High-affinity binding sites for the *Deformed* protein are required for the function of an autoregulatory enhancer of the *Deformed* gene

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The homeotic selector gene *Deformed* (*Dfd*) is required to specify the identity of head segments during *Drosophila* development. Previous experiments have shown that for the *Dfd* segmental identity function to operate in epidermal cells, the *Dfd* gene must be persistently expressed. One mechanism that provides persistent embryonic expression of *Dfd* is an autoregulatory circuit. Here, we show that the control of this autoregulatory circuit is likely to be directly mediated by the binding of Dfd protein to an upstream enhancer in *Dfd* locus DNA. In a 25-kb region around the *Dfd* transcription unit, restriction fragments with the highest binding affinity for Dfd protein map within the limits of the upstream autoregulatory element at approximately -5 kb. A minimal autoregulatory element, within a 920-bp segment of upstream DNA, has four moderate- to high-affinity binding sites for Dfd protein, with the two highest affinity sites sharing an ATCATTA consensus sequence. Site-specific mutagenesis of these four sites results in an element that has low affinity for Dfd protein when assayed in vitro and is nonfunctional when assayed in embryos.

**Key Words:** Deformed; autoregulatory circuit; homeo domain, homeotic protein

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The pattern of the *Drosophila* body plan along the anteroposterior axis is defined through a complex hierarchy of interactions among several classes of pattern formation genes (for review, see Akam 1987; Ingham 1988). At the bottom of this hierarchy are the homeotic selector genes that specify unique identities to specific body compartments along the anteroposterior axis (Garcia-Bellido 1977; Lewis 1978; Wakimoto and Kaufman 1981). Genes of the homeotic selector class exert regulatory effects on each other and themselves to maintain their respective expression patterns in the embryo (Struhl 1982; Hafen et al. 1984; Carroll et al. 1986; Bienz and Tremmel 1988; Kuziora and McGinnis 1988). They are also believed to regulate the transcription of downstream “realisator” genes, which would be responsible for the formation of particular structures in individual segments (Garcia-Bellido 1977; Gonzalez-Reyes and Morata 1990; Immergluck et al. 1990).

The homeotic selector genes of the Antennapedia and Bithorax complexes share similar 180-bp sequences called homeo boxes (McGinnis et al. 1984a,b; Scott and Weiner 1984; Regulski et al. 1985). Homeo box sequences are found in many other genes involved in the regulation of *Drosophila* development (Poole et al. 1985; Frigerio et al. 1986; for review, see Scott et al. 1989) and are highly conserved in many higher and some lower animals (Carrasco et al. 1984b; McGinnis et al. 1984; McGinnis 1985; Way and Chalfie 1988; Scott et al. 1989). Homeo box sequences encode a family of sequence-specific DNA-binding protein domains, collectively referred to as homeo domains (Desplan et al. 1985, 1988; Fainsod et al. 1987; Hall and Johnson 1987; Hoey and Levine 1988; Muller et al. 1988). Homeo domain proteins can mediate transcriptional activation or repression when tested in cotransfection assays in tissue culture cells, or in transcription assays in vitro (Jaynes and O’Farrell 1988; Thali et al. 1988; Biggin and Tjian 1989; Hans et al. 1989; Krasnow et al. 1989; Winslow et al. 1989). However, most tissue culture and in vitro experiments have been done with DNA sequences whose relevance to embryonic regulatory function(s) is questionable or nonexistent.

At present, the best described embryonic DNA targets for *Drosophila* homeo domain proteins are those that map just upstream of the *hunchback* and *fushi tarazu* gene transcription initiation sites. A ~300-bp element of *hunchback* upstream DNA directs expression of *hunchback* transcripts in the anterior half of the early blastoderm embryo in a *bicoid* (*bcd*)-dependent manner and
has multiple binding sites for the homeo domain-containing bcd protein [Tautz 1988; Driever and Nüsslein-Volhard 1989a; Struhl et al. 1989]. Deletions of portions of the element that include bcd protein-binding sites inactivate its function. In addition, multimerized fragments of the element that include little additional sequence flanking the bcd protein-binding sites are capable of providing 

hunchback-like expression patterns that are sensitive to the levels of bcd protein [Driever et al. 1989b; Struhl et al. 1989]. An ~300-bp element of fushi tarazu upstream DNA provides expression in the posterior half of the early embryo [Dearolf et al. 1989]. This fushi tarazu element has two pairs of binding sites for the homeo domain-containing caudal protein. The mutation of either pair of binding sites abolishes the function of the element [Dearolf et al. 1989].

