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Activity regulation of a Hox protein and a role for the homeodomain in inhibiting transcriptional activation

Xuelin Li, Cornelis Murre and William McGinnis[1]

Department of Biology, University of California, San Diego, La Jolla, CA 92093, USA

[1]Corresponding author
e-mail: mcginnis@biomail.ucsd.edu

Hox proteins are transcription factors that assign positional identities along the body axis of animal embryos. Different Hox proteins have similar DNA-binding functions *in vitro* and require cofactors to achieve their biological functions. Cofactors can function by enhancement of the DNA-binding specificity of Hox proteins, as has been shown for Extradenticle (Exd). We present results supporting a novel mechanism for Hox cofactor function: regulation of transcriptional activation function. First, we provide evidence that the Hox protein Deformed (Dfd) can interact with simple DNA-binding sites in *Drosophila* embryos in the absence of Exd, but this binding is not sufficient for transcriptional activation of reporter genes. Secondly, either Dfd or a Dfd-VP16 hybrid mediate much stronger activation in embryos on a Dfd–Exd composite site than on a simple Dfd-binding site, even though the two sites possess similar Dfd-binding affinities. This suggests that Exd is required to release the transcriptional activation function of Dfd independently of Exd enhancement of Dfd-binding affinity on the composite site. Thirdly, transfection assays confirmed that Dfd possesses an activation domain, which is suppressed in a manner dependent on the presence of the homeodomain. The regulation of Hox transcriptional activation functions may underlie the different functional specificities of proteins belonging to this developmental patterning family.

Keywords: *Deformed*/*Drosophila*/homeotic/*Hox*

Introduction

During the embryonic development of multicellular organisms, thousands of transcription factors are required to generate elaborate temporal and spatial patterns of gene expression. The homeodomain proteins represent a large and important class of transcription factors underlying such patterning decisions (Gehring *et al*., 1994). The founding members of the homeodomain class, the Hox proteins, assign positional identities along the embryonic body axis in animals ranging from arthropods to vertebrates (McGinnis and Krumlauf, 1992; Lawrence and Morata, 1994; Manak and Scott, 1994) and provide a paradigm to study mechanisms of morphological evolution (Carroll, 1995).

Different Hox proteins can direct cells onto markedly different developmental pathways, even though they possess similar *in vitro* DNA binding preferences as monomers (Laughon, 1991; Dessain *et al*., 1992; Ekker *et al*., 1994). Many types of evidence suggest that diversity of Hox function *in vivo* is crucially dependent on cofactors (Kornberg, 1993; Mann and Chan, 1996). For example, simple arrays of Hox-binding sites are not enough to generate specific response elements in embryonic cells (Vincent *et al*., 1990; Gross and McGinnis, 1995). Instead, Hox-binding sites in naturally occurring Hox response elements are flanked by DNA sequences that contain required cofactor-binding sites (Mann and Chan, 1996; Biggin and McGinnis, 1997).

A key issue then arises of how Hox proteins act together with co-factors to generate specific functions. One possibility is that cofactors act by increasing the DNA-binding specificity of Hox proteins. In this 'selective binding' model, the crucial step is to target Hox proteins to a subset of their potential DNA-binding sites. This has been proposed to be the function of the Extradenticle (Exd)/Polybithorax family of the homeodomain proteins (Peifer and Wieschaus, 1990; Rauskolb *et al*., 1993; van Dijk *et al*., 1993). Consistent with this idea, Exd/Pbx proteins can interact with most Hox proteins and raise Hox binding affinity on specific DNA sites (Chan *et al*., 1994; van Dijk and Murre, 1994; Chang *et al*., 1995; Pöpperl *et al*., 1995; Neuteboom and Murre, 1997). In addition, specific Exd–Hox composite sites do exist in some naturally occurring Hox regulatory elements and are required for Hox-dependent activation (Mann and Chan, 1996).

Not surprisingly, naturally occurring Hox regulatory elements also contain functionally important high affinity Hox-binding sites that are not closely juxtaposed to high affinity Exd half sites (Regulski *et al*., 1991; Capovilla *et al*., 1994; Chan *et al*., 1994; Zeng *et al*., 1994; Manak *et al*., 1995; Pöpperl *et al*., 1995; Sun *et al*., 1995). Can Hox proteins bind *in vivo* to these sites independently of Exd/Pbx proteins or other cofactors? Some recent evidence is consistent with such a possibility. For example, *in vivo* UV cross-linking has been used to show that the *Drosophila* homeodomain proteins Eve and Ftz, close relatives of the Hox family, have binding specificities in embryos that are very similar to their monomer binding specificities *in vitro* (Walter *et al*., 1994; Walter and Biggin, 1996). Recent results have also indicated that the phosphorylation state of the Hox protein Antp strongly influences its biological activities in embryos (Jaffe *et al*., 1997). Thus it is possible that Hox proteins may occupy a very wide range of binding sites *in vivo*, and have their activities instead of their binding occupancies regulated on response elements. In this 'activity regulation' model, cofactors such as Exd/Pbx might act by converting Hox proteins

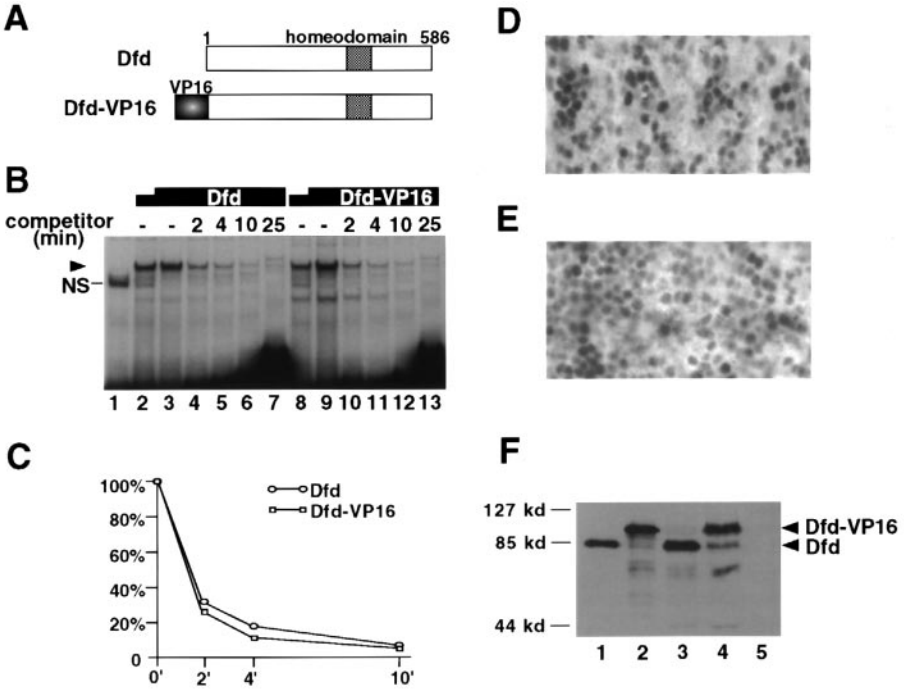

Fig. 1. Dfd and Dfd-VP16 DNA-binding properties and expression in *Drosophila* embryonic cells. (**A**) Diagram of Dfd and Dfd-VP16 proteins. (**B**) Binding of Dfd and Dfd-VP16 to the 2×D site (see Figure 4 for sequence) in EMSAs. The amount of proteins in each lane was as follows: lane 1, 2 μl of reticulocyte lysate; lane 2, 1 μl of Dfd and 1 μl of reticulocyte lysate; lanes 3–7, 2 μl of Dfd; lane 8, 1 μl of reticulocyte lysate and 1 μl of Dfd-VP16; lanes 9–13, 2 μl of Dfd-VP16. In lanes 4–7 and 10–13, after the binding reaction, 500-fold unlabeled 2×D site was added as the specific competitor for the indicated times. The arrowhead denotes shifted complexes of the 2×D site with Dfd or Dfd-VP16. NS represents non-specific complexes formed by the reticulocyte lysate. (**C**) Quantitation of the dissociation result in (B). Binding of Dfd and Dfd-VP16 in the absence of the competitor is taken as 100% (time point 0) and compared with the binding in the presence of the competitor at various time points. (D and E) Immunostaining of Dfd (**D**) and Dfd-VP16 (**E**) in *arm*-GAL4^4/UAS-Dfd (D) and *arm*-GAL4^4/UAS-Dfd-VP16 (E) embryos with anti-Dfd antibodies. Each field shows a close-up of the lateral epidermis of a stage 13 embryo. Notice the variable levels, but near-ubiquitous expression pattern for both proteins, and the nuclear localization of both proteins. (**F**) Immunoblotting of Dfd and Dfd-VP16 with anti-Dfd antibodies: lane 1, *in vitro*-translated Dfd; lane 2, *in vitro*-translated Dfd-VP16; lanes 3–5, embryonic extracts from *arm*-GAL4^4/UAS-Dfd (lane 3), *arm*-GAL4^4/UAS-Dfd-VP16 (lane 4) and wild-type (lane 5) embryos.

from neutral states into states capable of transcriptional activation or repression (Biggin and McGinnis, 1997; Pinsonneault *et al*., 1997).

Here we test whether the function of the *Drosophila* Hox protein Deformed (Dfd) is 'activity regulated'. Wild-type Dfd protein does not activate reporter transcription from simple Dfd-binding sites in either tissue culture cells or embryonic cells. However, Dfd fused to a constitutive activation domain from VP16 can activate transcription through these simple Dfd-binding sites. This strongly suggests that the simple binding sites can be occupied by wild-type Dfd, but the normal protein does not provide detectable transcriptional activation from these sites. We design regulatory elements that allow an uncoupling of the binding enhancement effects of Exd from its proposed role in activity regulation, and provide evidence that Exd function is required to release a covert transcriptional activation function in the Dfd protein. We further find that the Dfd activation domain function in transfected cells is inhibited by the presence of the Dfd homeodomain in the same polypeptide.

