Development 126, 5581-5589 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
DEV5364

A sequence motif distinct from Hox binding sites controls the specificity of a Hox response element

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Accepted 5 October; published on WWW 24 November 1999

SUMMARY

Hox transcription factors, in combination with cofactors such as PBC proteins, provide diverse developmental fates to cells on the anteroposterior body axis of animal embryos. However, the mechanisms by which the different Hox proteins and their cofactors generate those diverse fates remain unclear. Recent findings have provided support for a model where the DNA binding sites that directly interact with Hox-PBC heterodimers determine which member of the Hox protein family occupies and thereby regulates a given target element. In the experiments reported here, we test the function of chimeric Hox response elements and, surprisingly, find evidence that runs counter to this view. A 21 bp cofactor binding sequence from an embryonic *Deformed* Hox response element, containing no Hox or Hox-PBC binding sites, was combined with single or multimeric sites that bind heterodimers of *Labial*-type Hox and PBC proteins. Normally, multimerized Labial-PBC binding sites are sufficient to trigger a Labial-specific activation response in either *Drosophila* or mouse embryos. Here we find that the 21 bp sequence element plays an important role in Deformed specificity, as it is capable of switching a Labial-PBC binding site/response element to a *Deformed* response element. Thus, cofactor binding sites that are separate and distinct from homeodomain binding sites can dictate the regulatory specificity of a Hox response element.

Key words: Hox specificity, Homeotic, Exd, Deformed, Labial, *Drosophila*

INTRODUCTION

Hox transcription factors play instructive roles in generating morphological diversity along the anteroposterior body axis during animal development (McGinnis and Krumlauf, 1992; Lawrence and Morata, 1994; Manak and Scott, 1994). Recent findings support the idea that the different developmental fates specified by the Hox proteins are partly due to their abilities to regulate different sets of downstream regulatory genes (Mann and Chan, 1996), and partly due to their abilities to regulate a largely overlapping set of genes in different ways (Biggin and McGinnis, 1997). Evidence that Hox proteins require cofactors for specificity has been accumulating for some time. For example, the different members of the Hox family have very similar DNA binding properties as monomers (Ekker et al., 1994), and many of the amino acid residues that diversify the segmental identity functions of Hox proteins map in the homeodomain region, but far from the DNA binding interface (Kornberg, 1993).

At present, the best understood cofactor at the genetic and molecular level is *Drosophila* Extradenticle (Exd), which acts in parallel with Hox proteins to assign segmental identities (Peifer and Wieschaus, 1990). The Exd protein and its mammalian Pbx homologues (the PBC proteins) are members of the homeodomain superfamily, albeit highly diverged from the Hox family (Flegel et al., 1993; Rauskolb et al., 1993). In vitro, PBC proteins cooperatively interact with a wide range of Hox type (as well as a few non-Hox type) homeodomain proteins on composite binding sites, where they influence the occupancy and activity of Hox proteins in the context of larger response elements (Chan et al., 1994; van Dijk and Murre, 1994; Pöpperl et al., 1995; Chan et al., 1997; Pinsonneault et al., 1997; Li et al., 1999). There are other potential cofactors, such as Teashirt (Röder and Kerridge, 1992), which influence Hox specificity in a manner apparently independent of Exd, but their mechanism(s) of action are still being mechanistically dissected.

The functions of Hox proteins and their cofactors converge on the cis-regulatory elements of target genes, and the dissection of such elements in both *Drosophila* and mouse has provided important insights concerning Hox protein function (Regulski et al., 1991; Vachon et al., 1992; Gould and White, 1992; Appel and Sakonju, 1993; Capovilla et al., 1994; Chan et al., 1994; Zeng et al., 1994; Manak et al., 1995; Mastick et al., 1995; McCormick et al., 1995; Pöpperl et al., 1995; Sun et al., 1995; Chan et al., 1997; Gould et al., 1997; Grieder et al., 1997; Haerry and Gehring, 1997; Maconochie et al., 1997; Pinsonneault et al., 1997; Li et al., 1999). Some general lessons emerge from these studies. Many Hox response elements are under the control of multiple Hox activators and/or repressors

in multiple segments. Elements that can be activated or repressed by Hox proteins in embryos usually contain several Hox binding sites of varying affinities, most of which make a detectable contribution to the overall activity of the element. There is rarely a correlation between the functional importance of the sites in embryos and the in vitro affinity of the sites for Hox monomers. However, there is a good correlation between the affinity that an element has for Hox-PBC heterodimers binding sites, and a robust activation response. Cofactor binding regions that are adjacent to Hox or Hox-PBC binding sites are also required for the proper activity levels of Hox response elements, but searches for common, functionally relevant, consensus sequences in adjacent regions have largely been futile. One exception is that Ftz-F1 binding sites are often adjacent to Ftz binding sites (Schier and Gehring, 1993; Han et al., 1993; Florence et al., 1997; Guichet et al., 1997; Yu et al., 1997). As judged by biological function, Ftz is quite distinct from the Hox proteins, but its homeodomain region is very similar to Hox type homeodomains. The use of FtzF1 and PBC cofactor binding sites is similar to the way that homeodomain protein functional specificity is achieved in yeast, where response elements for the Matα2 homeodomain protein also contain highly conserved cofactor binding sequences overlapping the Matα2 binding site (e.g. Johnson and Herskowitz, 1985).

Composite sites that interact with both Hox and PBC proteins have been intensively studied. A multimerized 20 bp element containing a composite site that binds both mouse Hoxb-1 [or its *Drosophila* ortholog Labial (Lab)] and a PBC protein, provides a Hoxb-1 or Lab-dependent reporter expression in either mouse or *Drosophila* embryos (Pöpperl et al., 1995; Chan et al., 1996). A change of two basepairs in each multimer of this element has been reported to switch its functional specificity from a Lab responsive to a Deformed (Dfd) responsive element in *Drosophila* embryos (Chan et al., 1997). This alteration in functional specificity is paralleled in vitro by a switch in binding preference of the composite site, from one that prefers to bind Lab-Exd complexes (TGATGGAT sites), to one that prefers to bind Dfd-Exd complexes (TGATTAAT sites). Could it be that the specificity code is as simple as switching one or two base pairs in a composite site? Unfortunately, the simple correlation between Hox-PBC binding and specificity breaks down if further analyzed. Other Hox proteins like Ultrabithorax (Ubx) bind cooperatively with Exd to TGATTAAT sites in vitro, but do not activate these multimerized sites in embryos (Chan and Mann, 1996; Chan et al., 1997).