Though many cross- and autoregulatory relationships exist among the homeotic selectors, the target sequences that mediate these effects are largely undefined. The two selector regulatory elements whose limits are best defined are autoregulatory elements at Ultrabithorax (Ubx) and Deformed (Dfd). The Ubx autoregulatory element maps between -3.1 and -1.7 kb from the Ubx transcription start and confers late expression in parasegment 7 of the visceral mesoderm [Bienz and Tremml 1988; Muller et al. 1989]. The Dfd autoregulatory element maps between -6.6 and -3.9 kb from the Dfd transcription start and normally provides expression in the maxillary and mandibular epidermis [Bergson and McGinnis 1990].

**Dfd** is a homeotic selector gene that specifies the identity of mandibular and maxillary lobes in *Drosophila* embryos [Chadwick and McGinnis 1987; Merrill et al. 1987; Regulski et al. 1987]. We have shown previously that persistent embryonic expression from Dfd is required for its segment identity function and that persistent expression is maintained by autoregulation through a distant upstream enhancer [Kuziora and McGinnis 1988; Bergson and McGinnis 1990]. Here we provide evidence consistent with a direct interaction between the Dfd protein and the Dfd autoregulatory element. Using stringent DNA-binding conditions to test fragments in a 25-kb region, including the Dfd transcription unit and flanking regions, we find that the region with the highest binding affinity for Dfd protein is located within the Dfd autoregulatory element. Site-specific mutagenesis of some of the high-affinity Dfd protein-binding sites abolishes the activity of a minimal autoregulatory element.

### Results

**Dfd protein**

Current results are consistent with either a direct or an indirect effect of Dfd protein on Dfd autoregulation. If Dfd protein directly mediates its positive regulatory effect on the Dfd transcription unit, we expected to find restriction fragments in Dfd genomic sequences with significant binding affinity for Dfd protein. To test for such binding fragments, we used Dfd protein that had been overproduced in either bacterial cells or *Drosophila* embryos. Dfd encodes a homeo domain protein of 586 amino acids [Regulski et al. 1987]. To produce full-length Dfd protein in *Escherichia coli*, we used a T7 expression system [Studier and Moffatt 1986] and the expression plasmid pAR3040Dfd [Jack et al. 1988]. After induction, Dfd protein constitutes ~5% of total protein in a soluble extract from these cells. For some of the following experiments, bacterially produced Dfd protein was purified using sequence-specific DNA affinity chromatography [Fig. 1, lane 4]. The identity of Dfd protein in the bacterial extract and in affinity chromatography fractions was confirmed by Western blot analysis [Fig. 1A, lanes 2'-4'] using anti-Dfd antiserum [Jack et al. 1988]. We also prepared a *Drosophila* nuclear extract from

**Figure 1.** Dfd protein and extracts. [A] Purification of full-length Dfd protein from *E. coli* extracts. [Lane 1] Molecular weight markers, with their sizes in kilodaltons indicated on the left; [lane 2] 8 µg of soluble protein extract prepared after induction of an *E. coli* strain containing the control pAR3040 plasmid; [lane 3] 8 µg of soluble protein extract prepared after induction of an *E. coli* strain containing pAR3040Dfd plasmid; [lane 4] 350 ng of purified Dfd protein from a 0.6 M NaCl fraction of a DNA-affinity column. (Lanes 2'-4') The equivalent of lanes 2'-4' was transferred to nitrocellulose and the Dfd protein was detected with anti-Dfd antiserum [Jack et al. 1988]. [B] Identification of the Dfd protein in the nuclear extract from *Drosophila* embryos. The extract was prepared from transgenic embryos carrying hsp70-Dfd construct after heat shock induction of Dfd protein expression. [Lanes 1'] Molecular weight markers; [lane 2'] 13 µg of soluble bacterial protein extract containing the Dfd protein; [lane 3'] 20 µg of embryonic nuclear protein extract. (Lanes 2' and 3') The equivalent of lanes 2 and 3, respectively, was transferred to nitrocellulose and reacted with anti-Dfd antiserum to detect Dfd protein. The arrow indicates the position of the Dfd protein.
hsfp70-Dfd embryos [Kuziora and McGinnis 1988] to compare the binding specificity of the E. coli-produced Dfd protein to that produced in embryos [Fig. 1B].

