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A model for *extradenticle* function as a switch that changes HOX proteins from repressors to activators

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The *Drosophila* EXD protein and its mammalian counterparts, the PBX proteins, have been proposed to function in HOX target selectivity. Here we show that *exd* function is required for the autoactivation phase of *Dfd* expression in the posterior head. Mutations that change the affinity of a small autoactivation element for EXD protein result in corresponding changes in the element's embryonic activity. Our data suggest that the EXD and DFD proteins directly activate this element in maxillary cells without cooperatively binding to a specialized heterodimer binding site. Based on the types of homeotic transformations and changes in gene expression observed in *exd* mutant embryos, we propose a new model for EXD/PBX action in which these proteins are required for HOX protein transcriptional activation functions, but dispensable for HOX transcriptional repression functions. Although the selection of a specific target gene by a HOX protein versus another may be explained in some cases by the selective modulation of HOX binding specificity by EXD, we favor the idea that EXD interacts in a more general sense with most HOX proteins to switch them into a state where they are capable of transcriptional activation.

Keywords: *Deformed*/*Drosophila*/*extradenticle*/homeotic/HOX/PBX

Introduction

In *Drosophila*, as in many other animals, the members of the Homeotic complex (*Hox)* gene family are expressed in successive domains of cells on the anterior–posterior (A/P) axis of the embryonic body plan (Akam, 1987). Within each A/P domain, a different HOX protein assigns positional identities which are eventually realized in diverse morphological structures on the segmented A/P axis of the *Drosophila* embryo (McGinnis and Krumlauf, 1992). The HOX proteins are homeodomain-containing transcription factors, which regulate the expression of many downstream target genes (Botas, 1993). Some of these downstream genes are regulated by multiple HOX proteins, but the ability of HOX proteins to differentially regulate downstream gene expression underlies their function in diversifying A/P morphology (Vachon *et al.*, 1992; Jones and McGinnis, 1993; Capovilla *et al.*, 1994).

The DNA-binding function of HOX proteins resides within their similar homeodomain regions, and not surprisingly, the AT-rich DNA sequences recognized by most HOX protein monomers *in vitro* are correspondingly similar (Odenwald *et al.*, 1989; Affolter *et al.*, 1990; Dessain *et al.*, 1992; Ekker *et al.*, 1994). A variety of experiments indicate that the minor differences in DNA-binding specificity that distinguish HOX monomers *in vitro* have little influence on their ability to target different downstream genes (Hayashi and Scott, 1990; Mann, 1995). Instead, like many other eukaryotic transcription factors that act through distant enhancers, HOX proteins apparently act with the help of cofactors in larger nucleoprotein complexes (Mann, 1995). The protein–protein contacts within these complexes are believed to include crucial interactions that allow HOX proteins to discriminate among target regulatory elements.

One protein that is strongly implicated as a HOX cofactor involved in regulating target specificity is the protein product of the *extradenticle (exd)* gene. Peifer and Wieschaus (1990) originally suggested that *exd* might encode a homeotic cofactor since embryos which lacked zygotic *exd* function had homeotic transformations in the thoracic and abdominal cuticle, and their genetic experiments indicated that *exd* acted in parallel with many *Hox* genes. There is also a maternal component to *exd* function, and embryos that lack both maternal and zygotic *exd* have no obvious homeotic transformations, instead developing rudimentary thoracic and head segments which have no specialized structures/identities (Peifer and Wieschaus, 1990). Thus, the homeotic transformations seen in zygotic mutants apparently require the presence of wild-type *exd* function early in embryogenesis and its absence or depletion at later embryonic stages. It has been suggested that these *exd* dose-dependent homeotic transformations are due to some HOX proteins assuming the regulatory specificity of other members of the family (Peifer and Wieschaus, 1990). For example, in *exd* mutants, UBX protein might regulate downstream target genes in a way that is normally characteristic of ABD-A protein. However, direct evidence for this model is not yet available, and there are other mechanisms by which *exd* zygotic loss of function could result in homeotic transformations. The EXD protein is ubiquitously expressed in early embryonic cells during the period when segmental identities are being determined (Rauskolb *et al.*, 1993), and may act in parallel with most or all of the *Hox* family genes as well as with other homeodomain-containing proteins (Peifer and Wieschaus, 1990; Gonzalez-Crespo and Morata, 1995; Rauskolb *et al.*, 1995).

The EXD protein contains an atypical homeodomain embedded in a highly conserved 300 amino acid region

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that shares extensive similarity with the vertebrate PBX class proteins (Flegel *et al.*, 1993; Rauskolb *et al.*, 1993). The use of gel mobility shift assays has shown that EXD/PBX class proteins can cooperatively bind DNA as heterodimers with many of the *Drosophila* or mammalian HOX class proteins, suggesting that interactions between EXD/PBX and HOX proteins modulate the strength and/ or selectivity of HOX response elements (Chan *et al.*, 1994; van Dijk and Murre, 1994; Chang *et al.*, 1995; Phelan *et al.*, 1995; Popperl *et al.*, 1995; van Dijk *et al.*, 1995). The biological significance of these observations is supported by experiments showing that EXD-binding sites and the genetic function of *exd* are required for the activity of some HOX response elements in embryonic cells (Chan *et al.*, 1994; Rauskolb and Wieschaus, 1994; Sun *et al.*, 1995). Also, at least two HOX response elements contain multiple binding sites which preferentially interact with specific HOX–EXD heterodimers, consistent with the idea that HOX–EXD heterodimers function as co-activators in combination with specific composite binding sites (Chan *et al.*, 1994, 1996; Popperl *et al.*, 1995).

We have investigated the effects of *exd* on the function of *Deformed (Dfd)* and DFD protein response elements. *Dfd* is a homeotic selector gene which is responsible for specifying the identity of the maxillary and mandibular segments of *Drosophila* embryos (McGinnis *et al.*, 1990). Previous results have suggested that *Dfd* function is at least partially retained in *exd* zygotic mutants, since normal maxillary structures develop on the partially involuted head segments of these mutants (Peifer and Wieschaus, 1990). However, when both maternal and zygotic *exd* functions are removed, *Dfd* function also appears to be abolished, as the rudimentary maxillary segment is smaller and develops no segment-specific sensory organs or cuticular structures (Peifer and Wieschaus, 1990).

We find that unlike *Hox* genes that act in the trunk, *Dfd* expression is changed dramatically in *exd* maternal and zygotic mutant embryos. Most *Dfd* response elements also exhibit a requirement for *exd* genetic function which could be exerted directly or indirectly. A minimal DFD response element seems likely to be regulated directly by EXD protein, as an increase or decrease in the affinity the element has for EXD protein results in parallel changes in its embryonic activity. This element appears to be regulated directly by the combination of DFD and EXD without the need for a specialized heterodimer-binding site for the two proteins. Therefore, EXD may not play an important role in the selection of this element by DFD versus other HOX proteins. The same minimal DFD response element also exhibits ectopic activation in late stage *exd* mutant embryos. Our results are consistent with a model where EXD protein acts as a cofactor that is required for HOX proteins in their roles as activators, but where EXD is not required for the repressive functions of HOX proteins.

Results

exd interacts genetically with *Dfd* loss-of-function mutations

Previous modifier screens have identified *Dfd* modifier mutations based on their enhancement of the lethality of partial loss-of-function *Dfd* mutant alleles (Harding *et al.*, 1995). One of the mutations (*S136*) isolated in such a screen for *Dfd*-interacting genes on the X chromosome (B.Florence and W.McGinnis, unpublished) has been assigned to the *exd* locus since it maps to 52 cM and fails to complement mutations in *exd.* Individuals heterozygous for exd^{S136}, in combination with two weak hypomorphic alleles of *Dfd*, survive at only 40% of the frequency of controls with two wild-type copies of *exd*. In addition, two previously isolated *exd* alleles (exd^{XP11} and exd^{Yo12}) also have a similar effect on viability in combination with *Dfd* hypomorphic alleles (Table I). These genetic interactions support the idea that the dose of *exd* function is critical for the expression and/or function of *Dfd*.