Results

Function of Dfd and Dfd-VP16 in Drosophila embryos

The activity regulation model predicts that Hox proteins can bind to even high affinity sites without transcriptionally activating adjacent promoters. One way to detect the presence of such non-functional binding is to generate a protein variant (Jimenez *et al*., 1996) that to some extent can bypass the normal requirement for cofactors to activate transcription. To this end, we attached a VP16 activation domain (Triezenberg *et al*., 1988) to the N-terminus of Dfd to generate a Dfd-VP16 protein (Figure 1A). The addition of the VP16 domain does not influence the DNA-binding function of the fusion protein detectably. In electrophoretic mobility shift assays (EMSAs) shown in Figure 1B, both Dfd and Dfd-VP16 proteins exhibited similar binding affinities for a Dfd-binding site (D site; Regulski *et al*., 1991). When protein–DNA complexes were challenged by high levels of unlabeled D-site oligo-nucleotides, both Dfd and Dfd-VP16 also exhibited very similar dissociation rates (Figure 1B, compare lanes 3–7 with lanes 9–13, and Figure 1C).

Both the *Dfd* and *Dfd-VP16* genes were then introduced into the *Drosophila* genome, and conditionally expressed in embryos using the GAL4/UAS system (Brand and Perrimon, 1993). After induction via the ubiquitously expressed *armadillo* (*arm*)-GAL4^4 driver, both Dfd and Dfd-VP16 proteins are localized properly in nuclei (Figure 1D and E) and accumulate to similar levels (Figure 1F) as detected by anti-Dfd antibodies. Interestingly, in the *arm*-GAL4^4/UAS-Dfd-VP16 embryonic extract, the anti-Dfd antibodies detect not only Dfd-VP16 but also a protein that corresponds to Dfd in size (Figure 1F, lanes 3 and

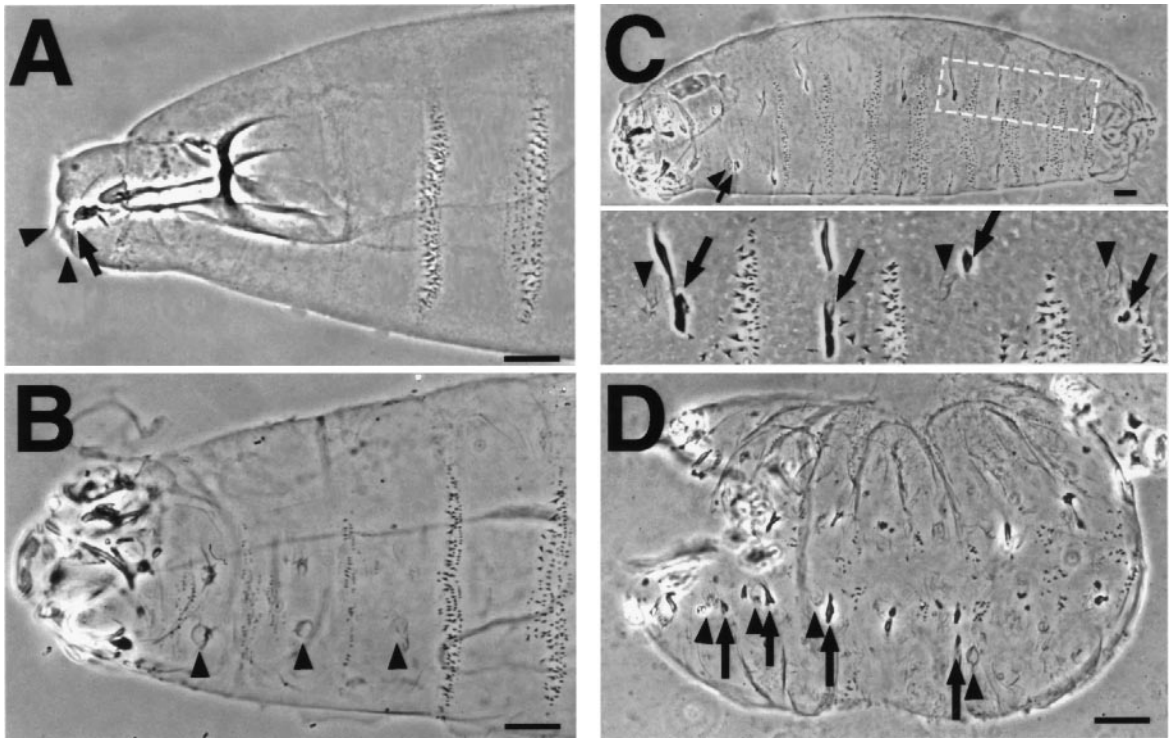

Fig. 2. Cuticular phenotypes induced by ectopic expression of Dfd and Dfd-VP16. (**A**) Anterior cuticle of a wild-type embryo. (**B**) Anterior cuticle of an embryo from a cross of female UAS-Dfd and male *arm*-GAL4^4 parents. (**C**) Cuticle of an embryo from a cross of male UAS-Dfd and female *arm*-GAL4^4 parents. (**D**) Cuticle of a *arm*-GAL4^4/UAS-Dfd-VP16 embryo. Ventral views of the cuticles are shown, with anterior to the left. Arrowheads point to cirri, either normal (A) or ectopic (B–D), and arrows point to mouth hook cuticle, either normal (A) or ectopic (C and D).

4). Using the same immunoblotting conditions, Dfd protein is not detectable in the wild-type embryonic extract (Figure 1F, lane 5), presumably due to the wild-type pattern of Dfd expression in only a small percentage of embryonic cells (Jack *et al*., 1988). We believe that the extra band in the Dfd-VP16 lane represents the widespread activation of the endogenous *Dfd* gene due to Dfd-VP16's ability to mimic the autoregulatory function of Dfd (Kuziora and McGinnis, 1988).

In wild-type *Drosophila* embryos, *Dfd* is expressed in maxillary and mandibular head segments and is necessary for the development of maxillary and mandibular structures such as mouth hooks, cirri and lateralgräten (Merrill *et al*., 1987; Regulski *et al*., 1987; Jack *et al*., 1988). Both cirri and mouth hook fragments are ectopically produced in the labial and thoracic segments after uniform expression of *Dfd* under heat shock promoter control (Kuziora and McGinnis, 1988). The same ectopic structures are induced after ubiquitous and persistent UAS-Dfd expression by the *arm*-GAL4^4 driver (Figure 2A–C). Interestingly, when the *arm*-GAL4^4 driver is provided paternally, the development of ectopic maxillary structures is restricted largely to the labial and thoracic segments (Figure 2B). However, when the *arm*-GAL4^4 driver is provided maternally, ectopic maxillary structures are produced throughout the trunk, including the abdominal segments (Figure 2C). This may result from higher and/or earlier levels of *Dfd* expression induced by the maternally expressed *arm*-GAL4^4 driver. Whether the driver is maternally or paternally derived, the number and segment-specific features of the thoracic and abdominal cuticle develop in a nearly normal manner. Thus, persistent, abundant accumulation of Dfd protein is capable of generating Dfd-dependent structures in all segments without severely disrupting other developmental pathways.

Ubiquitous expression of Dfd-VP16 results in extreme morphological defects in cuticular features along the entire A/P axis, whether the *arm*-GAL4^4 driver is provided paternally or maternally (Figure 2D). The *arm*-GAL4^4/UAS-Dfd-VP16 embryos are less than half the length of normal embryos, due to a dramatic narrowing and partial fusion of many body segments. Each of these rudimentary segments develops *Dfd*-dependent maxillary cirri and fragments of maxillary mouth hooks. The ectopic maxillary structures indicate that both Dfd-VP16 protein and Dfd protein regulate many of the same downstream genes in embryos. The segments in Dfd-VP16 embryos develop only fragments of the normal head and trunk structures, which in the abdominal region corresponds to only a few denticles per segment. The rudimentary nature of the segments indicates that ectopic Dfd-VP16 protein disrupts many developmental pathways of the head, thorax and abdomen that are unaffected by ectopic Dfd.

The *arm*-GAL4^4/UAS-Dfd-VP16 phenotype might be explained by the ability of the hybrid protein to activate the normal target genes of Dfd ectopically in many more cells than normal or at much higher levels within those cells. Normal downstream genes of Dfd (O'Hara *et al*., 1993; Vanario-Alonso *et al*., 1995), such as *paired* (*prd*) (Figure 3A–C) and *Distalless* (*Dll*) (data not shown), are indeed activated in a few more embryonic cells by Dfd-VP16 than by wild-type Dfd, especially in abdominal regions. However, it seems unlikely that such modest differences account for the dramatic differences in embryonic phenotypes between UAS-Dfd and UAS-Dfd-VP16.