Naturally evolved Hox response elements appear to be quite complex, as would be expected since most are activated or repressed by Hox proteins in a manner that integrates both the developmental history of a cell and the extracellular signals that impinge upon it. For example, a 550 bp Lab autoactivation enhancer (Chouinard and Kaufman, 1991; Tremml and Bienz, 1992), contains a Lab-Exd consensus site that is required for its embryonic function, as well as other cofactor binding regions that are also required (Grieder et al., 1997). The cofactor binding regions include sequences that mediate the effects of the Dpp signal that is also required for the function of the 550 bp Lab enhancer (Tremml and Bienz, 1992; Grieder et al., 1997; Kim et al., 1997). The preference of the element for Lab, of all the Hox proteins that could potentially activate it, is not strictly controlled by the sequence of the Hox/Exd composite site, since the switch of the TGATGGAT composite site to a TGATTAAT composite site did not change its specificity (Grieder et al., 1997). Therefore, the selective responsiveness of this and other Hox target elements might depend on sequences that are not part of the Hox or Hox-PBC composite sites.

In the present study, we have asked whether a DNA sequence that is derived from a natural response element and which binds neither Hox proteins nor Hox-PBC dimers could play an instructive role in Hox specificity. Previously, we identified the region 6 (21 bp) inverted repeat sequence, that was required for the embryonic activity of a 120 bp Dfd autoregulatory element (Zeng et al., 1994; Gross and McGinnis, 1996). Here we show that, when the region 6 inverted repeat sequence is juxtaposed to one or more repeats of a composite Lab-Pbx site (a TGATGGAT site), the composite site is no longer activated by Lab, but is activated by Dfd protein in embryos. Our results suggest that cofactor binding sites can determine the specificity of Hox response elements in a manner that is largely independent of the binding preferences for certain Hox proteins, or certain Hox-Exd heterodimers.

MATERIALS AND METHODS

Construction of regulatory elements

Oligonucleotides containing a Hox binding site, a GAL4 binding site, or a region 6 inverted repeat were annealed in proper combinations and ligated into *Pst*I-*Hin*dIII of pBluescript KS$^+$. The resulting regulatory elements were sequenced to ensure fidelity and subsequently transferred as *Bam*HI-*Xho*I fragments into pCaSpeR hs43 *lacZ*III vector (Zeng et al., 1994). 4xBZ was made as described (Zeng et al., 1994). Transgenic lines carrying these constructs were then established by standard procedures (Rubin and Spradling, 1982).

Immunostaining of β-galactosidase

The immunostaining procedure was as described in Li et al. (1993). For each trangenic construct, embryos from three to ten transgenic lines were stained for β-galactosidase to test whether they provided similar or identical maxillary expression patterns. Occasionally, one or two lines exhibited no staining, or staining in other locations, presumably reflecting P element insertion position effects. For accurate comparison of *lacZ* expression levels, embryos from multiple representative homozygous lines from each construct were collected, fixed, stained and photographed in parallel. The embryos were staged as described (Campos-Ortega and Hartenstein, 1985).

Genetic control of 4xBZ expression

To test 4xBZ expression in *Dfd* mutant embryos, 4xBZ was recombined onto the *Dfd*^{w21} mutant chromosome and balanced over *TM3 Sb* with a *Ubx-lacZ* transgene. Thus, all *Dfd* mutant embryos carry two copies of 4xBZ and could be identified by the lack of *Ubx-lacZ* staining. 4xBZ expression in *exd* maternal/zygotic mutant embryos was analyzed as described (Rauskolb et al., 1993).

To test 4xBZ expression after ectopic *Hox* gene expression, flies homozygous for 4xBZ and *arm*-Gal4^{11} (Sanson et al., 1996) were crossed with flies carrying UAS-Dfd (Li et al., 1999), UAS-Ubx, or UAS-Abd-B (Castelli-Gair et al., 1994). All of the progeny embryos contained one copy of 4xBZ and were analyzed for the effects of ectopic Dfd, Ubx, and Abd-B protein on *lacZ* expression. The expression of Lab was provided by *hsp70*-Lab (Chouinard and Kaufman, 1991) as described (Chan et al., 1996). The embryos from