Table I. Genetic interaction between *Dfd* and *exd* mutants

exd allele	Interaction strength[a]
S136	39 ± 26%
XP11	48 ± 20%
YO12	70 ± 21%

[a]The interaction strength is defined as the ratio of *exd/+;* Dfd^{rc11}/Dfd^{RV8} adult survivors to +/+; Dfd^{rc11}/Dfd^{RV8} survivors. For details, see Materials and methods.

exd is required for *Dfd* autoactivation

We first asked whether diminution or loss of *exd* function influenced embryonic expression of *Dfd*. Transcription of *Dfd* is initiated in the maxillary and mandibular primordia of cellular blastoderm embryos under the control of coordinate, gap and pair-rule proteins (Jack and McGinnis, 1990). Once established, transcription is maintained in most epidermal cells by an autoactivation circuit (Kuziora and McGinnis, 1988; Bergson and McGinnis, 1990; Zeng *et al.*, 1994). Autoactivation also plays a role in amplifying the levels of *Dfd* expression within the CNS (Zeng *et al.*, 1994; Lou *et al.*, 1995). To assay *Dfd* expression, *exd* zygotic and maternal/zygotic mutant embryos were stained with affinity-purified antibodies directed against DFD protein, or reacted with antisense probes to detect *Dfd* transcripts. Using both techniques, identical changes in the *Dfd* expression pattern were observed, and the antibody staining results are shown in Figure 1.

In embyos lacking only zygotic *exd*, *Dfd* expression was normal at every embryonic stage (data not shown). However, in embryos which lack both maternal and zygotic *exd* (exd^{mz-}), only the establishment phase of *Dfd* expression is normal. Prior to stage 10 of embryogenesis (Campos-Ortega and Hartenstein, 1985), no difference can be detected between exd^{+} and exd^{mz-} mutants (Figure 1b). However, during stage 10 in the exd^{mz-} mutants, *Dfd* protein can no longer be detected in most of the epidermal and sensory progenitor cells of the maxillary and man-dibular segments, and remains undetectable for the remain-der of embryogenesis (Figure 1d, f and h). Occasionally, weak expression can still be detected in dorsal regions of the maxillary remnant of the exd^{mz-} embryos. We believe that these few dorsal cells correspond to the posterior compartment of the maxillary segment, since the same cells also stain with antibodies directed against the EN protein (Peifer and Wieschaus, 1990; and data not shown).

Dfd expression in the CNS is still detectable at all embryonic stages in the exd^{mz-} mutants, but the levels of

expression are significantly lower than controls (Figure 1g and h). The period during which *Dfd* expression is reduced or abolished in the cells of exd^{mz-} mutants corresponds to the period during which DFD protein begins to be required for transcriptional maintenance, suggesting that *exd* function is required for the autoactivation circuit. The exd^{mz-} mutant effect on *Dfd* expression also mimics the effect of loss of *Dfd* protein function in two other ways. First, exd^{mz-} mutants and *Dfd* protein null mutants both show the same effects on *Dfd* expression with respect to tissue specificity, with epidermal expression being eliminated in most cells and CNS expression being reduced. Second, exd^{mz-} mutants and *Dfd* protein null mutants both still retain retain weak expression in the same posterior epidermal region of the maxillary segment. All of this is consistent with exd^{mz-} mutations abolishing DFD protein function, which in turn results in a loss of the autoactivation circuit.

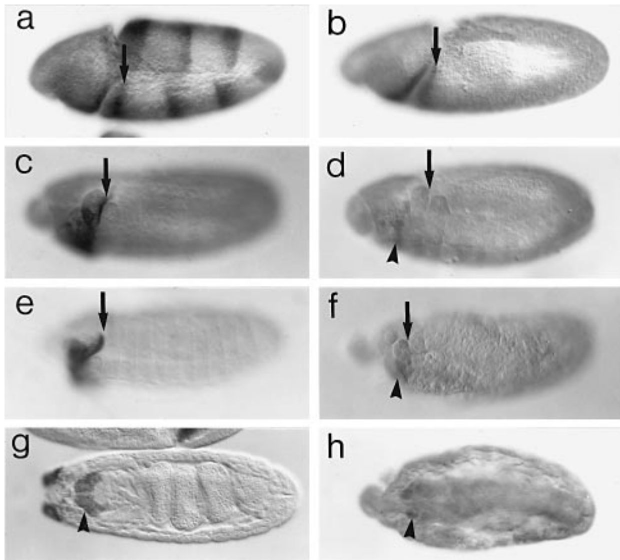

Fig. 1. The expression pattern of DFD protein in exd^{mz-} embryos. (a and b) Embryos at stage 9 of development. At this early stage, *Dfd* is expressed equally in both the wild-type (**a**) and *exd* maternal zygotic mutant (exd^{mz-}, **b**) embryos. The additional stripes of stained cells in (a) derive from a *ftz–lacZ* expression construct on a balancer chromosome, and indicate that this embryo is wild-type for *exd* function. (c and d) Embryos at stage 11. The epidermal expression pattern in the wild-type embryo (**c**) is absent in the exd^{mz-} embryo (**d**). The arrow marks the dorsal, posterior boundary of the maxillary segment in this and other panels. (**e** and **f**) Wild-type and exd^{mz-} mutant embryos at stage 13. *Dfd* expression is only detectable in the central nervous system (arrow) of exd^{mz-} mutant embryos. (**g** and **h**) Wild-type and exd^{mz-} mutant embryos at stage 16. The only cells in the exd^{mz-} mutants (h) that weakly express *Dfd* are in the mandibular neuromere (arrowhead).

The ectopic expression of DFD protein from a heat shock promoter is capable of inducing the *Dfd* autoactivation circuit in segments outside the normal *Dfd* expression domain (Kuziora and McGinnis, 1988). To test further whether *exd* function is required for *Dfd* autoactivation, we asked whether transcription from the endogenous *Dfd* gene could be induced by this exogenous source of DFD protein in an exd^{mz-} mutant background. At both 1 and 5 h after heat shock, the exd^{mz-} mutant embryos had strikingly lower levels of *Dfd* transcripts when compared with controls, both in the posterior head and other body segments (Figure 2). We conclude that *exd* is required for the *Dfd* autoactivation circuit in embryonic cells. This might be due to a requirement for *exd* to activate the expression of a required cofactor of DFD, to repress the expression of a repressor of *Dfd* or by a direct involvement of the EXD protein in the regulation of autoactivation elements targeted by DFD protein.

DFD response elements in exd mutant embryos

To explore the possibility that *exd* might be involved directly in the regulation of DFD autoactivation elements, we analyzed the activity of some of these elements in exd^{mz-} mutants. Previous work has shown that an epidermal autoregulatory enhancer (EAE) maps in a 2.7 kb fragment ~4 kb upstream of the *Dfd* transcription start (Bergson and McGinnis, 1990; Zeng *et al.*, 1994). This enhancer is composed of many semi-autonomous modules, and two of these were tested for activity in exd^{mz-} mutants.