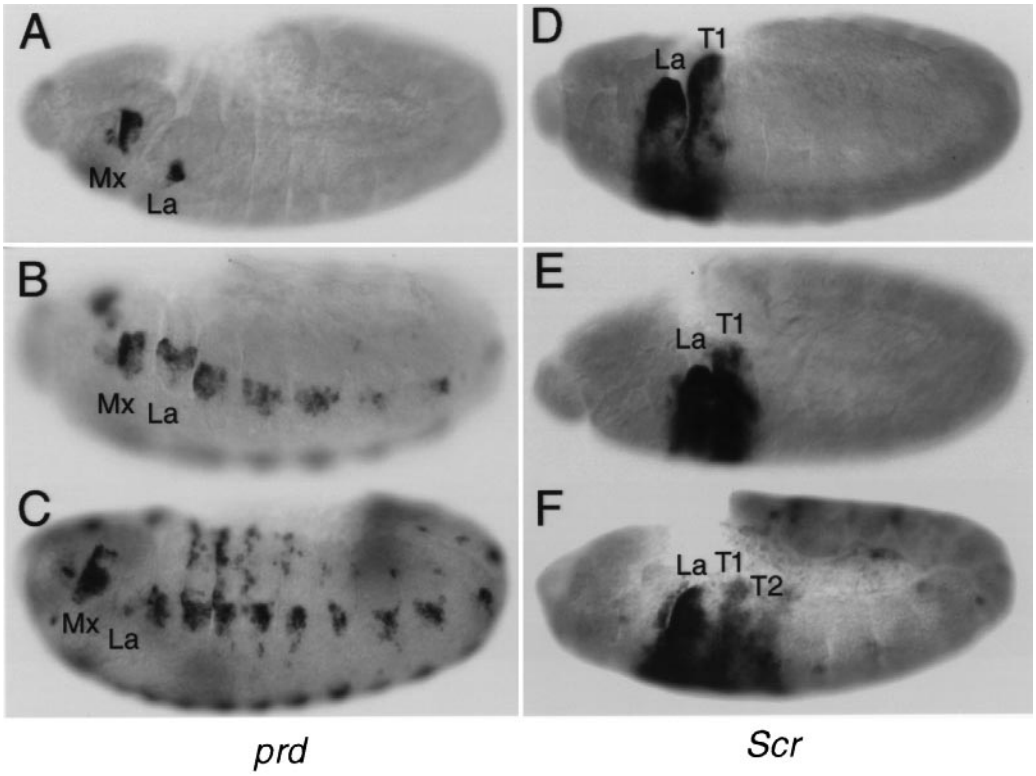

Fig. 3. Regulation of *prd* and *Scr* by UAS-Dfd and UAS-Dfd-VP16. (A–C) Lateral–ventral views of embryos hybridized with a *prd* probe. (**A**) In wild-type embryos, *prd* is expressed in maxillary (Mx) and labial (La) segments. (**B** and **C**) In both *arm*-GAL4^4/UAS-Dfd (B) and *arm*-GAL4^4/UAS-Dfd-VP16 embryos (C), *prd* is ectopically activated in the trunk segments. (D–F) Lateral–ventral views of embryos hybridized with a *Scr* probe. (**D**) In wild-type embryos, *Scr* is expressed in labial (La) and first thoracic (T1) segments. (**E**) In *arm*-GAL4^4/UAS-Dfd embryos, the *Scr* expression pattern remains wild-type. (**F**) In *arm*-GAL4^4/UAS-Dfd-VP16 embryos, *Scr* is activated in ectopic locations. All embryos are at stages 11 or 12, with anterior to the left.

The Dfd-VP16 protein might also be able to activate transcription of genes that are not normally regulated by Dfd. We tested for changes in the expression pattern of a variety of embryonic patterning genes that are not normally regulated by Dfd. Some gene expression patterns, e.g. *Antennapedia* (*Antp*) and *teashirt* (*tsh*), were unaffected by either Dfd or Dfd-VP16 (data not shown). However, *Sex comb reduced* (*Scr*) expression is ectopically activated by Dfd-VP16 but unaffected by normal Dfd (Figure 3D–F). Although we do not know whether *Scr* activation results directly from binding of Dfd-VP16 to its promoter, this result is consistent with the idea that in normal embryos Dfd protein also binds to the regulatory regions of some non-target genes without regulatory output. These non-target genes are possibly vulnerable to activation by the stronger Dfd-VP16 activator upon its binding, which may lead to the severe disruptions in development seen in Dfd-VP16 embryos.

Neutrality of Dfd on simple binding sites in Drosophila embryos

In view of the complexity of regulatory inputs influencing a gene such as *Scr*, we next decided to make use of simple regulatory elements to explore further the connections between binding affinity and activation function on Dfd targets. Previous experiments have indicated that *cis*-regulatory elements consisting solely of multimerized Dfd-binding sites are not activated in a Dfd-dependent fashion in embryonic maxillary cells (Gross and McGinnis, 1995). To test further the hypothesis that Dfd binding *per se* is inherently neutral in embryos, we decided to test whether high levels of Dfd or Dfd-VP16 proteins could activate transcription through simple Dfd recognition sites. *In vitro*, a DNA sequence consisting of two tandem copies of the simple D site (2×D), is bound by Dfd with high affinity but not detectably bound by Exd (Figure 4A and B, lanes 1–4). The affinity of Dfd protein for the 2×D-site is not enhanced by the inclusion of Exd protein (Figure 4B, compare lanes 2 and 3 with 5 and 6).

Next, integrated transgenes consisting of the 2×D sequence upstream of a *LacZ* reporter gene were combined with either *UAS-Dfd* or *UAS-Dfd-VP16* accompanied by *arm*-GAL4^4 or *hsp70*-GAL4 drivers in *Drosophila* embryos. The 2×D sequence does not induce detectable *LacZ* expression in the maxillary cells that accumulate Dfd protein in wild-type embryos (Figure 5A). When an *hsp70*-GAL4 driver induces UAS-Dfd expression, the 2×D reporter is active in only a few amnioserosal cells (Figure 5B). However, when the same *hsp70*-GAL4 driver uniformly expresses UAS-Dfd-VP16 in these embryos, the 2×D reporter is activated at high levels in all aminoserosal cells, and in a subset of embryonic epidermal cells (Figure 5C). As expected, a similar pattern of 2×D reporter activity is detected in Dfd-VP16 embryos that lack both maternal and zygotic *exd* gene function (Figure 5D), indicating that the regulatory effect of Dfd-VP16 on 2×D does not require the function of Exd protein. *hsp70*-GAL4 driven expression of Dfd-VP16 has a detectable effect on 2×D only in a subset of embryonic cells after stage 11 of embryogenesis. In addition, the level of Dfd-VP16

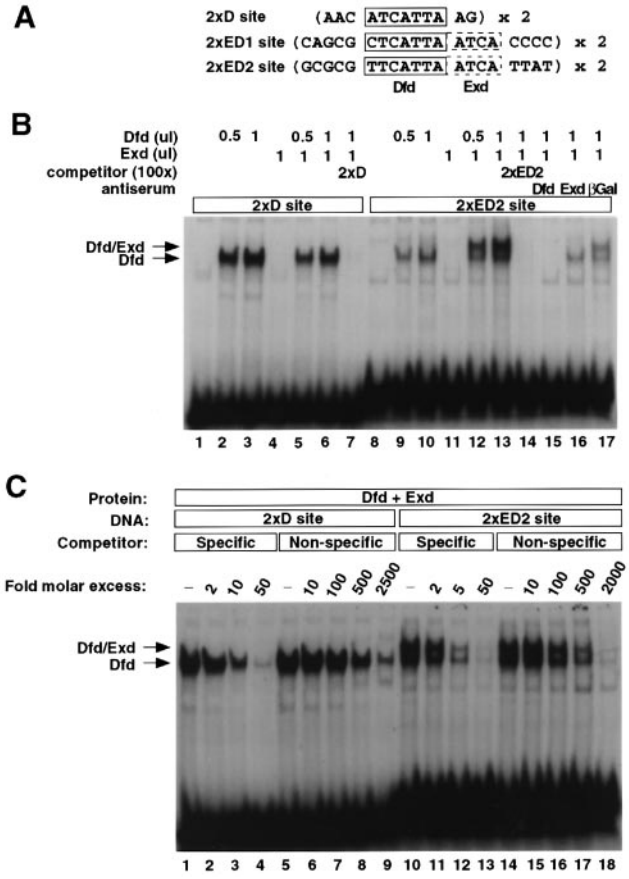

Fig. 4. Binding of Dfd and Exd to 2×D and 2×ED sites. (**A**) Sequences of the 2×D, 2×ED1 and 2×ED2 sites. The core of the binding sites for Dfd and Exd proteins (Dessain *et al*., 1992; Chan *et al*., 1997) are indicated with solid and dotted boxes, respectively. (**B**) Binding of the 2×D and 2×ED2 sites by Dfd and Exd proteins from reticulocyte lysate. The composition of the binding reactions was as indicated on top of the EMSA lanes. Reticulocyte lysate amounts were adjusted so that a total of 2 μl of lysate was present in each binding reaction. (**C**) Competitive binding of Dfd and Exd to the 2×D and 2×ED2 sites in the presence of specific and non-specific oligos (see Materials and methods). All reactions included 1 μl of Dfd lysate and 1 μl of Exd lysate.

expression driven by the *arm*-GAL4[4] driver is not sufficient for activation of the 2×D reporter (data not shown), suggesting that Dfd-VP16 activates transcription only at high concentrations, and that the *arm*-GAL4[4] driver provides lower levels of GAL4 expression than the *hsp70*-GAL4 driver. The limited ability of Dfd-VP16 to activate the 2×D reporter gene in embryos is consistent with the VP16 activation function being context dependent when attached to this Hox protein (see Discussion). However, these results also suggest that D sites are accessible to Dfd protein in amnioserosal and epidermal cells, but that wild-type Dfd protein is incapable of activating transcription from D sites in those cells even at the high concentrations produced after heat-shock induction.

Activity regulation of Dfd and Dfd-VP16 by Exd

To assay whether Exd protein might contribute to the activity regulation of Dfd, we tested the embryonic function of two variations of the D site reporter constructs which contain two tandem copies of a core sequence that Dfd and Exd bind together (2×ED1 and 2×ED2 sites, Figure 4A and B; Chan *et al*., 1997). The DNA binding and *in vivo* functional characteristics for 2×ED1 and 2×ED2 are very similar; therefore, only the results for 2×ED2 are shown. *In vitro*, the 2×ED2 site is bound weakly by Dfd protein alone, but is not bound detectably by Exd alone (Figure 4B, lanes 8–11). Binding of Dfd to the 2×ED2 site is enhanced in the presence of Exd as shown by the formation of an abundant complex that contains Dfd, Exd and 2×ED2 (Figure 4B, lanes 12 and 13). The evidence that the complex contains both Dfd and Exd rests on the ability of either anti-Exd or anti-Dfd antibodies specifically to abolish the formation of the complex (Figure 4B, lanes 15–17). The affinity of the Dfd–Exd heterodimer for the 2×ED2 site is approximately the same as the affinity of the Dfd protein alone for the 2×D site (Figure 4B, compare lanes 2 and 3 with 12 and 13).