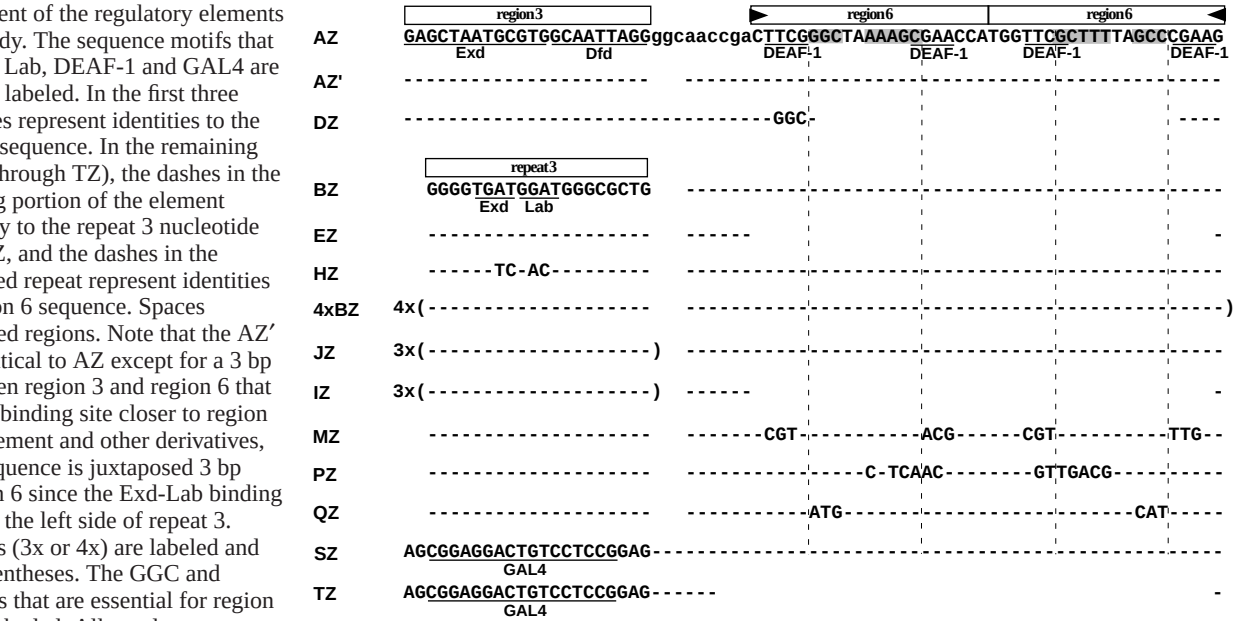

Fig. 1. Alignment of the regulatory elements used in this study. The sequence motifs that bind Exd, Dfd, Lab, DEAF-1 and GAL4 are underlined and labeled. In the first three lines, the dashes represent identities to the AZ nucleotide sequence. In the remaining elements (BZ through TZ), the dashes in the 5′, Hox binding portion of the element indicate identity to the repeat 3 nucleotide sequence of BZ, and the dashes in the region 6 inverted repeat represent identities to the AZ region 6 sequence. Spaces represent deleted regions. Note that the AZ′ element is identical to AZ except for a 3 bp deletion between region 3 and region 6 that brings the Dfd binding site closer to region 6. In the BZ element and other derivatives, the repeat 3 sequence is juxtaposed 3 bp closer to region 6 since the Exd-Lab binding site is closer to the left side of repeat 3. Tandem repeats (3x or 4x) are labeled and flanked by parentheses. The GGC and AAAGC motifs that are essential for region 6 function are shaded. All regulatory elements were inserted 39 bp upstream of the TATA box of the basal *hsp70* promoter-*lacZ* reporter gene. The sequence that joins TATA to each regulatory element is CTTATCGATACCGTCGACCTCGAGGAAGAGCGCCGGAG.

the cross of *hsp70*-Lab and 4xBZ flies were analyzed for effects of Lab on 4xBZ expression.

EMSA of DEAF-1

BZ and MZ regulatory elements in pBluescript KS$^+$ were released by digestion with *Pst*I and *Xho*I and labeled with ^{32}P by fill-in reactions. DEAF-1 protein was produced by the TNT coupled transcription/translation reticulocyte lysate system (Promega). Binding reactions were performed at room temperature for 30 minutes as described (Neuteboom and Murre, 1997) except 50 ng herring sperm DNA was used to replace 1 μg poly(dI-dC) as non-specific competitor DNA in each reaction.

RESULTS

Required sequences in a minimal *Dfd* response element

From cellular blastoderm until late stages of *Drosophila* embryogenesis, the *Dfd* gene is expressed in an evolving pattern that is limited to the maxillary and mandibular segments (Jack et al., 1988). One of the mechanisms that maintain *Dfd* transcription in these gnathal cells is an autoregulatory circuit, which involves Dfd protein functioning as a transcriptional activator on upstream and intronic enhancers of the gene (Zeng et al., 1994; Lou et al., 1995). A small upstream autoactivation element (module E), that directs Dfd-dependent transcription in maxillary epidermal cells, has been subjected to a systematic structure/function analysis (Zeng et al., 1994; Gross and McGinnis, 1996; Pinsonneault et al., 1997). The 120 base pairs (bp) of module E contain two regions crucial for its function. The first of these regions is the 22 bp of region 3, which include a strong Dfd binding site and a weak Exd binding site. Both of these binding sites are required for the module's ability to activate high levels of reporter gene transcription in embryonic maxillary cells. The second important region of the natural version of module E is region 5/6, which consists largely of an imperfect palindrome sequence. Mutations in either the 5 half or the 6 half of the palindromic region either reduce or abolish the activity of module E in embryos (Zeng et al., 1994; Gross and McGinnis, 1996). When this region is mutated to generate a perfect 6/6 palindrome in the context of a 120 bp module with region 3, the ability to function as a Dfd responsive element is retained and slightly enhanced (Gross and McGinnis, 1996).

To further define the role of the palindromic repeat region, we deleted the sequences from regions 1, 2 and 4 of module E, leaving only an inverted repeat of 21 bp from region 6 combined with 22 bp of region 3, linked by a 9 bp spacer. In embryos, this minimal element (AZ, Fig. 1) is capable of activating Dfd-dependent reporter gene transcription in the stage 13 maxillary lobe, and by stage 15 (Fig. 2C) the pattern of Dfd-dependent activity is similar to that provided by the entirety of module E in one copy (Zeng et al., 1994). In controls that possess no regulatory sequence insertions upstream of the *hsp70* basal promoter/*lacZ* reporter gene, only two or three cells in the posterior maxillary lobe accumulate low levels of β-galactosidase from late stage 15 onward (Fig. 2A), with a few more cells exhibiting reporter expression at later stages. The late expression of the reporter-only construct is independent of *Dfd* function as it is not abolished in *Dfd* mutant embryos (data not shown). Therefore, we have analyzed expression patterns only at stages 15 and earlier in our functional tests of regulatory sequence inserts. A regulatory construct with a single copy of region 3 alone (DZ, Fig. 1) is not expressed above the background level (Fig. 2B). The spacing between region 3 and the region 6 inverted repeat is apparently important, as a deletion variant of AZ called AZ′ (Fig. 1) that brings region 3 and region 6 sequences closer together by 3 bp exhibits background levels of activity (Fig. 2D). The inactivity of the AZ′ construct also indicates that the region 6 inverted repeat alone possesses no intrinsic Dfd-dependent activity. From the preceding results, we conclude