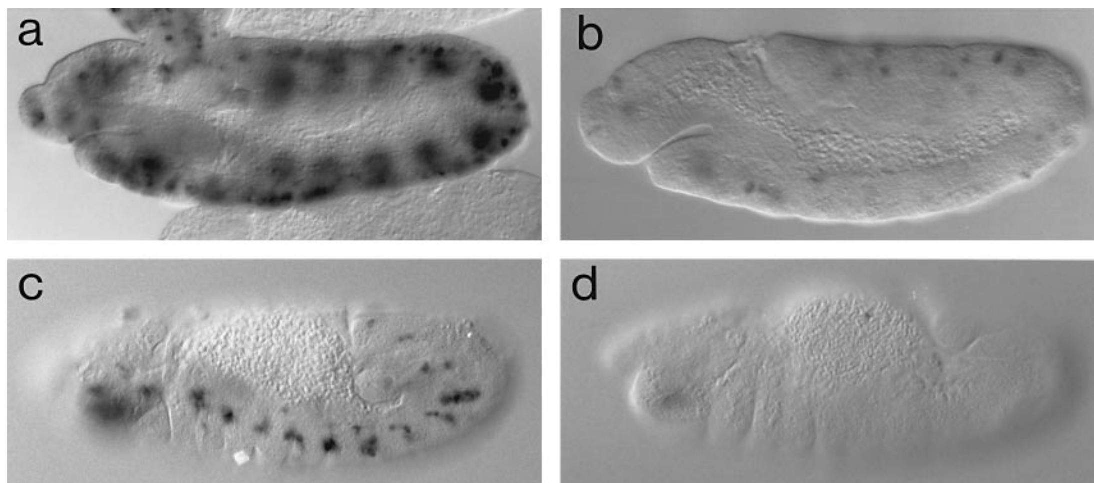

Fig. 2. *hsDfd*-induced ectopic expression in *exd* mutant embryos. After a pulse of ectopic DFD protein expression during early embryogenesis using a *hsp70* promoter–*Dfd* cDNA construct, wild-type and exd^{mz-} mutant embryos were allowed to recover for 1 (a and b) or 5 h (c and d) before fixation. Previous experiments have shown that transcripts induced from the *hsDfd* construct are undetectable 30 min following a heat shock (Kuziora and McGinnis, 1988), thus *in situ* hybridization thereafter can reveal where endogenous *Dfd* transcripts are produced via the autoactivation circuit. In the wild-type controls (**a** and **c**), the endogenous *Dfd* transcription unit has been activated inappropriately in other segments. In *exd* mutants (**b** and **d**), only a few scattered cells show weak activation of *Dfd* transcription.

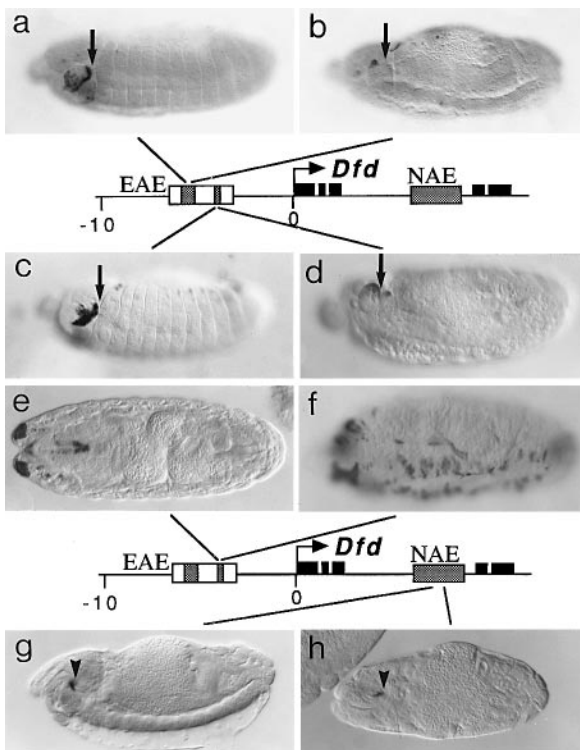

Fig. 3. The function of *Dfd* autoactivation enhancers in exd^{mz-} mutants. The panels on the right (b, d, f and h) show *exd* mutants stained for β-gal reporter expression, the panels on the left (a, c, e and g) show wild-type controls. The diagrams of the *Dfd* transcription unit show the normal location of the enhancers tested in the *exd* mutant background. (**a** and **b**) Reporter expression provided by element C, a 570 bp part of the 2.7 kb *Dfd* epidermal autoregulatory element (EAE; Zeng *et al.*, 1994). The activity of module C is abolished in most maxillary cells in exd^{mz-} mutants (b). (c–f) Reporter expression provided by the multimerized (4×) 120 bp module E (Zeng *et al.*, 1994). (**c**) and (**d**) show the function of module E in stage 13 embryos. In the *exd* mutant, the activity is abolished in nearly all maxillary cells, save for a small amount in posterior compartment cells (located dorsally in the mutants) that is not DFD dependent (see text). (**e** and **f**) show module E function in stage 16 embryos. In the exd^{mz-} background, module E is ectopically expressed in the ventral thorax and abdomen. (**g** and **h**) show the expression of the *Dfd* neural autoregulatory element (NAE). The activity of this element is only modestly reduced in *exd* mutants (g).

As expected, the enhancer activities of the 570 bp module C (Figure 3a and b), and the 120 bp module E (Figure 3c and d) of the EAE were largely abolished in the exd^{mz-} background. Both provided only a small amount of reporter expression in a few dorsally located maxillary cells (which correspond to the posterior compartment, see previous section) of stage 12 mutant exd^{mz-} embryos. Interestingly, the smaller of these elements becomes activated in many more cells in the exd^{mz-} mutants at later embryonic stages (Figure 3f). Based on their shape, size and position, most of these cells appear to be ventral muscle precursors. A similar, slightly weaker pattern of ectopic expression of the 120 bp element is detected in embryos that lack only the zygotic expression of *exd* (although the maxillary activity of the 120 element is unaffected in the *exd* zygotic mutants, data not shown). Evidence exists that the 120 bp module E is activated directly by DFD protein through a single binding site (Zeng *et al.*, 1994).

Based on these results, *exd* function on the 120 bp module E appears to be dependent on segmental context. In maxillary cells where DFD is normally expressed, *exd* is required for the activity of the 120 bp DFD response element, and maternal *exd* alone is sufficient to supply this function. This positive effect could be exerted indirectly by EXD action on other elements required for persistent DFD protein expression, directly by EXD binding to the 120 bp module E or by a combination of these two mechanisms. In contrast, in some posterior embryonic cells outside the normal *Dfd* expression domain, *exd* function is required to repress module E. Both maternal and zygotic *exd* contribute to this repressive function.

We also tested the function of a neural autoregulatory enhancer (NAE; Lou *et al.*, 1995) from the *Dfd* locus in exd^{mz-} mutants. The function of the *Dfd*-NAE is affected only quantitatively in these mutants, still providing reporter gene expression at somewhat lower levels at both early and late stages (Figure 3h) in mandibular neuromere cells of the CNS. This is consistent with the CNS expression of the endogenous *Dfd* transcription unit being reduced to only a moderate extent in the exd^{mz-} mutant background.

Sequences required for the derepression of module E in exd mutants

The interesting dual response of the 120 bp module E to *exd* loss-of-function mutations prompted us to explore in more detail which sequences were required for the activating and repressing effects of *exd* on this element. Previous studies have generated numerous mutant versions of the 120 bp element (Zeng *et al.*, 1994), and we placed many of these into exd^{mz-} backgrounds. The crucial sequences for the homeotic response of this element reside in regions 3, 5 and 6, which are outlined in Figure 5.