DNA fragments in the Dfd locus that bind Dfd protein

We used a modified version of an immunoprecipitation assay [McKay 1981] to identify DNA fragments with high affinity for the Dfd protein within the Dfd genomic region. As the input DNA we used digests of two λ phage DNAs that contain 25 kb of Antennapedia complex DNA: λ100, which contains 8 kb of Dfd upstream sequence and the 5’ half of the 11-kb Dfd transcription unit; and λ99, which contains the 3’ half of the transcription unit and 6 kb of downstream sequences [Regulski et al. 1987]. Restriction fragments of these two phage DNAs were incubated with bacterial or embryonic nuclear extracts containing Dfd protein, and bound fragments were immunoprecipitated with anti-Dfd antiserum. As a control for the specificity of the immunoprecipitation we used bacterial extract without Dfd protein. Figure 2A shows the results of an experiment in which the input DNA was digested with AccI and HindIII endonucleases and the immunoprecipitated fragments were separated on an agarose gel. Within λ100 DNA, an AccI–HindIII fragment of 540 bp had the highest affinity for Dfd protein produced either in bacteria [lane 2] or embryos [lane 3]. No fragments are immunoprecipitated by control bacterial extracts that lack Dfd protein [lane 4]. Within λ99 DNA, the highest affinity fragment is a 2.3-kb HindIII fragment that was bound strongly by Dfd protein from embryonic extracts but was bound weakly by Dfd protein produced in E. coli.

Because small DNA fragments might go undetected on agarose gels, we also separated immunoprecipitated fragments on polyacrylamide gels [Fig. 2B]. In the experiment shown, λ100 and λ99 DNAs were digested with EcoRI and HindIII. Again, both protein preparations showed similar binding specificity on λ100 fragments, with highest affinity for a 900-bp HindIII fragment. This fragment includes the 540-bp AccI–HindIII fragment immunoprecipitated in Figure 2A. In the lanes containing λ99 digests, the same 2.3-kb HindIII fragment is preferentially bound by embryonic extract [lane 9]. When used in relatively large amounts, the bacterially produced Dfd protein exhibits the same binding specificity on λ99 fragments as does embryonic extract [Fig. 2B, lane 7]. However, at low concentrations, the bacterially produced Dfd protein has an apparent higher affinity for the 900-bp fragment than for the 2.3-kb HindIII fragment.

The location of one of the high-affinity binding regions correlates nicely with sequences that we have previously shown can provide autoregulated expression of Dfd [Bergson and McGinnis 1990]. A Dfd autoregulatory enhancer maps between −3.9 and −6.6 kb from the transcription start [Fig. 3]. In wild-type embryos, this en-