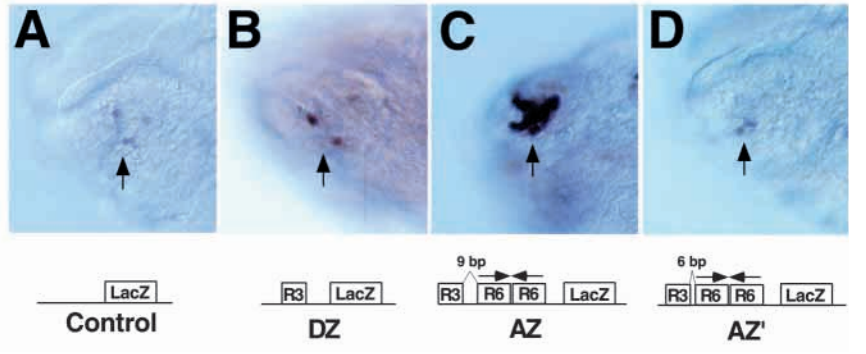

Fig. 2. Region 6 confers Dfd response from a Dfd binding site. The heads of the embryos at stage 15 are shown in lateral view with dorsal up and anterior to the left. Arrows point to the maxillary lobe. (A) Background expression of the *lacZ* reporter from the control vector without regulatory element. (B) Region 3 (R3) does not activate *lacZ* expression in DZ. (C) *lacZ* expression is activated in AZ where the inverted region 6 (R6) repeat is placed 9 bp apart from region 3. Note the anchor-like shape of *lacZ*-expressing cells. (D) *lacZ* expression is not activated in AZ′ where region 6 and region 3 are 6 bp apart.

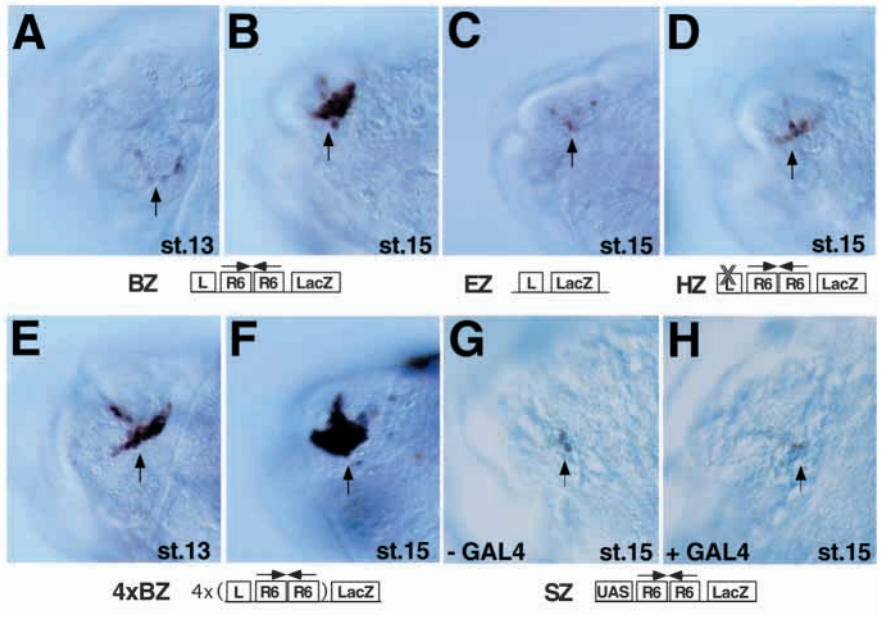

Fig. 3. The Lab-Exd site mediates *lacZ* expression in maxillary cells in the presence of region 6. The heads of the embryos at stages 13 or 15 are shown in lateral view with dorsal up and anterior to the left. Arrows point to the maxillary lobe. (A,B) *lacZ* expression is activated in BZ where the inverted region 6 (R6) repeat is adjacent to the Lab-Exd binding site (L). (C) The Lab-Exd binding site does not activate *lacZ* expression in EZ. (D) *lacZ* expression is severely reduced in HZ where the Lab-Exd site is mutated. (E,F) Four copies of BZ (4xBZ) mediate stronger *lacZ* expression than one copy (A,B). (G,H) *lacZ* expression is at background levels in SZ embryos, which have a single GAL4 homodimer binding site (UAS) adjacent to the inverted region 6 (R6) repeat, either in the absence (G) or the presence (H) of GAL4.

that the 22 bp of region 3, together with an appropriately spaced 21 bp inverted repeat from region 6, constitute a Dfd response element.

Region 6 endows a Lab-Exd composite site with a *Dfd* response function

Although the activity of the AZ element requires both region 3 and region 6 sequences, it is possible that the specificity of the response of this element to Dfd is encoded only in region 3 (the Dfd and Exd binding region), or only in region 6 sequences, or is dependent on specificity determinants encoded in a combination of these two sequences. To discriminate among these possibilities, we constructed hybrid regulatory elements with sequences derived from other Hox response elements.

One of the best characterized and smallest Hox target is the 20 bp 'repeat 3' (*rpt3)* element (Pöpperl et al., 1995; Chan et al., 1996). This element was discovered in a mouse *Hoxb-1* autoactivation enhancer, and contains an 8 bp sequence (TGATGGAT) that cooperatively binds either Hoxb1-Exd or Lab-Exd heterodimers in vitro (Pöpperl et al., 1995; Chan et al., 1996). The TGATGGAT sequence has low affinity for Dfd protein monomers or Dfd-Exd heterodimers (Chan et al., 1997). When *rpt3* is multimerized, the resulting *3Xrpt3* element can activate reporter transcription in many Hoxb-1-expressing cells in mouse embryos, as well as Lab-expressing cells in *Drosophila* embryos (Pöpperl et al., 1995; Chan et al., 1997). In *Drosophila*, the *labial*-dependent activation of *3Xrpt3* elements is easily detected in a subset of Lab-expressing midgut endoderm cells that are just anterior to the 2nd midgut constriction (Diederich et al., 1989; Immerglück et al., 1990; Chan et al., 1996). To test the function of the region 6 sequences, we substituted one copy of a Lab-dependent *rpt3* sequence for the copy of the Dfd-dependent region 3 in the AZ element to generate the BZ hybrid regulatory element shown in Fig. 1.