Region 3 of the 120 bp module E contains a DFD-binding site, denoted by the ATTA in Figure 4, plus a nearby TAAT sequence motif that interacts weakly with a variety of homeodomain proteins, including EXD. When the TAAT motif in region 3 is mutated, the ectopic activity of the 120 element is nearly abolished in the exd^{mz-} mutants (Figure 4c and d). Mutant versions of module E with the ATTA motif changed are affected less drastically, but also show ectopic activity in fewer cells in the *exd* mutant background (Figure 4e and f). This result suggests that *exd* exerts a repressive effect by preventing other homeodomain activators in thoracic and abdominal cells from activating this element via the ATTA and TAAT HOX-binding motifs. Since the pattern of ectopic expression differed between thorax and abdomen in *exd* mutants, we wished to test whether the ectopic activity was under the control of HOX proteins that had inappropriate activation function in the *exd* mutant background. To test this, we analyzed the expression of the 120 bp DFD response element in a genetic background that lacked zygotic *exd* and all *Hox* functions of the thorax and abdomen (quintuply mutant in *Scr*, *Antp*, *Ubx*, *abd-A* and *Abd-B*). Such embryos still exhibited ectopic expression of the 120 bp element (data not shown), which indicates that the ectopic expression of the 120 bp element is *Hox* independent.

Region 5 comprises 32 bp near the 3′ end of module E, and introduction of clustered base substitutions in region 5 (4×E5 mutants) leads to lower levels of element activity in the maxillary cells of wild-type embryos. Region 6 comprises 22 bp at the 3′ end of module E, and

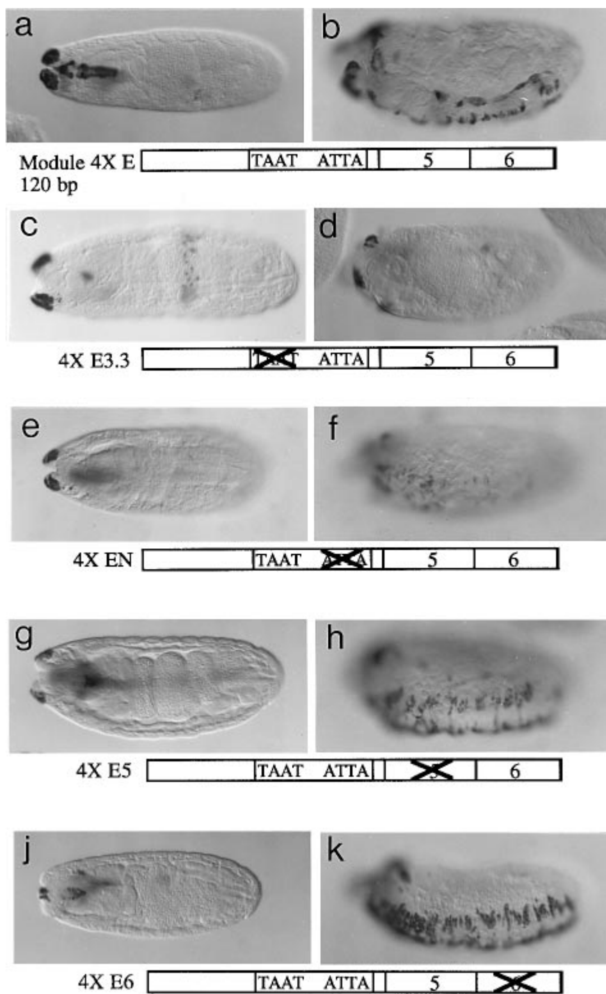

Fig. 4. Activity of 120 bp DFD response element mutants in exd^{mz-} embryos. The panels on the right (b, d, f, h and k) show *exd* mutants stained for β-gal reporter expression, the panels on the left (a, c, e, g and j) show wild-type controls. The plane of section of the embryos in the left panels shows the maxillary lobe expression of the 120 bp element, the plane of section of the embryos on the right shows the ectopic ventral expression of the 120 bp element. Below each pair of panels is a diagram of the 120 bp DFD reponse element variant tested in the *exd* mutant background. The sequence of this element is shown in Figure 5b. All reporter constructs contained 4× multimers of the 120 bp element (4×E as in Zeng *et al.*, 1994). (**a** and **b**) Reporter expression provided by 4×E, the wild-type element, which is ectopically activated in exd^{mz-} mutants (b). (**c** and **d**) Reporter expression provided by the 4×E3.3 element, which has the TAAT sequence replaced by GGCC. This sequence change abolishes ectopic reporter expression in the exd^{mz-}mutants (d). (**e** and **f**) Reporter expression provided by the 4×EN element, which has the DFD-binding site (GCAATTA) replaced with ACGTTAGGA. This sequence change diminishes ectopic reporter expression in the exd^{mz-}mutants (f). (**g** and **h**) Reporter expression provided by the 4×E5 element, which has a 25 bp substitution in region 5 (Zeng *et al.*, 1994). This sequence change results in a slight enhancement of the ectopic reporter expression in the exd^{mz-}mutants (h). (**j** and **k**) Reporter expression provided by the 4×E6 element, which has a 19 bp substitution in region 6 (Zeng *et al.*, 1994). This sequence change results in a significant enhancement of ectopic reporter expression in the exd^{mz-} mutants (k).

introduction of clustered base substitutions in region 6 (4×E6 mutants) leads to complete loss of maxillary activity. Neither of these sequence changes influences the ability of the 120 element to be ectopically activated in exd^{mz-} mutants (Figure 4h and k); in fact the opposite is observed with 4× E6 which is ectopically activated in even more ventral thoracic and abdominal cells (Figure 4k). Previous experiments have shown that regions 5 and 6 of the 120 bp element have *in vitro* binding sites for a novel protein, DEAF-1 (Gross and McGinnis, 1995). The mutant substitutions in both the E5 and E6 elements reduce their affinity for DEAF-1 protein in *in vitro* binding assays. It is possible that *exd* and DEAF-1 collaborate in non-maxillary cells to prevent ectopic activation of this head-specific HOX element.

EXD binding to the 120 bp DFD response element

The opposing effects that *exd* exerts on the 120 bp DFD response element could be direct or indirect. We were interested in addressing two hypotheses: first whether EXD might be acting directly to help DFD protein activate the 120 bp element in maxillary cells, and second whether EXD might be acting directly to repress the function of the same element in some thoracic and abdominal cells. The version of the 120 bp element on which further binding and functional studies were based is the ES element (Zeng *et al.*, 1994), used since it contains minimally sufficient wild-type sequences for *Dfd*-dependent, maxillary-specific expression (only regions 3, 5 and 6 are wild-type). DNase I protection analysis of the ES sequence indicates a weak binding site for EXD protein centered over the TAAT motif at position 2175 (Figure 5a). The mutation of this TAAT to GGCC, in element E3BS, results in a version of the 120 bp element that is more poorly protected by comparable amounts of EXD protein (Figure 5a). Conversely, a 5 bp substitution to generate a consensus high-affinity EXD binding site in the same region generates, in element E3CS, a 120 bp element that is protected by lower levels of EXD protein. We conclude from these studies that the original ES element does have modest affinity for EXD protein *in vitro*, with an extended binding region centered over a TAAT motif that maps adjacent to the well-characterized Dfd binding site in region 3 (GCAATTA). Since the binding affinity of an element can be improved by the changes in E3CS and diminished by the changes in E3BS, the TAAT motif in the wild-type ES element is important for EXD binding (Figure 5b).

Enhancement of DFD binding by EXD

Previous results have indicated that EXD or PBX proteins can interact cooperatively *in vitro* with many HOX proteins on ATCAATCAA heterodimer-binding sites, as well as on variants of such sites (Chan *et al.*, 1994; van Dijk and Murre, 1994; Chang *et al.*, 1995; Phelan *et al.*, 1995; Popperl *et al.*, 1995; van Dijk *et al.*, 1995). Since the ES element normally has some affinity for both DFD and EXD proteins in the same small region, we wished to test whether EXD would bind cooperatively with DFD on ES sequences, or on the ES variants with lower and higher affinity for EXD.

