tissues not examined in this study. For example, protein isoforms of Ubx are known to induce specific transformations in the nervous system, although they produce identical transformations in the larval epidermis (Mann and Hogness, 1990; Subramaniam et al., 1994).

Deletion of the acidic and C-tail regions had little effect on regulation of the *ems*-ARFE or on Deformed autoregulation. In contrast however, the Dfd Δ AC/Abd-B protein exhibits strikingly different regulatory activity towards the *Dll NRE-BX* when compared to the intact protein. Additional evidence that the acidic and C-tail regions can contribute to regulatory activity is suggested by the distinct alteration in homeotic expression patterns resulting from misexpression of each deletion protein. Based on these results, we conclude that the acidic and C-tail region might function to modulate the activation activity of the N domain in the regulation of some target genes.

The mechanism by which the acidic and C-tail region could influence transcriptional activity is unknown. One possibility

is that these regions could govern protein-protein interactions with free or promoter bound transcriptional cofactors which are expressed in a cell-specific manner. For example, the product of the *cap'n'collar* gene and the DEAF-1 protein have been proposed to interact with Dfd and alter its regulatory activity (Zeng et al., 1994; Mohler et al., 1995; Gross and McGinnis, 1996). The protein domains described by this paper may provide a framework for the assembly of a multi-protein complex at target gene promoters, the composition of which determines regulatory activity.

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