In embryos carrying the BZ construct, *lacZ* reporter expression is detected in maxillary epidermal cells from stage 13 onward (Fig. 3A). By stage 15, more than 20 maxillary cells express β-galactosidase in the typical anchor-like pattern (Fig. 3B). The pattern and amounts of reporter expression provided by the BZ element are only slightly less than that provided by AZ (Fig. 2C). The BZ expression pattern is dependent on region 6, as control elements containing only the Lab-Exd site (EZ, Fig. 1) exhibit background levels of activity (Fig. 3C). No BZ reporter activity is detected in the midgut endoderm cells that accumulate Lab protein.

To test whether the function of BZ is dependent on the Lab-Exd site, the TGATGGAT core was mutated to TGTCGACT (HZ, Fig. 1). This mutation of the composite site abolishes the

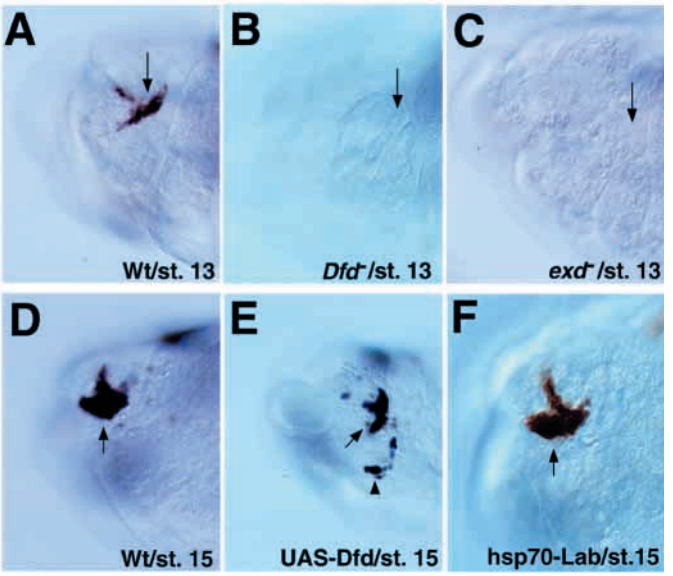

Fig. 4. Regulation of 4xBZ function by Dfd and Exd. The heads of the embryos are shown in lateral view with dorsal up and anterior to the left. Arrows point to the maxillary lobe. (A) *lacZ* expression of 4xBZ at stage 13 in wild-type (Wt) embryos. (B,C) *lacZ* expression of 4xBZ at stage 13 in *Dfd* zygotic (B) and *exd* maternal/zygotic (C) mutants is abolished. (D) *lacZ* expression of 4xBZ at stage 15 in wild-type embryos. (E) *lacZ* expression of 4xBZ in UAS-Dfd/*arm*-Gal4[11] embryos. Arrowhead points to the ectopic expression in the labial lobe. (F) No ectopic *lacZ* expression of 4xBZ is induced by uniform *lab* expression.

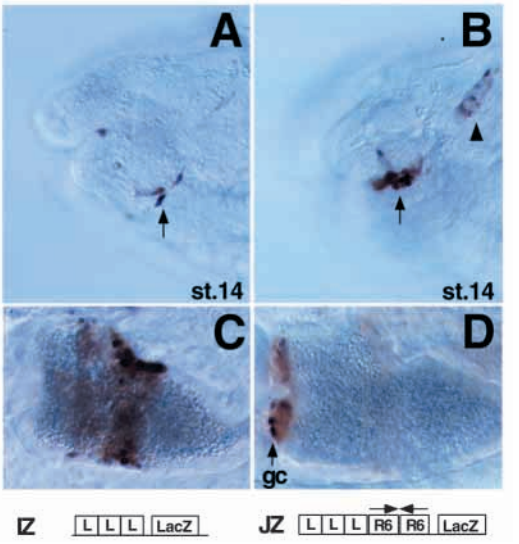

Fig. 5. Altered function of the Lab response element in the presence of region 6. (A,B) The heads of the IZ (A) and JZ (B) embryos at stage 14 are shown in lateral view with dorsal up and anterior to the left. *lacZ* expression in the maxillary lobe (arrow) and the dorsal ridge (arrowhead) in JZ (B) is much stronger than in IZ (A). (C,D) The midgut of the IZ (C) and JZ (D) embryos at stage 15 is shown in lateral view with dorsal up and anterior to the left. *lacZ* expression in IZ (C) is in the endodermal cells around the middle region (ps7) of the midgut whereas *lacZ* expression in JZ (D) is in the mesodermal cells at the tip of the midgut where the gastric caeca (gc) are forming.

binding of Lab-Exd heterodimers (Chan et al., 1996). As shown in Fig. 3D, the mutated HZ element provides much reduced levels of maxillary expression when compared to the BZ element.

Strong reporter expression can be provided by an element that consists of 4 tandem repeats of the BZ element (4xBZ, Fig. 1). In 4xBZ embryos, reporter expression can be detected as early as late stage 11, and is present in many maxillary cells at stage 13 and later. At the stages when it can be compared with one copy of BZ, 4xBZ also supplies more abundant expression on a per cell basis (Fig. 3E,F). No reporter gene expression is detected in cells that accumulate Lab protein.

The maxillary pattern of expression of the BZ element strongly suggests that the Lab-Exd composite site has come under the control of Dfd. As one test of this, we assayed *Dfd* mutants for 4xBZ activity and found it to be severely reduced. No 4xBZ-reporter expression is detected in stage 13 *Dfd* mutant embryos, while 4xBZ-reporter expression is robust in wild-type embryos (compare Fig. 4B with Fig. 4A). Weak 4xBZ-reporter expression in maxillary cells was observed from stage 14 in *Dfd* mutant embryos (data not shown), indicating that additional activators also contribute to BZ expression. The *Dfd* autoactivation circuit is interrupted in embryos that lack maternal/zygotic *exd* function (Pinsonneault et al., 1997) and, not surprisingly, 4xBZ function is also abolished in such embryos (Fig. 4C). In the *Dfd* and *exd* mutant embryos, the loss of reporter expression in the posterior maxillary segment is not due to cell loss, as staining these mutants with probes that reveal *engrailed* or *hedgehog* expression patterns indicate that lateral-posterior maxillary cells are still present (Peifer and Wieschaus, 1990, unpublished results). We also tested if ectopic expression of Dfd protein can induce expression of the 4xBZ element in ectopic locations. When Dfd protein is ubiquitously expressed by using the UAS/GAL4 system (Brand and Perrimon, 1993), 4xBZ is ectopically activated (compare Fig. 4E with Fig. 4D) in one or two mandibular cells and a subset of labial segment cells (despite the name, the Labial protein is not expressed in the labial segment). A similar pattern of ectopic reporter expression was also observed from the multimerized module E element after ectopic Dfd protein was induced from a *hsp70-Dfd* expression construct (Zeng et