When EXD protein is added to a DFD/ES element binding reaction, the ability of DFD to bind DNA is modestly enhanced (Figure 6). The enhancement effect of EXD on DFD binding was tested on elements ES, E3BS and E3CS in order to assay whether EXD affinity differences influenced the enhancement effect. When the higher amount of EXD is added to the binding reactions, approximately twice the amount of DFD–DNA complex

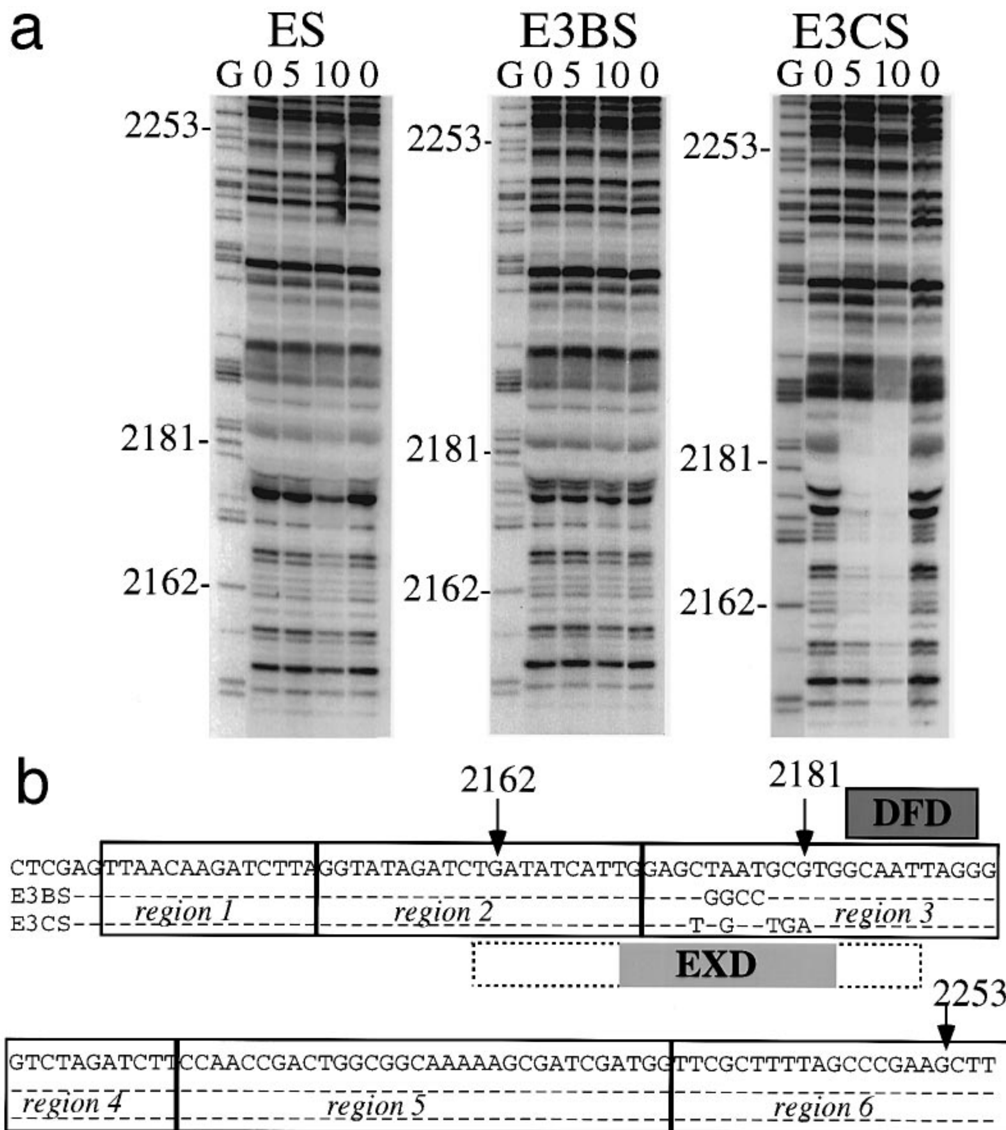

Fig. 5. EXD footprints on 120 bp DFD response elements. (**a**) A DNase I protection assays of 5′ end-labeled ES, E3BS and E3CS DNAs. Lanes 0, no added protein; 5 μl (1.5 μg); and 10 μl (3.0 μg) respectively of EXD protein produced in *E.coli*. Lanes labeled G are the same probes cleaved at guanine bases. (**b**) The sequence in the ES, E3CS and E3BS constructs, using the numbering system in Zeng *et al.* (1994). Specific G positions in the sequence are designated in both (a) and (b). The shaded and dotted bar in (b) denotes the sequences that are protected from DNase I digestion by EXD protein, the darker bar denotes the site protected by DFD protein which was previously mapped in these sequences (Zeng *et al.*, 1994). The open boxes enclose the extent of sequence in regions 1, 2, 3, 4, 5 and 6.

is formed as when DFD alone is present in the binding reaction (Figure 6). When E3CS is the labeled probe, the amount of DFD–DNA complex formed is increased further, and when E3BS is the probe, the amount of complex formed is decreased slightly. These mobility shift data indicate that the amount of enhancement of DFD binding to ES elements does correlate with the affinity of the elements for EXD protein, indicating that the enhancement effect on DFD binding activity is achieved on E3 DNA sequences, not off DNA, even though we did not observe the formation of mobility shift complexes indicating cooperative binding of DFD, ES sequences and EXD in ternary complexes.

To eliminate the possibility that this failure to detect cooperative binding might be due to producing and partially purifying the binding proteins from bacteria, we also used DFD and EXD produced from *in vitro* translation reactions. In conditions where HOXC-6 and PBX1 cooperatively bound in ternary shift complexes, DFD and EXD from *in vitro* translation reactions still failed to cooperatively bind ES or E3CS sequences (data not shown). This failure of DFD and EXD (or PBX1) to form cooperative complexes on EXD high-affinity consensus binding sites has been noted previously by C.Murre and co-workers (personal communication).

The regulatory activity of ES, E3CS and E3BS elements in embryos

The three different versions of the ES element have the same inherent affinity for DFD monomer binding *in vitro*, but differ in their affinity for EXD and in the amount of enhanced affinity that DFD protein shows in the presence of EXD. If EXD protein acts directly on element ES in embryos to activate expression in maxillary cells, and to

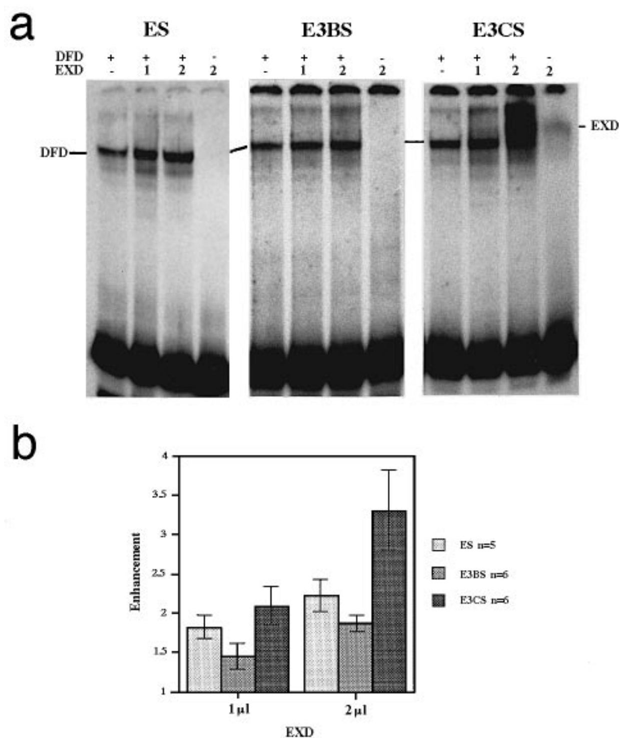

Fig. 6. EXD enhancement of DFD-binding affinity on 120 bp module E sequences. (**a**) In each mobility shift experiment, the labeled probe consisted of an oligomer including regions 2, 3 and 4 from the respective elements (see Figure 5). The DFD and EXD proteins were produced in *E.coli* and partially purified as described in Materials and methods. The binding reactions labeled '+' each contain 10 ng of DFD protein. EXD protein was added at 1 µl (60 ng) or 2 µl (120 ng) per binding reaction. ES is the 'sufficient' version of the 120 bp DFD response element. E3CS has base substitutions in region 3 that result in higher affinity for EXD binding. E3BS has base substitutions in region 3 that reduce EXD binding affinity. To calculate the enhancement of EXD on DFD binding (**b**), the amount of radiolabeled DNA in the DFD–ES, DFD–E3BS and DFD–E3CS complexes was quantified with a PhosphorImager as described in Materials and methods, and the counts from the shifted complex in the EXD+DFD lanes were divided by the counts from the shifted complex in the DFD alone lanes. For each different probe, the experiment was performed five or six times and the enhancement effect was averaged.