Figure 2. DNA fragments binding Dfd protein within the Dfd genomic region. DNA from phages λ100 and λ99 was digested with restriction enzymes, end-labeled with 32P, incubated with protein extract, and immunoprecipitated with the anti-Dfd antiserum. Products were resolved on agarose (A) or polyacrylamide (B, C) gels. [A] Lanes 1–4 show the results of an immunoprecipitation experiment with λ100 DNA digested with AccI and HindIII endonucleases; lanes 5–8 show the results with λ99 DNA, also digested with AccI and HindIII; (lanes 6–9) the input DNA fragments; (lanes 2–6) DNA fragments bound and precipitated in presence of 0.25 μg of a soluble E. coli protein extract containing the Dfd protein; (lanes 3–7) the fragments bound by 76 μg of Drosophila embryo nuclear extract. Lanes 4 and 8 are controls, showing that no fragments are bound by 0.25 μg of soluble E. coli protein extract without Dfd protein. The sizes of fragments with high affinity for Dfd protein are indicated: 900-bp HindIII and 540-bp AccI–HindIII fragments within λ100, and a 2.3-kb HindIII fragment within λ99. [B] Lanes 1–5 show the results of an immunoprecipitation experiment with λ100 DNA digested with EcoRI and HindIII endonucleases; (lanes 6–9) the results of an identical experiment with λ99 DNA presented in the same order except for the minus-Dfd lane, which was omitted. [Lanes 1 and 6] The input DNA fragments. [Lanes 2 and 7] The DNA fragments bound and precipitated in the presence of 0.5 μg of a soluble E. coli protein extract containing the Dfd protein. [Lanes 3 and 8] the results when 0.25 μg of the same extract is used. [Lanes 4 and 9] The fragments bound by 76 μg of Drosophila embryonic nuclear extract. Lane 5 is a control, showing that no fragments are bound by 0.5 μg of soluble E. coli protein extract without the Dfd protein. The sizes of fragments with moderate to high affinity for Dfd protein are indicated: the 1.4-kb HindIII–EcoRI, the 900-bp HindIII, and the 820-bp EcoRI fragments of λ100, and the 2.3-kb HindIII fragment of λ99. The location of these binding fragments relative to the Dfd transcription unit is shown in Fig. 3. [C] H2Z7.7 DNA [Bergson and McGinnis 1990], which contains the 2.7-kb XbaI fragment from −3.9 to −6.6 (Fig. 3) was tested in an immunoprecipitation experiment after digestion with Alul. [Lane 7] The input DNA fragments; [lanes 2 and 3] the fragments precipitated with 0.5 and 0.15 μg, respectively, of a soluble E. coli extract containing Dfd protein. The 230- and 270-bp Alul fragments with highest affinity for the Dfd protein are marked by arrowheads. The third fragment visible in lane 3 derives from the vector PHZ50 [Hiromi and Gehring 1987].
Figure 3. Map of fragments that bind Dfd protein. The top half shows a simplified restriction map of the Antennapedia complex region included in clones A100 and A99. Solid blocks on the map represent the exons of the 11-kb Dfd transcription unit, and the arrow shows the direction of transcription. The scale over the upstream region measures the distance from the Dfd transcription start site in kilobases. (Bottom) A more detailed map of the Dfd autoregulatory enhancer (Bergson and McGinnis 1990). Asterisks mark fragments in the region with relatively high (**) and low (*) affinity for E. coli-produced Dfd protein in immunoprecipitation assays. (R) EcoRI; (X) XbaI; (H) HindIII; (Ac) Accl; (A) AluI.

hancer provides late, spatially localized expression in the epidermal cells of the maxillary and mandibular segment and in Dfd-expressing regions of the eye–antennal imaginal disc. In Dfd mutant embryos, the enhancer is inactive, and in hsp70–Dfd embryos it is ectopically activated (Bergson and McGinnis 1990). The smallest known subelement of the enhancer that provides maxillary-specific expression is contained within the same 900-bp HindIII fragment that has high binding affinity for Dfd protein. The 900-bp HindIII fragment containing this Dfd minimal element maps between −4.5 and −5.4 kb from the transcription start site in kilobases (Fig. 3).

The 2.3-kb HindIII fragment [Fig. 3], with high affinity for Dfd protein-containing embryonic extracts, is unlikely to have an important role in the regulation of Dfd expression based on the variant Dfd locus in the DfdRX17 chromosome. The DfdRX17 locus contains a 35-kb DNA insert that maps between the Dfd polyadenylation site and the 2.3-kb HindIII fragment, yet exhibits a normal Dfd expression pattern during embryonic and larval stages (Chadwick et al. 1990).

To further delimit regions within the 900-bp HindIII fragment for their relative affinity for Dfd protein, we analyzed binding of an AluI digest of a 2.7-kb XbaI subclone of A100 [Fig. 3], which contains the 900-bp fragment and surrounding sequences. As shown in Figure 2C, bacterially produced Dfd protein preferentially binds two subregions of the 900-bp HindIII fragment. The highest affinity binding region is within a 230-bp AluI fragment, and a lower affinity binding region maps within an adjacent 270-bp AluI fragment. The same pattern of binding preferences was obtained for the embryonically produced Dfd protein (data not shown).