To quantify further the binding specificity of 2×D and 2×ED2 sites, we measured the ability of Dfd + Exd to recognize specific versus non-specific binding sites. This was done by pre-incubating Dfd and Exd proteins with progressively higher amounts of unlabeled specific binding site DNA (2×D or 2×ED2 oligos) or non-specific DNA, before adding ^{32}P-labeled 2×D or 2×ED2 sites to the binding reactions (Figure 4C). For the labeled 2×D site, a 4-fold excess of unlabeled 2×D competitor is required to reduce the Dfd–[^{32}P]2×D complex to one-half normal levels, whereas a 325-fold excess of non-specific competitor is needed to achieve the same reduction of Dfd binding to the 2×D site. Thus, Dfd can distinguish between the 2×D site and unrelated DNA by a factor of 80. Similar experiments with Dfd in the presence of Exd indicate that Dfd–Exd can distinguish the 2×ED2 site from unrelated DNA by a factor of 100 (Figure 4C). Taken together, these binding studies indicate that the 2×ED2 site has approximately the same selective affinity for Dfd protein in the presence of Exd protein as the 2×D site has for Dfd protein in the presence or absence of Exd protein. Therefore, any large regulatory differences between the embryonic activity of Dfd on the 2×D and 2×ED2 reporters cannot be attributed to the small differences in their Exd-dependent *in vitro* binding on the two sites.

In wild-type embryos, the 2×ED2 reporter gene is expressed in the dorsal ridge and in a few non-epidermal cells of the maxillary/mandibular region (Figure 5E). These cells comprise a small subset of the cells that accumulate Dfd protein in the maxillary/mandibular region (Jack *et al*., 1988). When UAS-Dfd is ubiquitously expressed via the *arm*-GAL4[4] driver, the 2×ED2 reporter is activated additionally in amnioserosal cells and a few scattered cells in the germ band (Figure 5F). Since Exd protein is localized in the nuclei of these and most other embryonic cells at this stage (Aspland and White, 1997; Mann and Abu-Shaar, 1997; Azipiazu and Morata, 1998), the amnioserosal and germ band expression presumably represents the concerted regulatory effect of Dfd and Exd proteins on 2×ED2. In contrast, the ectopic expression of Dfd-VP16 via the *arm*-GAL4[4] driver activates the 2×ED2 reporter gene in many more embryonic cells derived from all three germ layers (Figure 5G). In Dfd-VP16 embryos lacking both maternal and zygotic *exd* gene function, the 2×ED2 reporter is not activated (Figure 5H). Therefore,

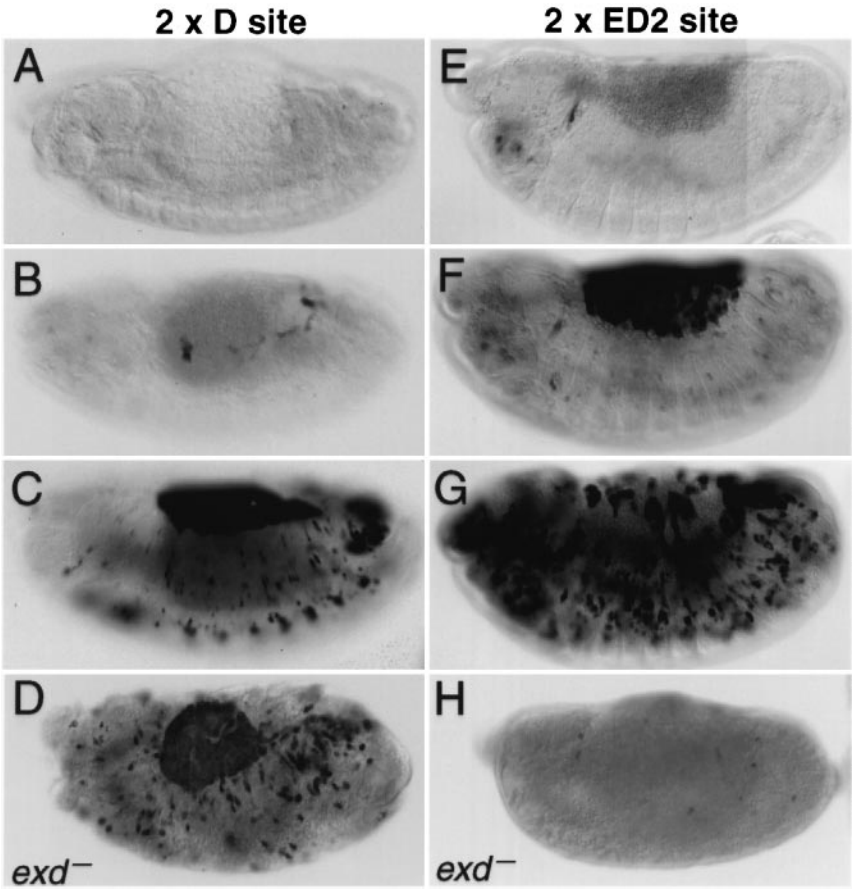

Fig. 5. Regulation of the 2×D and 2×ED2 site reporters by UAS-Dfd and UAS-Dfd-VP16. Immunostaining with anti-β-galactosidase antibodies shows the activity of the 2×D site (A–D) and 2×ED2 site (E–F) reporters in embryos. Embryos are shown at stage 13 from a dorsal lateral aspect with anterior to the left. (A–D) Expression of the 2×D site reporter in wild-type (**A**), after ectopic expression of UAS-Dfd by *hsp70*-GAL4 (**B**), after ectopic expression of UAS-Dfd-VP16 by *hsp70*-GAL4 (**C**) and after ectopic expression of UAS-Dfd-VP16 by *hsp70*-GAL4 in an *exd* maternal/zygotic mutant background (**D**). (E–H) Expression of the 2×ED2 site reporter in wild-type (**E**), after ectopic expression of UAS-Dfd by *arm*-GAL4 (**F**), after ectopic expression of UAS-Dfd-VP16 by *arm*-GAL4 (**G**) and after ectopic expression of UAS-Dfd-VP16 by *arm*-GAL4 in an *exd* maternal/zygotic mutant background (**H**).

the activation function of Dfd-VP16 on 2×ED2 requires the function of Exd protein.

We also tested if higher levels of Dfd or Dfd-VP16 driven by the *hsp70*-GAL4 driver can mediate even stronger activation on 2×ED2. In such embryos, the 2×ED2 reporter gene is activated in patterns similar to that in *arm*-GAL4^4/UAS-Dfd or *arm*-GAL4^4/UAS-Dfd-VP16 embryos (data not shown).

The foregoing results can be used for two interesting comparisons. In the first, we compare the amount of 2×ED2 activation provided by Dfd-VP16 with that provided by Dfd. Since Dfd-VP16 mediates activation on 2×ED2 in a large number of cells (Figure 5G) whereas Dfd activates the 2×ED2 reporter in only a small number of cells (Figure 5F), we believe that Dfd and Exd can co-occupy the 2×ED2 in a large number of cells. Such occupancy results in 2×ED2 activation in only a subset of those cells, further supporting the idea that Dfd is held in a neutral state in most cells on Dfd–Exd composite binding sites, even when Exd is present. In the second, we compare the function of 2×ED2 with that of 2×D. The 2×ED2 site is activated in many cells by Dfd-VP16 induced by the *arm*-GAL4 driver (Figure 5G) whereas the 2×D site is activated only in some cells at limited stages by Dfd-VP16 provided by the even stronger *hsp70*-GAL4 driver (Figure 5C). Since the Exd-dependent 2×ED2 site and the Exd-independent 2×D site each possess very similar *in vitro* Dfd-binding affinities in the presence of Exd, we conclude that the abundant activation of the 2×ED2 reporter by Dfd-VP16 results from an enhancement of the activation function of Dfd-VP16 by Exd on the 2×ED2 site. Similarly, Dfd activates the 2×ED2 reporter in more cells (Figure 5F) than the 2×D reporter (Figure 5B), suggesting that Dfd activity is also enhanced by Exd on the 2×ED2 site.

Regulated activity of Dfd in transfected cells

To gain further evidence of Dfd activity regulation, Dfd and Dfd-VP16 proteins were expressed in mouse NIH 3T3 cells and assayed for their ability to activate a luciferase reporter gene attached to a promoter containing the 2×D site. Both Dfd and Dfd-VP16 proteins accumulate to approximately the same levels in cells as measured by immunoblotting of transfected cell lysates with anti-Dfd antibodies (data not shown). As shown in Figure 6A, the Dfd protein does not activate transcription through the 2×D sites. In contrast, Dfd-VP16 activates the 2×D reporter gene ~5-fold when compared with controls. This activation is mediated by the 2×D site, as a reporter gene lacking the 2×D site in its promoter is not activated. The