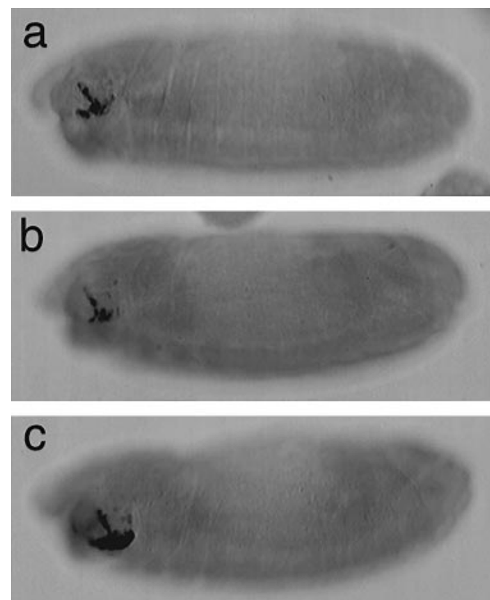

Fig. 7. Embryonic activity of 120 bp elements with different EXD-binding affinity. Transgenic embryos containing homozygous inserts of basal promoter–lacZ reporter constructs attached to one copy of either the ES (**a**), E3BS (**b**) or E3CS (**c**) elements were stained for β-gal antigen. Typical embryos at stage 14 from the three transgenic lines are shown, with anterior to the left and dorsal up. The staining for all three lines is always limited to maxillary epidermal cells at this stage, and E3BS has the fewest, and most weakly staining maxillary cells, and E3CS the most numerous and intensely staining cells.

repress ES activity in thoracic and abdominal cells, the differences in EXD affinity for the ES variants might confer differences in the activity or pattern of expression provided by ES enhancer function in developing embryos. To test this, transgenic embryos were generated carrying constructs with one copy of ES, E3CS or E3BS fused to basal promoter/reporter genes. As shown in Figure 7b, the four base substitution in E3BS results in a regulatory element that provides reporter expression in fewer cells, and at lower levels, when compared with ES. Conversely, the 5 bp substitution in E3CS expression generates an element that provides more abundant levels of reporter expression in more maxillary cells. Neither of the mutated constructs (E3BS or E3CS) exhibit any reproducible patterns of ectopic expression, indicating that the segmental specificity of the mutant elements is unaltered.

Discussion

EXD as a co-activator of DFD response elements

The EXD protein function has a critically important role in the maintenance of *Dfd* expression, being required in parallel with DFD protein for the function of the autoactivation circuit. When EXD is removed from embryos by mutation of both maternal and zygotic function, *Dfd* expression is abolished in the same temporal, spatial and tissue-specific pattern that is observed when DFD protein function is removed from embryos (Zeng *et al.*, 1994). The restoration of DFD protein function with an exogenous promoter is unable to restore the autoactivation function, indicating that DFD is incapable of activating autoactivation enhancers when EXD protein is absent. Since the zygotic *exd* mutants show no detectable changes in *Dfd* autoactivation, EXD protein provided from maternally expressed transcripts is sufficient to supply the DFD co-activator function in embryos.

On the 120 bp ES autoactivation module that is both *Dfd* and *exd* dependent, our results suggest that the *exd* co-activator effect is exerted directly. A four base substitution in this element that diminishes its *in vitro* binding affinity for EXD protein results in weaker activity of the mutant element in maxillary embryonic cells that are expressing both DFD and EXD. Conversely, a 5 bp substitution that increases the *in vitro* affinity of the element for EXD protein results in an element that has stronger embryonic activity. Though it is formally possible that the mutant base pairs introduced into these elements fortuitously generated regulatory elements with different activities that have nothing to do with EXD binding, it seems unlikely. Therefore, we conclude that EXD is binding directly to the 120 bp module E in maxillary cells and activating its function in combination with DFD protein. The different activities of the element with variant EXD-binding sites could be mediated by the differential enhancement of DFD protein binding that they exhibit in

the presence of EXD, but our current results do not allow us to address this point definitively.

Despite numerous attempts using different protein preparations, binding conditions and gel electrophoresis conditions, we and others (C.Murre personal communication) have so far been unable to detect the cooperative formation of DFD–EXD mobility shift complexes on the binding sites shown in this study. Cooperative binding complexes have been detected for many other HOX proteins in combination with EXD/PBX proteins, including a HOX protein that is a DFD ortholog (Phelan *et al.*, 1995). However, simple enhancement of DNA-binding activity, without the formation of ternary complexes, has been observed previously with homeodomain proteins in mobility shift assays (Grueneberg *et al.*, 1992; Chan *et al.*, 1994). These results do not rule out the possibility that stable EXD–DFD complexes exist in embryos on 120 bp element sequences, perhaps requiring other unknown embryonic factors for stability.

Our experiments provide no support for the idea that a specialized heterodimer-binding site which would bind a DFD–EXD complex stably and tightly is required for the specific activation of the 120 bp element in embryos. Mutations that subtly alter the EXD-binding region (as shown here), the DFD-binding region (Zeng *et al.*, 1994) or the spacing between the two (Zeng, 1995) without completely abolishing the binding affinity for either protein are still compatible with maxillary-specific, *Dfd*-dependent activity from the 120 bp element. The E3BS 120 bp element retains some maxillary activity and specificity even when EXD-binding affinity is reduced to even lower levels than that found in the wild-type element. However, even when EXD monomer binding affinity is nearly abolished on E3BS, the inclusion of EXD protein in binding reactions still induces a modest enhancement (1.5- to 2-fold) of DFD binding to the E3BS element *in vitro* (Figure 6B), which might explain the residual activity in embryos of E3BS. It is possible that other factors such as DEAF-1 (Gross and McGinnis, 1996) might also contribute to the small residual amount of E3BS element activity in embryos.