Dfd-binding sites within the 270/230-bp AluI fragments

The overlap between the position of the Dfd autoregulatory element and fragments with high binding affinity for Dfd protein is consistent with the hypothesis that Dfd protein might be directly involved in autoregulation. To begin to test this, we first needed to define the specific DNA sequences binding the Dfd protein. As the 270- and 230-bp AluI fragments had the highest affinities for Dfd protein in immunoprecipitation assays, we used purified Dfd protein and DNase I protection assays (Galas and Schmitz 1978) to identify Dfd protein-binding sites in this region. Footprint data for the highest affinity sites is shown in Figure 4. There is one high-affinity binding site, A, in the 270-bp AluI fragment [Fig. 4A] and three moderate- to high-affinity sites, B, C, and D, within the 230-bp AluI fragment [Fig. 4B, C]. The map in Figure 4.
Regulski et al.

4 shows the location of these sites within a 920-bp HindIII-A/III fragment.

The DNA sequence of the region containing these four binding sites is shown in Figure 5A. Sites A and D share a perfect sequence match of 7 bp: ATCATTA. The A and D sites appear to have higher affinity for the Dfd protein in the footprinting experiments than sites B and C, as they are protected at slightly lower concentrations of protein (data not shown). Figure 5B shows the ATCATTA high-affinity consensus sequence aligned with the consensus binding sequences of some other homeodomain-containing proteins. At least within the autoregulatory element, Dfd protein appears to prefer sites with ATC just 5' to an ATTA core (or GAT just 3' to a TAAT core), while other homeodomain proteins have different preferred bases adjacent to the core in their consensus sequences.

Dfd protein-binding sites are required for function of a minimal autoregulatory element

To test whether sites A–D in the minimal autoregulatory element are required for its function, we used in vitro site-specific mutagenesis (Kunkel et al. 1987) to alter the four Dfd-binding sites. In sites A and D the ATTA core was replaced with CCCC, and in sites B and C the sequence AATT was changed to CCCC. The quadruply mutated regulatory region was then cloned as a 920-bp HindIII-A/III fragment (from the left end of the 900-bp HindIII fragment to just downstream of the now mutated HindIII site, since site D overlaps the HindIII site at the left end of the 900-bp fragment), upstream of the hsp70 basal promoter–lacZ reporter gene in the P-element transformation vector HZ50 (Hiromi and Gehring 1987). We then generated germ-line transformants carrying the quadruply mutant autoregulatory element (Q920-HZ strains) and control strains carrying the wild-type 920-bp fragment in the HZ50 vector (920-HZ strains).

The mutation of sites A–D results in 920-bp fragments with little specific affinity for Dfd protein in vitro. As shown in Figure 6A, the wild-type fragment is strongly bound and immunoprecipitated by moderate concentrations of Dfd protein. Similar amounts of Dfd protein do not give detectable binding of the Q920 fragment. At higher concentrations of Dfd protein, some binding of the Q920 fragment can be detected, but the affinity appears to be not much greater than other nonspecifically bound fragments from the HZ50 host plasmid (Fig. 6).

Expression patterns in the 920-HZ strains are similar to that in the previously described HZ0.9 transformants (Bergson and McGinnis 1990). β-Galactosidase is expressed in a subset of the normal Dfd expression domain in the maxillary segment, from ~12 hr after egg laying (AEL) to the end of embryogenesis 8 hr later (Fig. 6B). We tested three Q920-HZ strains carrying the quadruply mutated regulatory constructs. None of these strains express β-galactosidase in the maxillary segment at any stage of embryonic development (Fig. 6C).

Surprisingly, the function of the entire epidermal autoregulatory element in the 2.7-kb XbaI fragment (Fig. 3; Bergson and McGinnis 1990) was not noticeably affected by mutations in sites A–D. Strains carrying Q2.7-HZ expression constructs exhibited a pattern of maxillary-specific expression identical to 2.7-HZ (Bergson and McGinnis 1990). However, the 2.7-kb XbaI fragment with mutations in sites A–D still possessed considerable specific binding affinity for Dfd protein in vitro, some...
pQ920-HZ, which is identical to p920-HZ except for the mutations in sites A–D sites (Bergson and McGinnis 1990). As we show here for the -5.6 to -4.5 fragment mapping from -3.9 to -6.6 kb (Bergson and McGinnis 1990). Within this large region, subelements have been defined that can provide subsets of the late pattern of autoregulated Dfd expression. The smallest element yet defined to have autonomous regulatory function is a 900-bp HindIII fragment (Bergson and McGinnis 1990). As we show here, this fragment also has the highest binding affinity for Dfd protein in a 25-kb DNA region including Dfd upstream and downstream sequences. When the four highest affinity Dfd protein-binding sites are mutated in a slightly larger 920-bp Dfd minimal element, its maxillary segment-specific enhancer activity is lost. Because previous studies have shown that similar homeo domain proteins will bind to similar DNA sites, these results prove only that the Dfd-binding sites are required, not that Dfd protein actually binds and activates these sites in the embryo. However, the above results, in combination with binding studies indicating that the 900-bp HindIII fragment preferentially binds Dfd protein over other similar homeo domain proteins (S. Dessain, unpubl.), strongly support the idea that these binding sites are embryonically relevant target sites for the regulatory activity of Dfd protein.