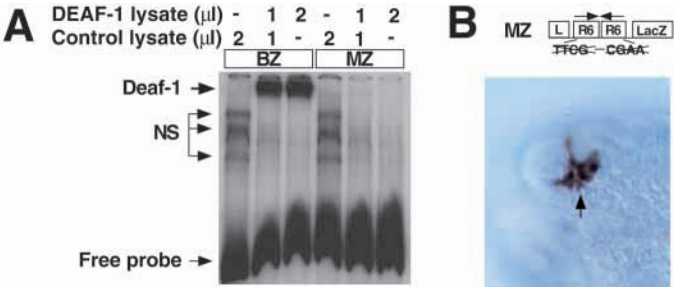

Fig. 6. DEAF-1 binding sites are not required for region 6 function. (A) EMSA of DEAF-1 on BZ and MZ. DEAF-1 binds strongly to BZ whereas no DEAF-1 binding is seen on MZ. NS represents nonspecific DNA binding by components in control reticulocyte lysate. (B) *lacZ* expression in maxillary cells (arrow) of the MZ embryo at stage 15. Note the normal level of expression in MZ as compared with BZ (Fig. 3B). The head of the embryo is shown in lateral view with dorsal up and anterior to the left.

al., 1994). Taken together, the above results strongly suggest that the BZ element responds to Dfd through the Lab-Exd composite site, but that the ability of Dfd to activate BZ is controlled by the region 6 inverted repeat.

To further test the specificity of the regulatory element consisting of region 6 plus the Lab-Exd composite site functions, we ubiquitously expressed several other Hox proteins including Lab, Ubx and Abdominal-B (Abd-B) in 4xBZ embryos and examined their effects on reporter expression. In each case, 4xBZ expression was not activated in any ectopic locations (Fig. 4F, data not shown), further suggesting that Dfd is the only known member of the Hox family capable of activating this hybrid element.

One possible way to explain the ability of Dfd to selectively activate BZ is to invoke a covert, region-6 DNA binding domain in the Dfd protein that is exposed only upon interaction with Exd protein. We tested for this hypothetical covert binding function in vitro and found no evidence for it. That is, Dfd + Exd has the same affinity for a Hox/Exd DNA binding site in isolation as Dfd + Exd has for a composite element consisting of a Hox/Exd binding region fused to the region 6 sequence (unpublished results). It therefore seems likely that the specific response of the Hox-Exd sites to Dfd but not to Lab depends on cofactors that bind region 6 and provide Dfd specificity to adjacent Hox-Exd sites.

Region 6 interacts specifically with Hox binding sites

The above results suggest that in 4xBZ and related Dfd response elements, the region 6 inverted repeat, rather than the specific Hox binding site, plays an instructive role in determining the response of these elements to Dfd. However, the results leave open the possibility that the maxillary specificity is entirely due to region 6, with the Hox binding site providing only a generic activation function. For example, it is possible that Dfd acts earlier in development to activate a maxillary-specific gene encoding a region 6 activating factor, but the later role of Dfd is to act solely as a generic activator on the Hox site adjacent to region 6. In this view, virtually any other generic activator protein that binds adjacent to region 6 would enhance the activity from a BZ-like element, and thereby provide significant reporter transcription in the maxillary segment.

To test the hypothesis that the Hox-Exd binding site is important for specificity, we tested whether the presence of a single GAL4 homodimer binding site could substitute for the Lab-Exd heterodimer binding site when juxtaposed to the region 6 inverted repeat. In *Drosophila* embryos, multiple GAL4 binding sites can activate transcription of downstream genes in all or nearly all cells (Brand and Perrimon, 1993), while in tissue culture cells even one GAL4 homodimer binding site is sufficient to mediate an activation response from a downstream promoter (Webster et al., 1988). We first tested a reporter construct that contained a single GAL4 binding site fused to a basal promoter/*lacZ* reporter gene (TZ, Fig. 1). The TZ-reporter is not detectably expressed in stage 13-15 embryos that lack GAL4 protein, and when GAL4 protein is provided by the *arm*-GAL4^4 driver (Sanson et al., 1996; Li et al., 1999), the TZ-reporter is activated to low levels in only a few scattered embryonic cells (data not shown). The SZ reporter differs from TZ by the addition of a region 6 inverted repeat adjacent to the GAL4 binding site (Fig. 1). In the SZ lines without GAL4, reporter gene expression is detected only at background levels in two or three cells of the maxillary segment (and occasionally other segments) in embryos from stage 15 (Fig. 3G). This background level of expression from SZ is unchanged in embryos in which GAL4 is provided by *arm*-GAL4^4 (Fig. 3H). The level of reporter expression of SZ in maxillary cells is dramatically lower than that provided by the BZ element (compare Fig. 3G,H with Fig. 3B), and is comparable to HZ in which the Lab-Exd site is mutated (Fig. 3D). Thus, the region 6 inverted repeat cannot use GAL4 as a general activation function to enhance maxillary-specific expression. Together with previous results, this indicates that the combination of Hox and Exd sites in AZ or BZ are interacting in a specific way with the sequences in region 6 composite element to produce maxillary-specific transcription.