Although EXD is not required for the maintenance of embryonic expression of the *Drosophila Hox* genes *Scr*, *Antp*, *Ubx* and *abd-A* (Peifer and Wieschaus, 1990), these genes do not use direct autoactivation as a mechanism for the maintenance of their post-establishment expression patterns, instead depending on the trithorax group of homeotic activators (Kennison, 1993). The *Hox* gene *labial* does use autoactivation to maintain its expression (Chouinard and Kaufman, 1991; Tremml and Bienz, 1992), and the work of Chan *et al.* (1996) has shown that *labial* requires *exd* function for persistent embryonic expression. The results from a variety of studies indicate that any enhancer that is activated directly by any of the HOX proteins from LAB through ABD-A, whether a regulatory element of a downstream gene or an autoregulatory element, requires *exd* for the activation function (Peifer and Wieschaus, 1990; Chan *et al.*, 1994, 1996; Rauskolb and Wieschaus, 1994; Popperl *et al.*, 1995; Sun *et al.*, 1995).

exd as a repressor of HOX target elements

The 120 bp DFD response element is the second HOX response element which has been shown to have ectopic activity in exd^{mz-} mutants. The first example was the *dpp*671 element, which is activated ectopically in visceral mesoderm anterior to PS7 in exd^{mz-} mutants (Rauskolb and Wieschaus, 1994). Interestingly, for both the 120 bp element and the *dpp*671 element, this ectopic activity is unchanged in *Hox* mutant backgrounds. The current results do not allow us to conclude whether the repressive effect of *exd* on the 120 bp DFD response element is direct or indirect, since the potential direct repressor (EXD)-binding site and the sequences required for ectopic activation overlap in the TAAT motif. The ectopic activity of HOX target elements in *exd* mutants may be thought of as *exd*-dependent homeotic transformations writ small. They are the only known examples of homeotic alterations in target activity in *exd* mutants, and both depend on *Hox*-independent repressor activity of *exd* on target elements that are regulated by HOX proteins in other segments on the body axis. Thus we believe that the repressor function of *exd* may regulate axial specificity of other HOX target elements, and may provide a partial explanation for the homeotic transformations seen in *exd* zygotic mutant embryos.

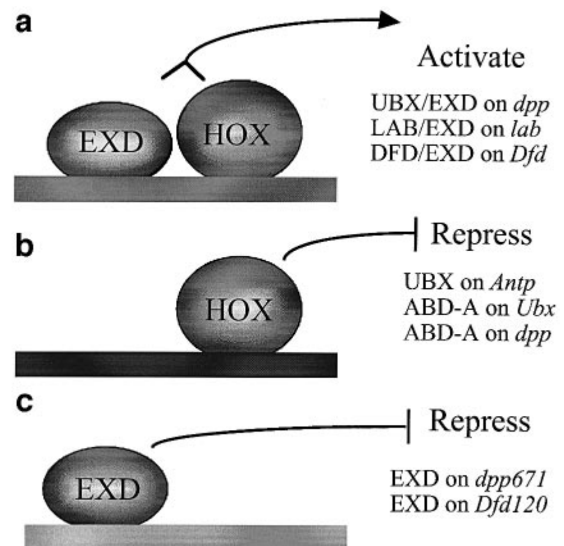

Fig. 8. A model for EXD action on HOX response elements. The three types of activity proposed for EXD and HOX proteins on HOX target elements, with examples of regulatory elements or downstream genes that respond in the manner proposed in the model. (**a**) On the first type of target element where both EXD and HOX proteins act together, they activate transcription. EN family members may also activate in combination with EXD proteins in a similar manner (Peifer and Wieschaus, 1990; Peltenburg and Murre, 1996). (**b**) On a second type of target element where HOX proteins act without EXD, they repress transcription. It seems likely that there are HOX co-repressors, not shown in the diagram, that contribute to the selection of HOX targets that are subject to repression. (**c**) The third part of the model proposes that EXD can act as a HOX-independent repressor of some HOX response elements outside the region of the body axis where it acts as a HOX-dependent activator of the same element. It seems likely that there are other factors, not shown in the diagram, that contribute to the selection of EXD targets that are subject to repression

Role of exd in HOX target specificity

We propose a three-part model that differs from those previously offered for EXD function in the HOX system, which has the advantage of explaining some previous results that were paradoxical (Figure 8). We believe that

the same model is likely to apply to PBX–HOX interactions in developing mammalian embryos.

First, the model proposes EXD as a required co-activator for most HOX target elements. All known elements activated in response to HOX proteins also require *exd* function (Chan *et al.*, 1994, 1996; Rauskolb and Wieschaus, 1994; Popperl *et al.*, 1995; Sun *et al.*, 1995). Based on the phenotypes of exd^{mz-} mutants and of clones of *exd* mutant cells in adult cuticular structures (Peifer and Wieschaus, 1990; Gonzalez-Crespo and Morata, 1995; Rauskolb *et al.*, 1995), it seems likely that a great many other HOX target elements also require *exd* for their activation. The only HOX protein that appears to have some *exd*-independent activation function is ABD-B (Peifer and Wieschaus, 1990). This correlates with the absence of a YPWM motif just upstream of the ABD-B homeodomain sequence. The YPWM or hexapeptide motif is required for the formation of HOX–EXD or HOX–PBX cooperative binding complexes (Chang *et al.*, 1995; Johnson *et al.*, 1995; Knoepfler and Kamps, 1995; Neuteboom *et al.*, 1995; Phelan *et al.*, 1995). The EN family of homeodomain proteins have an Trp-containing amino acid domain upstream of the EN homeodomain that is analogous to the HOX hexapeptide motif in its ability to confer cooperative binding with EXD/PBX family proteins (Peltenburg and Murre, 1996). Since EXD function is required for the EN-dependent autoactivation circuit in *Drosophila* embryos (Peifer and Wieschaus, 1990), perhaps the EN family proteins also require EXD in order to function as transcriptional activators.

As part of its HOX co-activator function, EXD may play a role in discriminating among HOX target elements through its ability to recruit HOX proteins selectively to different composite binding sites (Chan *et al.*, 1994, 1996; van Dijk and Murre, 1994; Chang *et al.*, 1995; Johnson *et al.*, 1995; Popperl *et al.*, 1995; van Dijk *et al.*, 1995), but our results on the 120 bp element suggest that this function is not essential to the generation of a specifically activated DFD target. Since the *in vivo* binding specificity of some homeodomain proteins seems nearly as indiscriminate as their *in vitro* binding specificity (Walter *et al.*, 1994), it is possible that selective cooperative binding of EXD–HOX heterodimers plays a rather modest role in discriminating among HOX targets to be activated. We favor the idea that EXD interacts generally with many HOX proteins to ensure that a wide variety of HOX targets are activated instead of repressed, and that cooperative binding might serve principally as a mechanism to achieve a more stably activated HOX conformation.

The second part of the model proposes that when HOX proteins act as repressors they do not require *exd* function. This explains why the repressive cross-regulatory interactions among the *Hox* genes are still functional in *exd* mutants. For example, *Antp* expression in the abdomen is still repressed normally by UBX and ABD-A in exd^{mz-} embryos (Peifer and Wieschaus, 1990). Another example of this *exd*-independent repressive activity is that of *abd-A* on *dpp* gene expression in the posterior visceral mesoderm (Rauskolb and Wieschaus, 1994). In an extreme version of the model, EXD is the only factor that discriminates between HOX proteins as transcriptional activators in embryos, and HOX proteins as transcriptional repressors. Presumably there are sequences in HOX target elements which allow the selection of activation functions versus repressive functions. In the simplest model, which we do not favor, HOX activation elements would contain EXD-binding sites, and the repression elements would lack EXD-binding sites. It seems likely that additional cofactors are involved, perhaps some that help specifically define HOX targets for repression in the same way that EXD apparently defines HOX targets to be activated.

An EXD-dependent switch of HOX activator to HOX repressor can also explain some of the homeotic phenotypes observed in *exd* zygotic mutant embryos, and it is helpful to consider this in the context of phenotypic suppression. Phenotypic suppression refers to the ability of more posterior *Hox* genes to functionally inactivate more anterior *Hox* gene functions (Gonzalez-Reyes and Morata, 1990; Macias and Morata, 1996), and has been proposed to involve a functional hierarchy among HOX proteins where more posterior proteins in the HOX complex are better competitors for downstream targets. In the context of our model, we suggest that phenotypic suppression may be explained partly by the ability of posterior HOX proteins to repress, in an *exd*-independent fashion, target genes that are activated by more anterior HOX proteins in an *exd*-dependent fashion.