Although the 900-bp fragment has the most compact cluster of moderate- to high-affinity Dfd-binding sites, the remainder of the epidermal autoregulatory element, which is currently defined by a 2.7-kb Xbal fragment spanning -6.6 to -3.9, has additional sites that Dfd protein binds in vitro (C. Gross and S. Dessain, unpubl.). Many of these are likely to be relevant to the function of the intact element, since the function of a 2.7-kb element is not strongly disrupted by the quadruple mutations in the A–D sites (Bergson and McGinnis 1990; M. Regulski, unpubl.). Our working model for the upstream autoregulatory element is that it is composed of largely independent Dfd-dependent modules that provide epidermal expression in different subregions of the maxillary and mandibular segments and are functionally redundant to some extent (Bergson and McGinnis 1990; C. Zeng, unpubl.). As we show here for the -5.6 to -4.5 minimal element, the function of many or perhaps all of these modules may be dependent on clusters of Dfd protein-binding sites. More detailed studies on the Dfd autoregulatory enhancer will be necessary to compare its overall structure and function to better characterized viral and mammalian enhancers, which are known to be organized from combinations of functional modules (for review, see Serfling et al. 1985; Dynan 1989).

As measured by immunoprecipitation assays, the binding specificity of the Dfd protein produced in E. coli appears to be similar to that of the Dfd protein present in the nuclear fraction of Drosophila embryo extracts. This indicates that other components of the nuclear extract do not appreciably change the selectivity of DNA binding by Dfd protein, at least on the 25 kb of DNA tested. However, because the 2.3-kb HindIII fragment had apparent higher affinity for the embryonic Dfd protein than for E. coli-produced Dfd protein, the absolute affinity for specific fragments may be altered by other embryonic factors or embryo-specific modifications of Dfd protein. Experiments on the DNA-binding properties of another homeotic selector protein, the Ubx protein, have shown that its ability to protect sequences from DNase digestion is indistinguishable whether the protein is produced in E. coli or in insect cells in tissue culture (Beachy et al. 1988).

**Figure 6.** Binding and function of a mutated autoregulatory enhancer element. (A) [Lanes 3–7] The results of immunoprecipitation experiments with p920-HZ plasmid DNA. This plasmid contains the wild-type 920-bp HindIII–AflII fragment upstream of the hsp70 basal promoter–lacZ reporter gene in the P-element transformation vector HZ50 (Hiromi and Gehring 1987). (Lanes 7–12) The results of similar experiments with pQ920-HZ, which is identical to p920-HZ except for the mutations in sites A–D (Fig. 5). Both plasmids were digested with BamHI and Xbal, which yields a 930-bp fragment that includes the 920-bp minimal autoregulatory element. (B) Ventral–lateral view of the anterior end of a Q920-HZ embryo. Staining for β-galactosidase shows that no expression of the lacZ reporter gene is detected in cells of the maxillary segment (mx) of this 14-hr embryo. (C) Ventral–lateral view of the anterior end of a Q920-HZ embryo. Staining for β-galactosidase shows expression of the lacZ reporter gene in cells of the maxillary segment (mx) of this 14-hr embryo. What lower than the wild-type 2.7-kb fragment but much higher than the Q920 fragment (Fig. 6, M. Regulski, unpubl.).