Reprogramming a *Lab* response element

Since the Lab-Exd site can become a functional Lab response element (*3Xrpt3*) when existing in three tandem copies (Chan et al., 1996), we went on to test whether the region 6 inverted repeat is capable of altering the function of such a multimerized element. Previous experiments have shown that *3Xrpt3* is expressed mainly in Lab-expressing cells, which include midgut endoderm cells adjacent to parasegment 7, some epidermal and neural cells from the intercalary region of the head, and some dorsal ridge cells (Chan et al., 1996). In addition, reporter gene expression was also activated by *3Xrpt3* in a *labial*-independent pattern in the gastric caeca primordia (Chan et al., 1996). In the context of our *hsp70* basal promoter/*lacZ* reporter construct, three copies of the 20 bp repeat 3 sequence (IZ, Fig. 1) provide expression in midgut endoderm cells just anterior to the 2nd midgut constriction, adjacent to parasegment 7 (Fig. 5C), but we did not detect any reporter expression in the head aside from a few maxillary cells from stage 14 onward (Fig. 5A). In addition, none of our IZ transformant lines exhibited reporter gene expression in the gastric caeca primordia (Fig. 5C).

When the region 6 inverted repeat was inserted adjacent to *3Xrpt3* sequences (JZ, Fig. 1), a regulatory element was generated that activates reporter expression in maxillary cells of stage 14 embryos in the typical anchor-like shape (Fig. 5B). The extent and amount of maxillary expression is approximately the same as provided by the BZ element, consistent with the idea that only one of the Lab-Exd sites in the JZ element contributes to the maxillary expression. In addition, the JZ element provides no detectable reporter expression in the Lab-expressing midgut endoderm cells adjacent to parasegment 7 (compare Fig. 5D with Fig. 5C). Instead, reporter expression is now detected in the gastric caeca mesoderm (Fig. 5D). These results suggest that, in the presence of an inverted repeat of region 6, the Lab-Exd composite site tandem repeat has lost the ability to respond to Lab and gained the ability to respond to Dfd, as well as to another unknown activator present in the visceral mesoderm of the gastic caeca. The *Sex comb reduced* (*Scr*) Hox gene is expressed in visceral mesoderm cells near the gastric caeca (Reuter and Scott, 1990), but the expression pattern provided by JZ does not overlap the *Scr* expression pattern in these cells (data not shown).

DEAF-1 binding sites are not required for region 6-dependent activation

In an attempt to identify cofactors that may act through region 6, we previously isolated the DEAF-1 protein, which binds to TTCG sequence motifs in regions 5 and 6 of module E (Gross and McGinnis, 1996). DEAF-1 has recently been discovered to have mammalian homologs, called NUDR (Huggenvik et al., 1998), or suppressin (LeBoeuf et al., 1998). To examine if DEAF-1 binding sites are required for the activation of the minimal BZ element, all four TTCG sequences in the inverted region 6 repeat were mutated to random sequences (MZ, Fig. 1). As measured by electrophoretic mobility shift assays (EMSA), these mutations abolish in vitro binding of DEAF-1 protein to the MZ element (Fig. 6A). When the function of the MZ element is assayed in embryos, the reporter expression pattern is similar to that of BZ (compare Fig. 6B with Fig. 3B). This result suggests that the DEAF-1 binding sites are not required for the activation of region 6, at least in the context of minimal elements such as BZ. In agreement with this, in zygotic mutants homozygous for *Df(3L)25-21* (which deletes DEAF-1 and four other adjacent genes), the 4BZ element is still activated in maxillary cells (A. V. and W. M., unpublished results).

Essential nucleotides for region 6 function

To define the sequences that are required for generating the activity of region 6, we mutated other blocks of nucleotides in region 6, including GGC and AAAGC motifs. These two motifs are both perfectly conserved as part of the inverted repeat in region 5 of module E (Zeng et al., 1994). When the two GGC motifs in the inverted region 6 repeat were mutated in the context of the BZ element (QZ, Fig. 1), reporter expression is significantly reduced and only detectable from stage 15 in a few maxillary cells (compare Fig. 7B with Fig. 7A). Likewise, the mutation of AAAGC in the MZ element (PZ in Fig. 1) results in a similar reduction of reporter expression in maxillary cells (Fig. 7C). However, in PZ embryos, ectopic reporter expression appears in amnioserosa and salivary gland cells (data not shown), suggesting that nucleotides in the AAAGC motif are also needed to prevent the BZ element from being activated in these cells. The diminished expression of both QZ and PZ in maxillary cells suggests that both GGC and AAAGC motifs are essential for region 6 activation function. Presumably, these motifs are part of a composite binding site, or are two separate binding sites for activating cofactors.

Interestingly, the combination of GGC and AAAGC motifs also exist in other Dfd response elements, and the motifs are near Dfd binding sites (Fig. 7D). These other elements include modules C and F from the *Dfd* autoregulatory region (Zeng et al., 1994) and a conserved Dfd response element from the human Dfd homolog HoxD4 that is capable of driving *Dfd*-dependent maxillary expression in *Drosophila* embryos (Malicki et al., 1992).

DISCUSSION

Specific Hox responses independent of specific Hox binding sites

Although different Hox proteins often exhibit only modest distinctions of DNA binding affinity for different monomer sites or Hox-Exd composite sites, much effort has been devoted to correlating these distinct binding properties with functional differences on normal response elements in vivo, with only limited success (Dessain et al., 1992; Ekker et al., 1992; Mann and Chan, 1996; Chan et al., 1997). Certainly, any binding differences that can be exploited for biological specificity will be important, and there is evidence that small binding site changes in multimerized Hox/Exd sites can have a strong influence on which Hox protein will activate such sites in embryos, and which will not (Chan et al., 1997). However, the results in this paper provide a counterpoint for this view and suggest that the binding distinctions provided by different Hox or Hox/Exd sites may have only modest importance in complex cis-regulatory elements.

In our study, an apparently optimal Lab-Exd composite binding site acquires a maxillary-specific, Dfd-dependent regulatory specificity when juxtaposed to a 21 bp inverted repeat taken from a natural Dfd response element. Strikingly, Dfd appears to activate reporter transcription from these hybrid elements to approximately the same levels whether they possess a Lab-Exd binding site, or the Dfd binding site from the naturally evolved element. Since these two sites exhibit very different in vitro affinity for Dfd or for Dfd-Exd (Chan et al., 1997; data not shown), a high level of binding affinity of Dfd or Dfd-Exd to Lab-Exd sites is apparently not crucial for discriminating Dfd from other Hox proteins in vivo. Mutation of the Lab-Exd binding site to random sequence, or to a GAL4 binding site, abolishes a Dfd-dependent activation response from the BZ element. Therefore some amount of Dfd binding specificity and affinity is required for function, but we presume that other factors bound to region 6 interact directly or indirectly with Dfd or Dfd-Exd to raise their affinities to BZ to a level compatible with transcriptional activation.