An example of homeotic phenotype in *exd* zygotic mutants which can be explained by the HOX activator to repressor switch is the transformation of embryonic first abdominal segment (A1) to A2/4-like morphology (Peifer and Wieschaus, 1990). We assume that the transformation of A1 to A2/4-like morphology involves the repression of some genes by ABD-A in A2/4 that normally are activated by UBX in A1. We propose that at later stages of embryogenesis when maternal EXD is depleted from zygotic mutants, UBX would be switched from an activator of A1 genes to a repressor. This switch would mimic the normal repression function of ABD-A protein on the A1 genes and the result would be a morphology that is A2/4-like in the region where UBX is expressed. The generation of a novel UBX repression function would explain why the phenotype of exd^{z-}, Ubx^{-} double mutants differs from that of exd^{z-} mutants.

Homeotic transformations are not seen in *exd* mutants that lack both maternal and zygotic *exd* function. In our model, this would be explained by the complete absence of the co-activation function of *exd.* The resulting embryonic segments would thus lack almost all knowledge as to their identity, except in the posterior abdomen (Peifer and Wieschaus, 1990).

Recent fascinating results have shown that an EXD-induced conformational change in LAB allows it to bind DNA with higher affinity, relieving an inhibitory activity of the YPWM motif (Chan *et al.*, 1996). Mutation in the YPWM motif from LAB protein results in a LAB-binding activity that is *exd* independent *in vitro*, and hyperactive in embryos (Chan *et al.*, 1996). However, Chan *et al.* (1996) have also shown that the constitutive binding-activated form of LAB still requires EXD for its transcriptional activation function, indicating that the binding enhancement conferred by the YPWM mutation is not sufficient for LAB activity in embryos. For some of the other HOX proteins, which do not obviously require EXD for the acquisition of monomer-binding activity *in vitro*, we propose that a mechanism involving an EXD interaction

with the YPWM motif of HOX proteins can switch HOX repressors into HOX activators.

The third part of the model proposes that EXD can act as a HOX-independent repressor of some HOX response elements outside the region where it acts as a HOX-dependent activator of the same element. The known examples of this repressive function have been observed on the 120 bp DFD response element and the *dpp*671 UBX response element (Rauskolb and Wieschaus, 1994). Embryos that lack zygotic *exd* function apparently have enough EXD to co-activate initially many of the normal HOX target genes in their normal positions (Peifer and Wieschaus, 1990), but at later stages the deficit of *exd* function may result in the ectopic expression of some HOX target genes. This derepression of HOX targets may also contribute to the homeotic transformations seen in *exd* zygotic mutants, or in clones of *exd* mutant cells in the adult cuticle.

Materials and methods

exd and *Dfd* genetic interactions

Flies of the genotype Dfd^{rC11}/Dfd^{rV8} have a reduced viability of ~50%, and reducing the copy number of certain modifier genes vital to *Dfd* function can result in further, precipitous reductions of viability (Harding *et al.*, 1995). To test the interaction of *exd* with *Dfd*, *exd/inscy, v; Ki* Dfd^{rV8}*red/+* females were mated to *v/Y;* Dfd^{rC11} p^p*/TM6B* males on standard corn meal-based fly food. At least three vials were set up for each experiment. The flies were mated initially at room temperature for 4 h and then the vials were moved to 29°C. After 2 days, the flies were transferred to new vials. The transfer was repeated once more. To determine the interaction strength of each allele, the ratio of the number of + *exd/v +; Ki* Dfd^{rV8} *red/*Dfd^{rC11} p^p to *exd/v +;* Dfd^{rC11} p^p*/+* progeny was calculated. This ratio was corrected using the same calculation for the *inscy, v* chromosome as an internal control. The exd^{S136} allele was mapped in meitoic recombination experiments; 16 of 384 recombinants separated *fused* (at 56.2 cM) and *S136*, which placed the *S136* lethal at ~52 cM.

Protein–DNA binding assays

DNase I protection experiments were carried out as described in Heberlein *et al.* (1985), except that 3 µl of a 1/4000 dilution of DNase (10 U/µl) were used. EXD protein was produced in *Escherichia coli*, from a 900 bp *Nco*I fragment of the *exd* cDNA which was subcloned into the pRSET (Invitrogen) T7 expression vector. The insoluble fraction was dissolved in 8 M urea and purified over a Ni^{2+} column. Fractions which contained EXD protein were pooled together and dialyzed against phosphate-buffered saline (PBS) plus 10% glycerol and 0.05 M β-mercaptoethanol. The partially purified protein was aliquoted and stored at –80°C. DFD protein was produced in *E.coli* and partially purified according to Dessain *et al.* (1992).

Binding reactions for mobility shift assays were carried out under the following conditions: 25 mM HEPES pH 7.6, 50 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 5 mM $MgCl_2$ and 5 mg/ml carrier DNA. Bacterially produced DFD (or DFD + EXD, or DFD + control proteins) was added to binding buffer containing ^{32}P-labeled double-stranded DNA probes. After incubating on ice for 15 min, the reactions were loaded onto running 8% native polyacrylamide gels, and run for 2 h 45 min at 200 V at 4°C. The amount of probe bound was quantified with the use of a PhosphorImager. For each experiment, counts were determined from boxes of identical size drawn around the DFD bands. Enhancement of binding was calculated by dividing the counts from the DFD plus EXD (or controls) lane by the counts from the DFD alone lane from the same experiment, and the values were averaged. Error bars mark one standard deviation from the mean values.

Regulatory constructs, germline transformations, germline clones

Constructs E3BS, E3CS and ES were generated from six pairs of oligos, subcloned into pBluescript, then into pCaSpeR as described in Zeng *et al.* (1994). The resulting reporter constructs (0.5 mg/ml) were co-injected with the P-element helper plasmid pπΔ2-3 (0.5 mg/ml) into w^1 embryos (Spradling, 1986). The numbers of independent inserts for each construct were: E3CS, nine; E3BS, 10; and ES, six. Embryos that were to be compared from these lines were always stained for the same lengths of time on the same day, using the same solutions.

Embryos lacking both maternal and zygotic copies of *exd* were created by crossing *exd* FRT18D; F38hsFLP females with ovo^D FRT18D; F38hsFLP males. After 2 days, flies were transferred to new vials. After two more days, vials containing larvae were placed in a 39°C water bath for 1 h. Larvae were allowed to recover and mature at room temperature. Adult virgin females were collected and crossed to males homozygous for reporter constructs, and the resulting embryos were stained with anti-β-Gal antiserum to visualize the reporter expression patterns in an *exd* maternal/zygotic mutant background.

Expression pattern detection with antiserum and antisense probes

Dfd or *lacZ* reporter gene expression patterns were detected by immuno-histochemical staining of whole-mount embryos. Embryos were collected for 24 h on grape juice plates, dechorionated in 100% bleach and fixed in vials containing 4 ml of 3.9% formaldehyde in PBS and 4 ml of heptane on a shaker for 15 min. After removing the aqueous layer, adding 4 ml of methanol and shaking, devitellinized embryos were stored in 100% ethanol. Rabbit anti-β-gal antibody (Cappel) diluted 1:10 000 was incubated with embryos overnight at 4°C. After two quick rinses, goat anti-rabbit antibody conjugated to horseradish peroxidase (HRP, Bio-Rad), diluted 1:300, was added and incubated for 1 h. After washing in 0.12 M Tris pH 7.6, the color reaction was developed in 0.5 mg/ml DAB and 1:1000 H_2O_2 at pH 7.6. *Dfd* expression was detected as above, except that affinity-purified guinea pig anti-DFD antiserum and goat anti-guinea pig HRP-conjugated antibody (Cappel) were used.

A 24 h collection of hsDfd/exd^{mz-} embryos was heat shocked at 38°C for 1 h, and allowed to recover for 1 or 5 h before fixation. *Dfd* transcripts were detected by *in situ* hybridization using digoxigenin-labeled RNA probes, as described in O'Neill and Bier (1994).

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