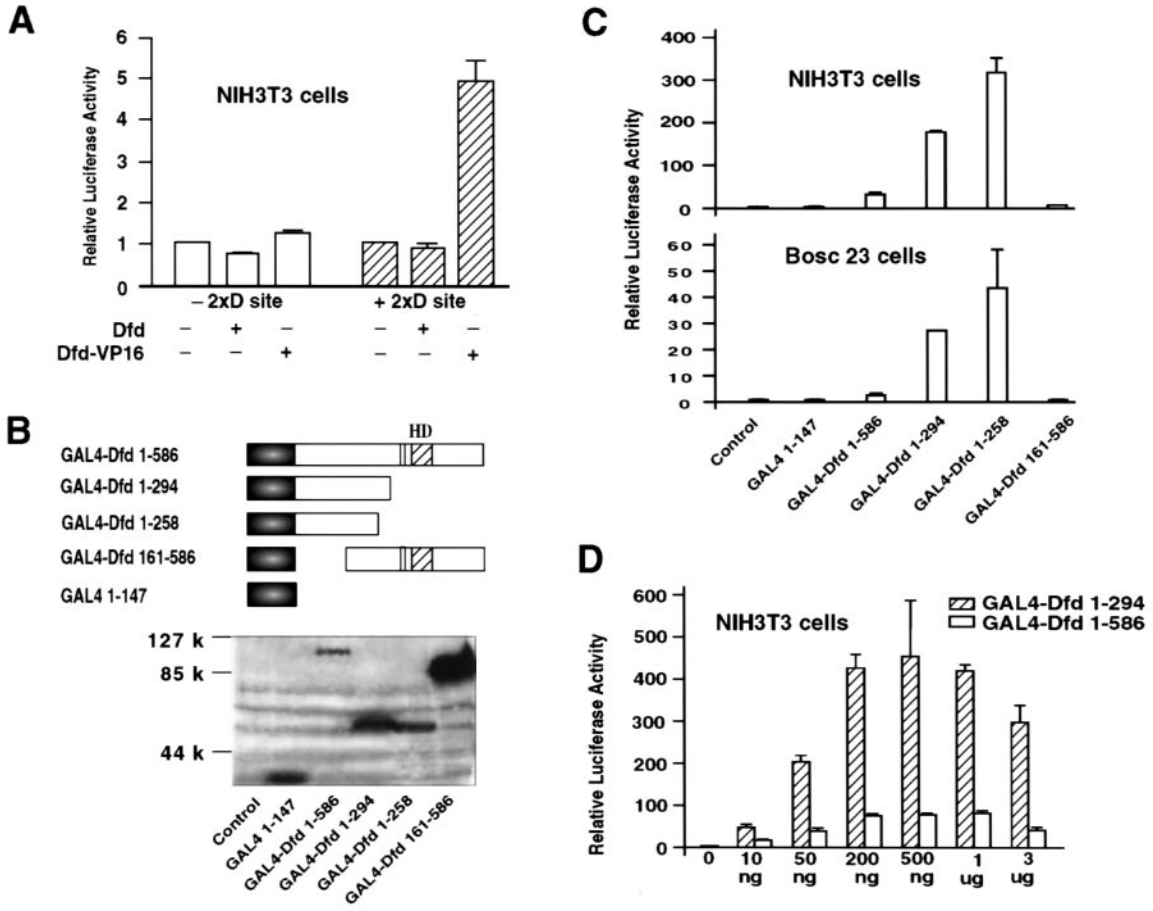

Fig. 6. Regulated activity of Dfd in tissue culture cells. (**A**) Transfection of Dfd and Dfd-VP16 in NIH 3T3 cells; 3 μg of Dfd or Dfd-VP16 expression plasmid was transfected. Their transcriptional activation on luciferase reporters with or without the 2×D site is reported in relative luciferase enzymatic activities. (**B**) Diagram of GAL4-Dfd fusion proteins (upper) and their relative protein levels in transfected Bosc 23 cells (lower) visualized by immunoblotting with a monoclonal antibody against the GAL4 DNA-binding domain. HD, homeodomain. (**C**) Transactivation of GAL4-Dfd fusions on the 5×GAL4-Luc reporter in NIH 3T3 (upper) and Bosc 23 (lower) cells. Expression plasmids were used at 50 ng for transfection of NIH 3T3 cells and at 17 ng for transfection of Bosc 23 cells. Notice that GAL4-Dfd 1–586 mediates much lower activation than GAL4-Dfd 1–258 and 1–294. (**D**) Dosage effects of GAL4-Dfd 1–294 and GAL4-Dfd 1–586 on the activation of the 5×GAL4-Luc reporter in transfected NIH 3T3 cells. In each transfection, the total amount of transfected DNA is adjusted to 5 μg with control plasmid pXS.

activation response provided by Dfd-VP16, combined with the same binding properties of Dfd and Dfd-VP16 (see Figure 1B and C), suggests that as in *Drosophila* embryos, Dfd protein occupies 2×D-binding sites in tissue culture cells, but has no effect on transcription of the adjacent reporter gene.

The normal Dfd protein does have an autonomous activation domain in the N-terminal half of the protein, which is capable of activating transcription when fused to the GAL4 DNA-binding domain in tissue culture cells (Zhu and Kuziora, 1996). Thus, the neutrality of Dfd in both embryos and tissue culture cells suggests that in the natural Dfd protein the activation domain might be prevented from activating transcription. To test this hypothesis, we fused different Dfd-coding regions in-frame to the GAL4 DNA-binding domain (GAL4 1–147) (Figure 6B) and analyzed their ability to activate a luciferase reporter gene from a promoter containing five copies of the GAL4-binding site (5×GAL4-Luc). In both tested cell lines, NIH 3T3 and Bosc 23, fusion proteins including the N-terminal 294 or 258 amino acids of Dfd (GAL4-Dfd 1–294 and GAL4-Dfd 1–258) mediate strong activation of the reporter gene, whereas a fusion containing the Dfd residues 161–586 (GAL4-Dfd 160–586) does not activate transcription (Figure 6C). These results confirm that the Dfd N-terminal half contains a transcriptional activation domain. However, when the entire Dfd-coding region is included in the fusion (GAL4-Dfd 1–586), it mediates much lower activation than the GAL4-Dfd activation domain fusion proteins (Figure 6C). Presumably, the lower activation function for GAL4-Dfd 1–586 is due partially to the lower levels that accumulate in cells when compared with GAL4-Dfd 1–294 or GAL4-Dfd 1–258 (Figure 6B). However, there is not a good correlation in these constructs between the amount of protein that accumulates and the amount of activation function, so this may not be the entire explanation (Figure 6B and C). In addition, GAL4-Dfd 1–586 has minimal effects on reporter gene expression even when much larger amounts of the expression construct are transfected into cells (Figure 6D). We concluded that the activation domain of Dfd was highly functional only when separated from the C-terminal half of the protein. This might be due to lower accumulation of GAL4 hybrids containing full-length Dfd protein, to suppression of activation domain function in the context of the full-length protein or to both.

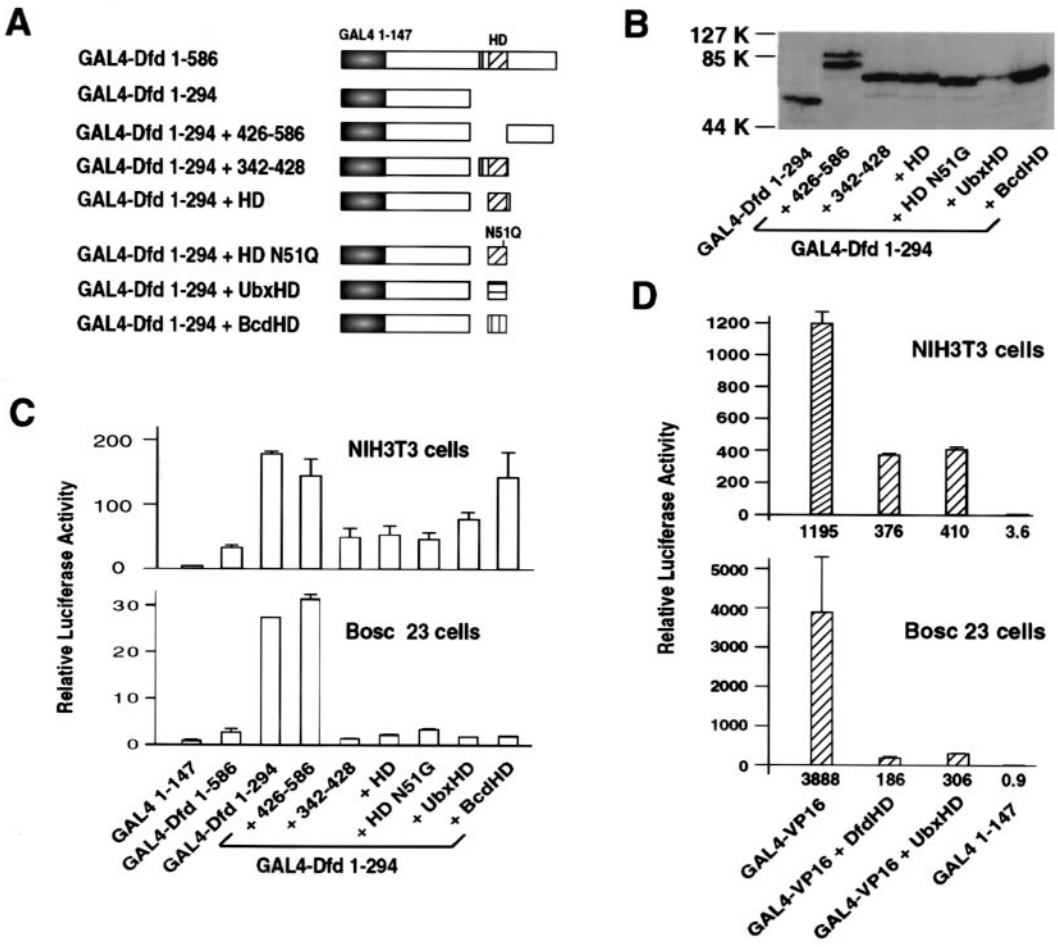

Fig. 7. An inhibitory role for the Dfd homeodomain on gene activation. (**A**) Diagram of GAL4-Dfd fusions. HD, homeodomain. (**B**) The relative protein levels of the GAL4-Dfd fusions in transfected Bosc 23 cells as visualized by immunoblotting with a monoclonal antibody against the GAL4 DNA-binding domain. (**C**) Transfection of GAL4-Dfd fusions in NIH 3T3 (top) and Bosc 23 (bottom) cells. (**D**) The effect of Dfd and Ubx homeodomains on the function of the VP16 activation domain. In each transfection (C and D), expression plasmids were used at 50 ng for transfection of NIH 3T3 cells and at 17 ng for transfection of Bosc 23 cells.

The Dfd homeodomain masks the Dfd activation function

Based on the hypothesis that some C-terminal sequences of Dfd protein mask its activation potential, different C-terminal regions were attached to the GAL4-Dfd 1–294 and tested for their effects on the ability of this construct to activate transcription (Figure 7A). All of the control and GAL4-Dfd 1–294-C-term domain variants accumulate to similar levels in transfected cells (Figure 7B). The attachment of the Dfd residues 426–586 has no effect on the activation function of GAL4-Dfd 1–294 (Figure 7C). However, two Dfd fragments containing either the homeodomain alone (365–434), or the YPWM motif region plus the homeodomain (342–428), significantly suppress the function of the Dfd activation domain in both NIH 3T3 and Bosc 23 cells (Figure 7C).

Since the homeodomain is a DNA-binding domain, its suppressive effect might arise from a sequestration of the fusion proteins to DNA sites other than those in the reporter constructs. To test this possibility, a mutant form of the Dfd homeodomain was generated with residue 51 changed from asparagine to glutamine (N51Q). Such a mutation has been shown to abolish the *in vitro* DNA-binding function and *in vivo* genetic function of a variety of homeodomain proteins (Hanes and Brent, 1991; Bertuccioli *et al*., 1996). In our suppression assay, the N51Q mutant is not distinguishable from a wild-type Dfd homeodomain (Figure 7C). Therefore, the Dfd homeodomain suppresses the function of the Dfd activation domain in a manner that is independent of both DNA-binding function and the stability of the hybrid proteins in transfected cells.