**Discussion**

Our results indicate that Dfd autoregulation is likely to be directly mediated by the binding of Dfd protein to specific sites in the upstream autoregulatory element. The Dfd autoregulatory element is largely contained within a 2.7-kb Xbal fragment mapping from -3.9 to -6.6 kb (Bergson and McGinnis 1990). Within this large element, subelements have been defined that can provide subsets of the late pattern of autoregulated Dfd expression. The smallest element yet defined to have autonomous regulatory function is a 900-bp HindIII fragment (Bergson and McGinnis 1990). As we show here,
The two binding sites in the 900-bp fragment with the highest in vitro affinity for Dfd protein share a 7-base consensus: ATCATTG. This sequence is related to, but different from, consensus sequences defined for other homeo domain proteins [Fig. 5B]. It appears to share the ATTA core that is part of many homeo domain-binding sites. However, the first 3 bases at the 5' end of this sequence are unique to the Dfd consensus-binding site. It is possible that these few bases adjacent to the ATTA core make a large contribution to the ability of Dfd protein to selectively bind the Dfd autoregulatory element. These putative discriminator bases may also provide a structure that allows closely related homeo domain proteins to distinguish their preferred sites.

Materials and methods

Dfd protein from E. coli and embryos

Construction of the pAR3040Dfd expression plasmid has been described [Jack et al. 1988]. Induction of Dfd protein expression in E. coli was carried out as described in Studier and Moffatt [1986]. Induced cells were washed with 60 ml of 20 mM HEPES [pH 7.6] and 200 mM NaCl at 4°C and resuspended in 6 ml of lysis buffer at 4°C: 20 mM HEPES [pH 7.6], 200 mM NaCl, 50 mM β-mercaptoethanol, 5 μg/ml of leupeptin, 5 μg/ml of pepstatin A, 2 μg/ml of aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium metabisulfite, 2 mM benzamidine, 10% (wt/vol) glycerol, 0.1 mM EDTA, and 0.5 mg/ml lysozyme. Cells were shocksonicated with a microtip cell disruptor at the highest setting at 4°C (Branson Sonic Power Co. no. 350). Lysates were spun in a microcentrifuge for 30 min at 4°C, and the soluble fractions were frozen at −80°C. The total protein concentration in the soluble extracts was 20−25 mg/ml as measured by the method of Bradford (1976). Aliquots were run on SDS-polyacrylamide gels (Laemmli 1970) and either stained with CBB G-250 or transferred to nitrocellulose for immunological detection of Dfd protein [Jack et al. 1988]. The concentration of Dfd protein in the soluble extract as estimated from stained gels was 0.2−1.0 mg/ml. Aliquots were kept at −80°C, thawed once, and used in the amounts described.

Nuclear extract from Drosophila hsp70−Dfd embryos was prepared as described by Biggin and Tjian (1988), as modified by Soeller et al. [1988]. Embryos from a 6-hr collection were heat-shocked at 37°C for 1 hr and then processed. Aliquots of nuclear protein extract were kept at −80°C, thawed once, and used in the amounts described.

Immunoprecipitations of DNA−protein complexes

These experiments were done with slight modifications of the protocol of McKay [1981]. DNA was digested with appropriate restriction enzymes, filled in with [32P] labeled nucleotides using Sequenase (U.S. Biochemicals, Sambrook et al. 1989), and DNA concentration was estimated by fluorometry in a Hoenfer TKO 100 fluorometer. Fifty-microliter binding reactions contained 1 fmole of labeled DNA fragment 25 μl of 2X binding buffer [Heberlein et al. 1985], 2.5 μg of poly[d(I-C)], and amounts of protein as indicated in the figure legends. After incubation for 30 min on ice, the binding reactions were treated with 20 ng of DNase I in 10 μl of 6X dilution buffer [10 mM Tris [pH 8.0], 1 mM EDTA, 15 mM CaCl2, 30 mM MgCl2], on ice for 8 min, and the reaction was stopped with 200 μl of 10 mM EDTA containing 20 μg of tRNA. DNA was then extracted with phenol/chloroform, and precipitated with ethanol. Samples were separated on 8% denaturing polyacrylamide gels and autoradiographed using Kodak AR film. The chemical sequencing protocol of Maxam and Gilbert [1977] was used to produce sequencing ladders of the same fragments used for footprinting.