The instructive role of factors bound to non-Hox binding sites in controlling Hox responses is probably a general mechanism by which different Hox proteins acquire distinct functions. Exd is a well-characterized example that is used in a subset of Hox-activated response elements. However, the influence of Exd on Hox specificity may be superseded in complex elements that contain sequences such as region 6. How the specificity code is generated in the average Dfd or Ubx response element is likely to vary depending on the cell type, the presence or absence of Exd in the cell, the stage of development, and the extracellular signals that are received by a given response element. The putative activating cofactor binding site(s) (GGC..AAAGC) in the region 6 element are present in other naturally derived Dfd response elements (Fig. 7D), so there may be an important subset of Dfd response elements that rely on these sites for maxillary specificity. At present, none of the known complex elements that respond to other Hox proteins contain good matches to the GGC.. AAAGC motifs (e.g. Manak et al., 1995; McCormick et al., 1995; Sun et al., 1995; Haerry and Gehring, 1997). The region 6 cofactor(s) that are required to elicit a Dfd-dependent activation response by interacting with the GGCnn(n)AAAGC motif are not yet known.

How might Dfd cofactor binding sites act?

One mechanism by which Dfd cofactors that bind to region 6 might act is by enhancing the DNA binding occupancy of

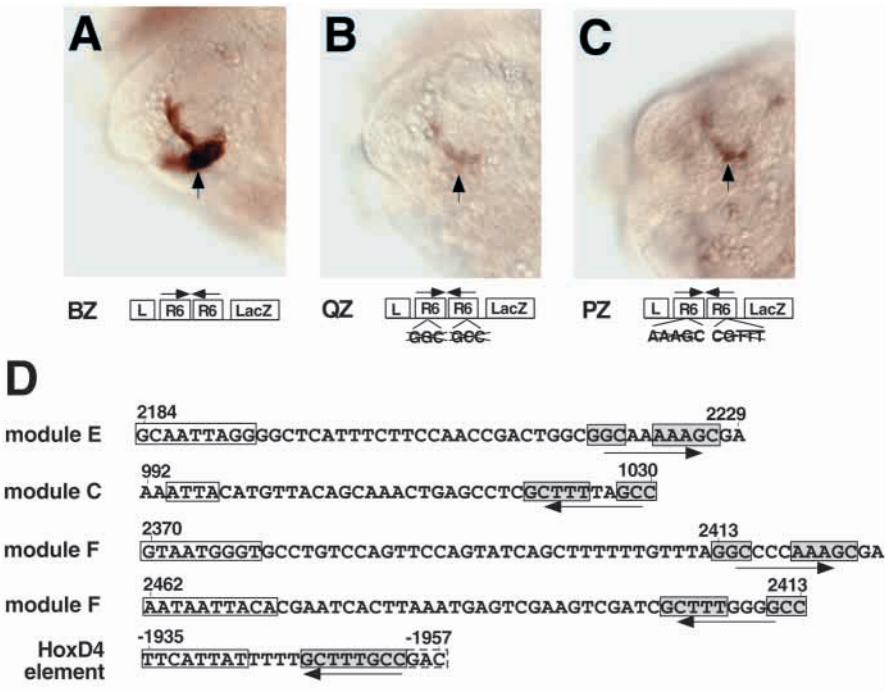

Fig. 7. Identification of essential motifs for region 6 function. (A-C) *lacZ* expression in maxillary cells (arrow) of the BZ (A), QZ (B) and PZ (C) embryos at stage 15. Notice that mutations of either GGC in QZ (B) or AAAGC in PZ (C) dramatically reduce the expression. Only the heads of the embryos are shown in lateral view with dorsal up and anterior to the left. (D) Alignment of Dfd binding sites (open boxes) and the putative cofactor binding motifs GGC and AAAGC (shaded boxes) in a variety of Dfd response elements. All of these Dfd binding sites have been previously reported (Malicki et al., 1992; Zeng et al., 1994).

Dfd-Exd, but not Lab-Exd. This general mechanism of assisting Hox proteins to occupy certain regulatory elements and not others is one way that Exd/Pbx proteins influence Hox specificity (Mann and Chan, 1996). The region 6 cofactor(s) might also act by selective regulation of Hox protein activities bound to nearby sequences. There is evidence that Dfd can occupy monomer Hox binding sites in *Drosophila* embryos independently of cofactors like Exd (Li et al., 1999). Yet binding site occupancy is not sufficient for gene activation in vivo, even though the N-terminal 294 residues of the Dfd protein display a strong intrinsic activation when fused to a heterologous DNA binding domain and tested in tissue culture cells (Zhu and Kuziora, 1996). The neutrality of Dfd on simple monomer binding sites is correlated with a homeodomain-mediated suppression of the activation domain(s) in the N-terminal half of the Dfd protein (Li et al., 1999). Therefore, region 6 cofactors might selectively release covert activation functions of Dfd, or interact with Dfd to form new activation functions. In this view, although multiple Hox proteins (e.g., Dfd and Lab) may bind to the region 3 Dfd binding site or the Lab-Exd composite site, only Dfd would functionally interact with the cofactors bound nearby on region 6 to activate transcription, while other Hox proteins would not. This might be one explanation why the multiple Lab-Exd binding sites in the region 6-containing JZ element are not activated by Lab protein (Fig. 5D). However, it is also possible that the multiple Lab-Exd sites in JZ might be exceptionally sensitive to distance from the promoter and fail to function in JZ because they are separated from the TATA box of the basal *hsp70* promoter by 81 bp instead of 39 bp.

We thank M. Akam for constructive criticism of the manuscript; the Bloomington stock center and T. Kaufman for fly stocks. A. V. was supported by an HHMI predoctoral fellowship. This research was supported by grant NICHD 28315 (to W. M.).

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