To test the specificity of the Dfd homeodomain suppression function, we assayed the suppressing effects of two other homeodomains. The first, Ultrabithorax (Ubx), is a close relative of Dfd in the Hox class. The other, Bicoid (Bcd), which acts as a morphogen in the *Drosophila* egg to establish the anterior–posterior axis (Driever, 1993), shares much less sequence similarity with Dfd. The Ubx homeodomain can suppress the Dfd activation function in both NIH 3T3 and Bosc 23 cells (Figure 7C). The Bcd homeodomain only shows slight suppression of Dfd activation function in NIH 3T3 cells, but in Bosc 23 cells it strongly suppresses Dfd activation function (Figure 7C). Thus, the suppressive function of the homeodomain is not specific to Dfd, but may be a more general property of Hox and other classes of homeodomains that is sensitive to the cellular context in which they are produced.

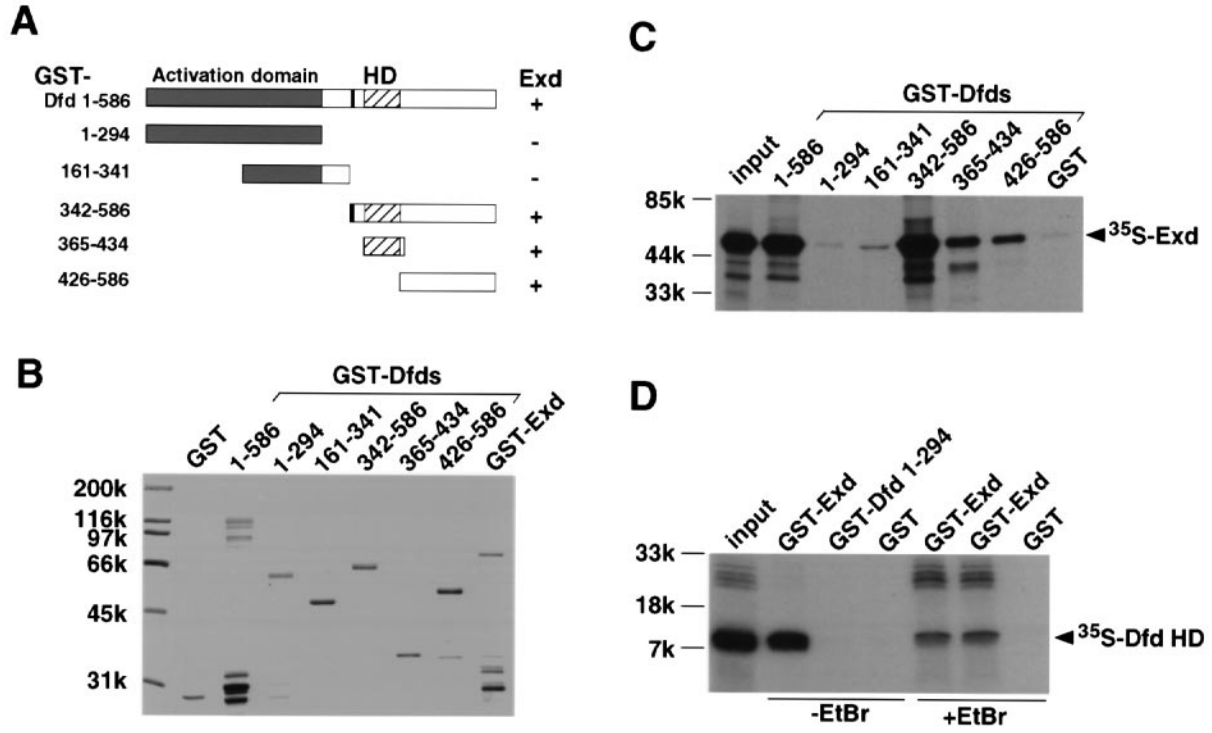

Fig. 8. Interaction of the Dfd homeodomain with Exd. (**A**) Diagram of GST–Dfd fusion proteins. Only the Dfd portions are depicted. Their abilities to interact with Exd are shown on the right. HD, homeodomain. (**B**) The relative amounts of GST–Dfd and GST–Exd fusion proteins that were bound to glutathione beads were assayed by being separated on a 10% SDS–polyacrylamide gel and then stained with Coomassie Blue. (**C**) Interaction of GST–Dfd fusions with ^{35}S-labeled Exd. The input lane represents 10% of the amount of the Exd protein used in the binding reactions. (**D**) Interaction of ^{35}S-labeled Dfd homeodomain (HD) with GST–Exd and GST–Dfd 1–294 in the absence or presence of ethidium bromide (EtBr). The input lane represents 25% of the amount of the Dfd HD peptide used in the binding reactions.

We further tested whether the Dfd homeodomain could suppress the function of other activation domains such as the VP16 activation domain. When homeodomains from Dfd or Ubx were attached to the GAL4-VP16 activation domain fusion, activation from GAL4-VP16 is reduced 3-fold in NIH 3T3 cells and as much as 20-fold in Bosc 23 cells (Figure 7D). However, even with this homeodomain-dependent suppression of VP16 function, the VP16 domain still provides activation levels >100-fold above background (Figure 7D).

Direct interaction of the Dfd homeodomain and Exd

As the activation function of Dfd is regulated negatively by the Dfd homeodomain in cultured cells, and positively by Exd in embryos, we further asked whether the suppression and enhancement might be associated with direct binding interactions among the Dfd homeodomain, the Dfd activation domain and Exd protein. The existence of such interactions was assessed by GST pull-down assays. There is already abundant *in vitro* evidence that Exd binds to Hox proteins in a homeodomain-dependent fashion (Mann and Chan, 1996).

We first mapped subregions of Dfd, including the homeodomain region, for their ability to bind Exd. Different portions of Dfd were fused to GST (Figure 8A and B) and incubated with the ^{35}S-labeled Exd protein. The fusion constructs were pelleted on glutathione–Sepharose beads, and the amount of Exd bound to the GST–Dfd variants and controls was detected by polyacrylamide gel electrophoresis. As shown in Figure 8C, in this assay Exd binds to full-length Dfd protein (1–586). The binding of deletion mutants indicates that the Dfd homeodomain alone (365–434) possesses most of the Exd-binding function, and the C-terminal 160 residues of Dfd (426–586) possess lower levels of affinity for Exd. However, the Dfd activation region (1–294) possesses no detectable binding affinity for Exd.

To test for *in vitro* binding between the Dfd homeodomain and the Dfd activation region, we assayed the ability of GST–Dfd (1–294) to bind ^{35}S-labeled Dfd homeodomain (Figure 8D). No binding interactions were detected between the homeodomain and the activation region of Dfd (Figure 8D). Under these conditions, a binding interaction was easily detected between the Dfd homeodomain and GST–Exd. The inclusion of ethidium bromide (EtBr) in the binding reactions (Lai and Herr, 1992) resulted in a reduction, but not a loss, of Exd–Dfd homeodomain binding. This indicates that the interaction is partially DNA independent. In summary, the Dfd homeodomain binds to Exd *in vitro*, but the Dfd activation domain does not interact *in vitro* with either the Dfd homeodomain or Exd.

Discussion

Regulation of Hox activities

Most studies directed at understanding the molecular mechanism of Hox functional specificity have focused on how Hox proteins acquire additional DNA-binding specificity (Mann and Chan, 1996). Here, we have found compelling evidence that binding of the Hox protein Dfd

to DNA is not sufficient for gene activation. This was based on the approach of using a Dfd protein fused to a strong activation domain to 'visualize' indirectly the interaction of Dfd with a simple target site (site 2×D) in both *Drosophila* embryos (Figure 5) and transfected cells (Figure 6A).

The neutral state of Dfd on simple binding sites indicates that additional regulatory steps and regulatory sequences are required for Dfd to activate gene expression. One factor required for the regulatory transition to an activated state in embryos is Exd. In previous studies, it has been shown that enhanced *in vitro* affinities of Hox proteins on specific Hox–Exd composite sites correlate with their ability to activate transcription from these sites (Chan *et al*., 1994, 1997; Pöpperl *et al*., 1995). In these studies, the function of Hox proteins on Exd–Hox composite sites was not compared with simple Hox-binding sites with similar *in vitro* binding affinities. Thus, it was not possible to separate a role for Exd in enhancing Hox DNA-binding affinity from a role in generating transcriptionally active Hox proteins, or a transcriptionally active Exd–Hox complex. We show here that the 2×D site and the 2×ED2 site have very similar *in vitro* affinity for Dfd in the presence of Exd (Figure 4). However, the 2×ED2 site is much more responsive than the 2×D site to either Dfd or Dfd-VP16 proteins in embryos (Figure 5). This strongly suggests that Exd is required to release the transcriptional activation function of Dfd in a way that is independent of the Exd enhancement of Dfd binding affinity on the 2×ED2 site. At present, the most widely accepted models propose Exd as a cofactor that has its effect on Hox specificity by acting to increase the binding affinity of different Hox proteins to different composite binding sites. Our results indicate that Exd has other regulatory effects on Hox proteins that may play a role in the diversification of function within the Hox family.

Dfd protein contains an autonomous activation domain that is functional in transfection assays when separated from the C-terminal half of the protein (Zhu and Kuziora, 1996; Figure 6C and D). On tandem repeats of simple Dfd-binding sites, the function of the Dfd transcription activation domain is suppressed both in cultured cells and in embryos. In embryos, this suppression can be partially relieved by the addition of Exd-binding sites to simple Dfd-binding sites. This is apparently due to the function of the Exd protein, since *exd* genetic function is required for the relief of the suppression of Dfd activation function on 2×ED2 sites. In cultured cells, the suppression of Dfd activation function can be conferred by the homeodomain regions from either Dfd or Ubx. Since we find no evidence that there is a direct intramolecular interaction between the Dfd homeodomain and its transcriptional activation region, we propose a model (Figure 9) that invokes a masking factor that suppresses the function of the activation domain by contacting the homeodomain region. In outline, this model is similar to the allosteric control model of Lefstin and Yamamoto (1998). In addition, we speculate that Exd may be required to alleviate the suppressive effect of the proposed masking factor by competing for overlapping protein–protein interaction sites on the homeodomain.