Construction and transformation of mutant autoregulatory elements

A 920-bp HindIII−AflII fragment containing the wild-type A-, B-, C-, and D-binding sites was subcloned into HZ50 [a P-element transformation vector containing hsp70 basal promoter−lacZ reporter gene; Hiromi and Gehring 1987]. This control construct, p920-HZ, was co-injected with pΔ2-3 into cn;ry506 embryos as described previously [Rubin and Spradling 1982]. Embryos (350) were injected, and three separate transformed lines (920-HZ lines) were obtained. One line was homozygous lethal on the second chromosome. The other lines were both homozygous viable, with one on the second chromosome and the other on the third chromosome.

The p920-HZ construct was made exactly as described above except that the A, B, C, and D-binding sites were mutated using 30-mer oligonucleotides according to the protocol of Kunkel et al. [1987]. The CCCCCC sequence in the middle of each oligonucleotide allowed a specific change at each binding site as shown in Figure 5 and was flanked by 12- and 13-bp "clamps" matching sequences around the binding sites. The mutant construct was co-injected with pΔ2-3 into cn;ry506 embryos. Embryos (497) were injected, yielding three separate Q920-HZ lines. All lines were homozygous viable, with one on the second, one on the third, and one on the fourth chromosome. To detect expression from the 920-HZ and Q920-HZ constructs, embryos were collected over 6 hr and aged for 12 hr at 25°C. They were then harvested, dechorionated, fixed in paraformaldehyde, and stained with mouse anti β-galactosidase antibodies as described previously [Bergson and McGinnis 1989].

The S. aureus cell/anti-Dfd antibody complex was prepared as follows: S. aureus cells [100 μl, formalin fixed, 10% solution, Calbiochem] were spun down, washed twice with binding buffer without competitor DNA, and resuspended in 500 μl of anti-Dfd antiserum [Jack et al. 1988]. Five hundred microliters of 100% glycerol was added to the mix and rotated on a nutator (Clay Adams) at 4°C for at least 1 hr. The suspension was washed twice in the binding buffer containing 1 μg/ml of hering sperm competitor DNA and resuspended in 200-250 μl of binding buffer.

DNase I protection

DNase I protection experiments were done according to Heberlein et al. [1985], with some modifications. Each 50-μl binding reaction contained 1 fmole of labeled DNA fragment, 25 μl of 2X binding buffer [Heberlein et al. 1985], 2.5 μg of poly[d(I-C)], and amounts of protein as indicated in the figure legends. After incubation for 30 min on ice, the binding reactions were treated with 20 ng of DNase I in 10 μl of 6X dilution buffer [10 mM Tris [pH 8.0], 1 mM EDTA, 15 mM CaCl2, 30 mM MgCl2] on ice for 8 min, and the reaction was stopped with 200 μl of 10 mM EDTA containing 20 μg of tRNA. DNA was then extracted with phenol/chloroform, and precipitated with ethanol. Samples were separated on 8% denaturing polyacrylamide gels and autoradiographed using Kodak AR film. The chemical sequencing protocol of Maxam and Gilbert [1977] was used to produce sequencing ladders of the same fragments used for footprinting.

The two binding sites in the 900-bp fragment with the highest in vitro affinity for Dfd protein share a 7-base consensus: ATCATTG. This sequence is related to, but different from, consensus sequences defined for other homeo domain proteins [Fig. 5B]. It appears to share the ATTA core that is part of many homeo domain-binding sites. However, the first 3 bases at the 5' end of this sequence are unique to the Dfd consensus-binding site. It is possible that these few bases adjacent to the ATTA core make a large contribution to the ability of Dfd protein to selectively bind the Dfd autoregulatory element. These putative discriminator bases may also provide a structure that allows closely related homeo domain proteins to distinguish their preferred sites.
1990. Three mutant and two wild-type strains were stained to eliminate the possibility that expression differences were due to insertion sites differences.

The quadruply mutated 900-bp HindIII fragment was also used to replace the wild-type HindIII fragment in an hsp70 basal promoter–lacZ vector that contained the 2.7-kb Xbal fragment (−3.9 to −6.6; Fig. 3). The pQ2.7-HZ construct that resulted is identical to p2.7-HZ [Bergson and McGinnis 1990] except for the mutations in sites A–D. Embryos from five independent transformed lines carrying the Q2.7-HZ fusion gene were tested for their pattern of expression of β-galactosidase.

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