Activity regulation of transcription factors by other proteins can be conferred by a variety of mechanisms. One

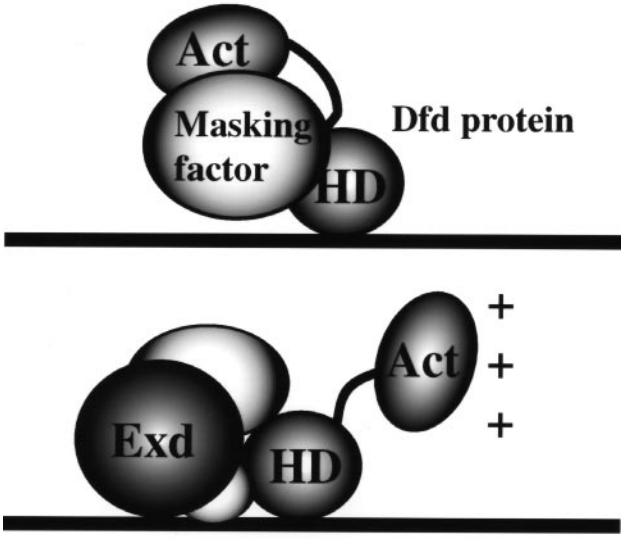

Fig. 9. A model for the activity regulation of Dfd. Dfd binds via the homeodomain (HD) to the regulatory regions of a variety of target genes; however, on some of these sites, no transcriptional activation is elicited because the function of Dfd activation domain (Act) is suppressed by the homeodomain. The suppression might be mediated by a masking factor(s). Activation domain function is released when Exd and other cofactors bind nearby and interact with the Dfd homeodomain. Attachment of the VP16 activation domain to Dfd can partially bypass the requirement for cofactors.

involves synergistic effects in which the transcriptional activation provided by two or more transcription factors in combination is much greater than the sum of their individual activities (Ptashne and Gann, 1997; Carey, 1998). In most such cases, each of the synergizing proteins possesses a domain that can mediate transcriptional activation at least weakly when the protein is bound individually to DNA. In other cases such as the T cell factor (TCF)–Arm interaction (Molenaar *et al*., 1996), the DNA-binding and activation functions are divided between two interacting proteins so that gene activation can only be achieved by their combination.

It is possible that Exd potentiation of Hox activation is due to combinatorial synergism of two activation domains, but this question currently is unresolved. Many studies have concluded that on simple binding sites, Pbx proteins have no detectable transcriptional activation function (van Dijk *et al*., 1993; Phelan *et al*., 1995; Lu and Kamps, 1996; Di Rocco *et al*., 1997). On the contrary, Pbx1 protein was found to act as a transcriptional repressor in transfected cells (Lu and Kamps, 1996). If the Exd protein resembles its vertebrate homologs in lacking autonomous transcriptional activation domains, then a direct interaction between Exd and the Dfd homeodomain (Figure 8) may be required for the release of the Dfd activation function. The conformational changes in Hox proteins that can be induced *in vitro* by Exd or Pbx (Chan *et al*., 1996; Sanchez *et al*., 1997) may also be an indication of the unmasking of the Dfd activation function. The Hox homeodomain region has been shown to possess most of the information that controls Hox protein functional specificity, and the residues that control specificity do not correspond to DNA-binding residues (Kornberg, 1993). Perhaps the specific informational content of the Hox homeodomains consists of the ability to allow regulatory proteins to

compete for homeodomain binding interactions, which results in different activation or repression functions on different targets.

Masking of covert transcriptional activation functions by other domains in the same transcription factor has been discovered previously in proteins from a variety of DNA-binding domain families (Baichwal *et al*., 1992; Kowenz-Leutz *et al*., 1994; Brown *et al*., 1995; Dennig *et al*., 1996; Dörfler and Busslinger, 1996; Li and Green, 1996). In the case of ATF-2, the suppression is mediated by the bZIP DNA-binding domain. Unlike Dfd, the ATF-2 bZIP domain interacts directly *in vitro* with the activation domain that it suppresses (Li and Green, 1996). In other cases, the inhibitory regions and activation domains apparently do not interact directly, and there is evidence for other cellular factors that are required for the suppression effect (Baichwal *et al*., 1992; Brown *et al*., 1995). Some of the release of activation domain function depends on extracellular signaling. For example, the activation function of transcription factor C/EBPβ is derepressed in response to cytokine signaling (Kowenz-Leutz *et al*., 1994). The signaling pathway results in the phosphorylation of the C/EBPβ inhibitory domain, which releases the function of the activation domain.

Activity regulation and Hox specificity

Like most transcription factors, Hox proteins have been believed to achieve their specific regulatory effects largely through their occupancy of distinct *cis*-regulatory elements *in vivo*. As the homeodomains of Hox proteins recognize simple sequences with an ATTA core and have relatively low DNA recognition specificity as monomers, Hox proteins such as Dfd may bind to many more DNA sites than they normally regulate (Biggin and McGinnis, 1997). How can Hox proteins restrict their function to their regulatory targets?

Extensive studies have shown that Exd/Pbx homeodomain proteins can enhance Hox-binding affinities to otherwise weak Hox-binding sites (Mann and Chan, 1996). Exd–Hox composite sites have been found in some naturally occurring Hox regulatory elements and some are bound preferentially *in vitro* by certain Hox protein(s) (Chan *et al*., 1994, 1997; Pöpperl *et al*., 1995; Grieder *et al*., 1997). Such composite sites in multimerized form are capable of generating Hox response elements that are activated by only one of many potential Hox proteins (Pöpperl *et al*., 1995; Chan *et al*., 1996, 1997).

However, many of the naturally occurring Hox regulatory elements that have been studied to date (Regulski *et al*., 1991; Capovilla *et al*., 1994; Chan *et al*., 1994; Manak *et al*., 1995; Pöpperl *et al*., 1995; Sun *et al*., 1995) contain functionally important sites on which Hox binding is unlikely to be significantly influenced by Exd/Pbx because of the absence of either high affinity Exd/Pbx homodimer-binding sites or high affinity Exd–Hox heterodimer-binding sites (van Dijk *et al*., 1993; Neuteboom and Murre, 1997). Those elements that contain cooperative binding sites for Hox and Exd class proteins also contain other sequences that control the specificity of the Hox response. For example, a recently described natural response element for the Hox protein Labial (Lab) requires both *lab* and *exd* genetic functions, as well as *dpp* signaling function, for its activation in the head and midgut. This element contains a site that is bound cooperatively by Lab and Exd proteins. Mutagenesis of this Lab–Exd preferred binding site to a Dfd–Exd preferred binding site neither abolished the Lab-dependent activation of the element nor switched it to a Dfd-responsive element (Grieder *et al*., 1997). We propose that the Exd sites and the other sequences required for the embryonic activity of the Hox response elements both play a role in releasing the covert activities present in Hox proteins such as Dfd.

On many of the Hox-binding sites, the inherent neutrality of Dfd, and perhaps other Hox proteins, may result in a failure to exert an independent influence on adjacent promoters and, therefore, keeps their functions under tight control. Activity regulation of Hox functions might provide a regulatory point for specificity determination. If Dfd protein remains neutral on many sites, and only activates those DNA regulatory elements that also bind a specific combination of cofactors, its ability to confer posterior head development may reside largely in the subset of DNA elements occupied by Dfd on which the protein is functionally activated. We believe this will generally involve multiple cofactors. Exd protein is necessary but not sufficient for the full release of Dfd activation function as the ED2 sites activate reporter gene expression in only a small subset of cells that accumulate both Dfd and Exd in nuclei (Figure 9). The neutral and low specificity binding of Hox proteins on a wide range of sites in the genome might provide a large reserve for evolution of new target elements. New target genes may be evolved easily from such non-functional sites by gaining additional binding sites for specific cofactors. The evolution of distinct functions within the Hox family may thus have occurred by regulating the same set of target genes in different ways, as well as by regulating different sets of target genes (Carroll, 1995).

Inhibitory role of the homeodomain in transcription

An unexpected finding of this study is the role of the Dfd homeodomain in suppressing transcriptional activation function. We have shown that the homeodomain is capable of inhibiting the Dfd activation function in transfection assays (Figure 7C), which apparently accounts for the neutrality of Dfd. The inhibitory effect was not detected in other protein domains appended to the Dfd activation domain, such as the C-terminal region of Dfd (Figure 7C). When the Dfd homeodomain is attached to GAL4-VP16, the homeodomain can also modestly attenuate VP16 activation function (Figure 7D). Consistent with these data, Dfd-VP16 only shows modest activation function in both *Drosophila* embryos (Figure 5) and transfected cells (Figure 6A), although the VP16 activation domain is perhaps the strongest activation domain known to date.

The mechanism by which the Dfd homeodomain masks the Dfd activation function is unknown. Although a direct association between the homeodomain and the activation domain might have provided a simple mechanistic explanation, the two domains do not interact detectably with each other *in vitro* (Figure 8D). Thus, we consider that a masking factor is probably involved in mediating the interaction of the homeodomain and the activation domain of Dfd (Figure 9).

The masking effect of the homeodomain in transcrip-

tional activation may be a common property of Hox proteins. For example, the homeodomain from another Hox protein, Ubx, is able to suppress activation domain functions of both Dfd and VP16 (Figure 7C and D). It is unclear whether this inhibitory effect applies widely to members of the homeodomain family. For example, in the same assay, the homeodomain from Bcd shows only a slight suppressive effect in NIH 3T3 cells, but it inhibits the Dfd activation function in Bosc 23 cells as well (Figure 7B). One explanation is that the Bcd homeodomain is able to mediate suppression of activation domain function provided that proper masking factors exist. Thus, in Bosc 23 cells, Bcd homeodomain may interact with masking factors that are not found in NIH 3T3 cells.

Materials and methods

Plasmid construction

Dfd-VP16 and Dfd expression constructs. To make pXS-Dfd-VP16 (for expression in tissue culture cells), a DNA fragment encoding the VP16 activation domain (codons 413–490) (Triezenberg *et al*., 1988), with the addition of an optimal translation start site and ATG codon upstream, was PCR amplified, ligated to the 5′ end of the Dfd open reading frame (ORF) (Regulski *et al*., 1987) via an *Eco*RI linker, and cloned into the expression vector pXS (generously provided by I.Engel). The entire Dfd ORF without the 78 codons of VP16 was also cloned into pXS to generate pXS-Dfd. pUAS-Dfd and pUAS-Dfd-VP16 (for expression in *Drosophila* embryos) were constructed by excising the Dfd and Dfd-VP16 ORFs as partial *Eco*RI–*Not*I fragments from pXS-Dfd and pXS-Dfd-VP16 and cloning into pUAST (Brand and Perrimon, 1993). pKS-Dfd-VP16 (for *in vitro* translation) was produced by inserting the Dfd-VP16 ORF as a partial *Eco*RI–*Not*I fragment from pXS-Dfd-VP16 into pBluescript KS+ (Strategene). pAR-Dfd (for *in vitro* translation) were described previously (Jack *et al*., 1988).

GST–Dfd or Exd fusion constructs for expression in Escherichia coli cells. All GST–Dfd and Exd fusion plasmids were made by subcloning Dfd- and Exd-coding sequences into pGex4T-1 (Pharmacia). Different coding regions were either isolated by convenient restriction enzyme digestions or PCR amplified with introduction of proper restriction sites.

GAL4-Dfd or VP16 fusion constructs for expression in tissue culture cells. pBXG-Dfd 1–586 was made by excising the Dfd ORF as a partial *Eco*RI–*Xba*I fragment from pXS-Dfd and cloning into pBXG-1 (Quong *et al*., 1993). pBXG-Dfd 1–294, 1–258 and 161–586 were made by internal excisions of *Bam*HI–*Xba*I, *Pst*I–*Xba*I and *Eco*RI fragments from pBXG-Dfd 1–586 respectively. pBXG-Dfd 1–294 + 426–586, 1–294 + HD and 1–294 + 342–428 were made by inserting the respective *Bam*HI–*Not*I fragment of pGex-Dfd 426–586, 365–434 and 342–428 into *Bam*HI–*Not*I of pBXG-Dfd 1–586. To make pBXG-Dfd 1–294 + UbxHD, 1–294 + BcdHD and 1–294 + DfdHDN51Q, DNA fragments encoding homeodomains of Ubx, Bcd and Dfd were PCR amplified with proper restriction sites and the N51G mutation and cloned into *Bam*HI–*Not*I of pBXG-Dfd1-586. pBXG-VP16, VP16 + DfdHD and VP16 + UbxHD were made by cloning the VP16 activation domain alone or together with DfdHD or UbxHD into pBXG-1.

Reporter gene constructs. The luciferase reporter plasmid containing the D site (pGL3-D site) was constructed by inserting site D in two directly repeated copies into the pGL3 promoter (Promega). The luciferase reporter plasmid containing five copies of the GAL4-binding site (5×GAL4-Luc) was a generous gift of M.E.Massari. The *LacZ* reporter constructs were cloned by inserting two copies of the sites shown in Figure 4A in direct repeat orientation into the reporter construct CZIII (Zeng *et al*., 1994).

Other constructs for in vitro translation. pSP64ATG-Dfd HD was prepared by PCR amplifying the Dfd homeobox sequence and subcloning into pSP64ATG (van Dijk and Murre, 1994). pSP64ATG-Exd was described in van Dijk and Murre (1994).

Cell transfection

NIH 3T3 cells were transfected with luciferase reporter plasmids at 1 μg and different expression plasmids at amounts specified in each figure, using lipofectamine (Gibco-BRL). A CMV-*LacZ* plasmid (1 μg) was also co-transfected to serve as an internal measure of transfection efficiency. Bosc 23 cells were transfected by the calcium phosphate precipitation method with one-third of the amounts of DNA used for NIH 3T3 cells. Luciferase and β-galactosidase activities were measured using the detection systems produced by Promega and Tropix, respectively. Relative luciferase activity from each luciferase reporter gene was calculated separately by dividing luciferase activity by β-galactosidase activity in each cell line.

EMSAs

Dfd, Dfd-VP16 and Exd proteins were produced by the TNT coupled transcription/translation reticulocyte lysate system (Promega). Control reactions using ^{35}S-labeled proteins were performed and analyzed on a protein gel to verify that proteins of the correct size were produced in similar quantities. D site and ED2 site oligos for binding reactions were ^{32}P-labeled using fill-in reactions to the same specific activities. Binding reactions were performed by incubating binding proteins with 20 000 c.p.m. of labeled oligos in binding buffer (Neuteboom and Murre, 1997) for 30 min at room temperature. The total volume of protein/reticulocyte lysate in each reaction was adjusted to 2 μl. In EMSA experiments to measure dissociation rates (Figure 1B), after a 30 min binding reaction, a 500-fold excess of unlabeled site D oligos was added to the reaction, and aliquots of binding reaction removed and loaded on a gel after 2, 4, 10 and 25 min. In reactions where antiserum was included (Figure 4B), 1 μl of antiserum was pre-incubated with proteins for 30 min before the labeled oligo was added to the reaction. To measure binding specificities of Dfd and Exd on the D and ED2 sites (Figure 4C), proteins were pre-incubated with unlabeled specific competitor or unspecific competitor nr1 (AAAGCATCAAGCGCGCGCAATCAATTTC) (Neuteboom and Murre, 1997) for 30 min before the labeled oligos were added for another 30 min. Binding complexes were resolved on a 5% polyacrylamide gel running in 0.5× TBE buffer and visualized by autoradiography. The quantities of shifted complexes were measured with a Phosphoimager.

GST pull-down assay

GST fusion proteins were purified and immobilized onto glutathione–Sepharose 4B beads (Pharmacia) according to the manufacturer's instructions. A 3 μg aliquot of GST fusion proteins was incubated with 2 μl of ^{35}S-labeled Exd or Dfd HD peptide in 200 μl of binding buffer (20 mM Tris–HCl, pH 7.6, 150 mM KCl, 1 mM EDTA and 0.5% NP-40) for 40 min at 4°C with nutation. The bed volume of glutathione–Sepharose 4B beads in each reaction was adjusted to 10 μl. The beads were then washed four times for 10 min each in 800 μl of the binding buffer. In reactions where EtBr was applied, 100 μg/ml of EtBr was included in the binding reaction and subsequent washing steps. The bound proteins were released after boiling the beads in the protein sample buffer for 4 min, fractionated on a 10% (for Exd) or a 16% (for Dfd HD) SDS–polyacrylamide gel and visualized by autoradiography.

Drosophila genetics

Transgenic lines of UAS-Dfd, UAS-Dfd-VP16 or reporter genes were established after injection of DNA into *Drosophila* w^{1118} embryos by standard procedures (Rubin and Spradling, 1982). The ubiquitous expression of UAS-Dfd and UAS-Dfd-VP16 in embryos was induced by crossing the transgenic lines with lines containing *arm*-GAL4 drivers (Sanson *et al*., 1996), which resulted in late embryonic lethality. Cuticles of the unhatched embryos were prepared according to Wieschaus and Nüsslein-Volhard (1986). Embryonic RNA *in situ* hybridization was performed as described (Tautz and Pfeifle, 1989).

To assay activation of the D site or ED sites reporters by Dfd and Dfd-VP16, transgenic flies were crossed such that the embryos had a combination of the transgenes of a *LacZ* reporter, UAS-Dfd or UAS-Dfd-VP16 and *arm*-GAL4 or *hsp70*-GAL4 drivers. In experiments involving heat shocks, embryos were collected on agar plates for several hours and aged for 3.5 h at 25°C. The plates were then placed in a 37°C water bath for 1–2 h for heat shock and returned to 25°C for 3–4 h before the embryos were washed off the plates and fixed for staining. Activation of the D site reporter by Dfd-VP16 was evident after a 1 h heat shock, but the stronger activation was seen after a 2 h heat shock. Such prolonged heat shocks (2 h) of control embryos using these conditions did not noticeably disrupt embryonic morphology, and had no effect on the function of the reporter constructs in wild-type genetic backgrounds. For all experiments, at least two different transgenic lines were tested.

To examine the role of *exd* in UAS-Dfd-VP16 function, *exd* germline clones were generated as described (Rauskolb *et al*., 1993). Female flies with *exd* mutant germline clones and heterozygous for UAS-Dfd-VP16

were crossed with males homozygous for both the D site reporter and *hsp70*-GAL4 (in Figure 5D) or males homozygous for both the 2×ED2 site reporter and *arm*-Gal4 (in Figure 5H). The resulting embryos were collected, heat shocked (only in Figure 5D) and immunostained (Li *et al*., 1993). The *exd* maternal and zygotic mutant embryos were identified unambiguously by staining with a monoclonal anti-Exd antibody (Aspland and White, 1997) and were stained further with anti-β-gal antibodies.

Western blot

For detection of Dfd and Dfd-VP16 expressed in embryos, embryos from parents of female UAS-Dfd or UAS-Dfd-VP16 and male *arm*-GAL4 flies were collected at 7–10 h of development. The embryonic extracts were obtained after homogenization of the embryos in 2 vols of buffer EE [40 mM HEPES, pH 7.4, 400 mM NaCl, 5 mM $MgCl_2$, 0.1% Tween-20, 10% glycerol, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 1 mM EDTA and 1 mM $Na_2S_2O_5$] followed by micro-centrifugation for 30 min at 4°C. Proteins (50 or 100 μg) were fractionated on a 7.5% SDS–polyacrylamide gel and transferred onto an Immobilon-P membrane. Proteins were then detected with anti-Dfd antibodies and visualized by the ECL Western blotting method (Amersham). For detection of the GAL4 fusion proteins in tissue culture cells, 2 μg of expression plasmids were each transfected in Bosc 23 cells by calcium phosphate precipitation. Cells were harvested 2 days later, directly resuspended in protein sample buffer and boiled before loading onto protein gels. GAL4 fusion proteins were detected with a mouse monoclonal antibody against the GAL4 DNA-binding domain (Santa Cruz Biotech.).

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