Mapping functional specificity in the Dfd and Ubx homeo domains

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To define homeo domain subregions that are important for embryonic targeting specificity of homeotic proteins, we generated a series of *Deformed/Ultrabithorax* chimeric genes in which parts of the *Deformed* homeo box region were substituted with *Ultrabithorax* sequences. Chimeric coding regions were attached to heat shock promoters and introduced into the *Drosophila* genome by P-element transformation. After heat-induced ectopic expression in embryos, we examined the cuticular phenotypes induced by the resulting chimeric proteins. We also tested the ability of the chimeric proteins to regulate transcription units that are normal targets of *Deformed* and *Ultrabithorax*. Our results indicate that specific amino acid residues at the amino end of the *Ultrabithorax* homeo domain are required to specifically regulate *Antennapedia* transcription; and in the context of a *Deformed* protein, these amino-end residues are sufficient to switch from *Deformed*- to *Ultrabithorax*-like targeting specificity. Although residues in the amino end of the homeo domain are also important in determining a *Deformed*-like targeting specificity, other regions of the *Deformed* homeo domain are also required for full activity.

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The family of homeotic selector genes residing in the Antennapedia and Bithorax complexes (ANT-C and BX-C, collectively referred to as the Homeotic complex or HOM-C), determine segmental identities during development of *Drosophila melanogaster* (Garcia-Bellido 1977; Lewis 1978; Struhl 1982; Kaufman et al. 1990). A dramatic demonstration of their functions is obtained from ectopic expression assays (Schneuwly et al. 1987; Gibson and Gehring 1988; Kuziora and McGinnis 1988; González-Reyes and Morata 1990; Mann and Hogness 1990), when homeotic selector proteins can induce the development of structures normally derived from one anterior-posterior axial position in ectopic positions (homeotic transformations). Sequence comparisons reveal that the proteins encoded by HOM-C selector genes conserve similar 60-amino-acid homeo domains with 60–90% sequence identity and have little sequence identity in regions outside the homeo domain (for review, see Gehring 1987; Scott et al. 1989). When tested in vitro, in tissue culture cells, in yeast cells, and in *Drosophila* embryos, homeo domain proteins can function as transcription activators or as repressors (for review, see Levine and Hoey 1988; Hayashi and Scott 1990), so the HOM-C proteins presumably exert their axial identity functions by transcriptional regulation of distinct sets of downstream genes.

How do the HOM-C proteins, which are all quite similar in homeo domain structure, achieve their diverse regulatory effects? Embryonic tests of mutant or chimeric versions of the *Deformed* (*Dfd*), *Sex combs* re-

duced (*Scr*), *Antennapedia* (*Antp*), *Ultrabithorax* (*Ubx*), and *Abdominal-B* (*Abd-B*) homeotic proteins have indicated that residues within or immediately flanking the homeo domain are responsible for the gross distinctions in their functional specificities (Kuziora and McGinnis 1989, 1991; Gibson et al. 1990; Mann and Hogness 1990). At present, there is no biochemical explanation for the homeo domain-dependent regulatory differences between HOM-C proteins. It seems likely that differences in DNA-binding affinities and protein-protein interactions both make some contribution (Hayashi and Scott 1990; Dessain et al. 1992; Ekker et al. 1991), but the subregions of HOM-C homeo domains that confer specific DNA-protein or protein-protein contacts that are relevant in vivo are unknown.

There is much useful information concerning HOM-C homeo domain DNA-binding interactions. The homeo domain region of HOM-C proteins apparently contributes most or all of their DNA-binding activity (Müller et al. 1988; Affolter et al. 1990; Ekker et al. 1991). This is in contrast to some other homeo domain subclasses, for example, paired and POU type, where the homeo domain regions contribute only a portion of DNA-binding activity of the entire protein (Sturm and Herr 1988; Kristie and Sharp 1990; Treisman et al. 1991). Structural studies of the *Antp*, *engrailed*, and *MATα2* homeo domains indicate that all fold into a triple helical domain in which the second and third α -helices are very similar in structure to the helix-turn-helix (HTH) motif of prokaryotic DNA-binding proteins (Laughon and Scott 1984; Otting

et al. 1988; Qian et al. 1989; Wolberger et al. 1991). Homeo domain/DNA cocrystals show that residues in the third α -helix interact with bases in the major groove of binding sites, and amino-terminal residues interact with bases in the minor groove, with many other residues making backbone contacts (Kissinger et al. 1990; Otting et al. 1990; Wolberger et al. 1991). In some homeo domains, residue 50, which maps in the third α -helix, has been singled out as a crucial determinant for DNA-binding specificity in vitro and in yeast cells (Hanes and Brent 1989, 1991; Treisman et al. 1989), but this position is occupied by glutamine in all HOM-C homeo domains (Fig. 1). Besides residues in the third helix, deletion and domain swap experiments have indicated that the amino-terminal end of the homeo domain is required for high-affinity interactions with DNA (Percival-Smith et al. 1990; Damante and Di Lauro 1991).

Our assays for mapping functionally important differences within the homeo domain regions of different HOM-C proteins rely on the distinct auto- and cross-regulatory interactions that are normally accomplished by *Dfd* and *Ubx*. *Dfd* protein positively autoregulates the *Dfd* transcription unit in epidermal cells of the maxillary segment (Bergson and McGinnis 1990; Regulski et al. 1991), and *Ubx* represses *Antp* transcription in epidermal cells of parasegment 6 (posterior T3–anterior A1; Hafen et al. 1984). These distinct targeting specificities are also reflected in the activities of *Dfd* protein and the *Dfd/Ubx*-HD protein [a chimeric protein with 66 amino acids of the *Ubx* homeo domain region substituted for

the analogous residues of *Dfd*] in embryos after heat shock-induced ectopic expression. Using this ectopic expression assay for homeotic regulatory function, *Dfd* protein specifically activates the endogenous *Dfd* transcription unit in epidermal cells of the thorax and abdomen but has no effect on *Antp* transcription (Kuziora and McGinnis 1988; Regulski et al. 1991). The *Dfd/Ubx*-HD protein has no regulatory effect on *Dfd* but, instead, activates the *Antp* transcription unit in the embryonic head (Kuziora and McGinnis 1989). Thus, the *Dfd/Ubx*-HD protein appears to have gained the targeting specificity of *Ubx* while retaining the ability of the *Dfd* protein to activate, instead of repress, transcription.

By systematic tests of a series of chimeric proteins in which homeo domain subregions were swapped, we wished to ask which subregions were most important in discriminating *Dfd* and *Ubx* homeo domain functions. By comparing the behavior of each hybrid protein with the *Dfd* and *Dfd/Ubx*-HD parental proteins, we find that the amino end of the *Ubx* homeo domain is both required and, to some extent, sufficient for the selective regulation of *Antp*.

Results

In the 66-residue homeo domain region of *Ubx* protein that was substituted to generate the *Dfd/Ubx*-HD chimera, there are 22 amino acid residues that are *Ubx* specific. Using these *Ubx*-specific residues and restriction sites that already were shared, or were intentionally en-

Figure 1. *Dfd/Ubx* homeo domain chimeras. (Top) The embryonic expression vector for the proteins containing chimeric *Dfd/Ubx* homeo domains. The P-element vectors that were used contained *hsp70* upstream sequences fused to a *Dfd* cDNA and *Dfd* 3' sequences, including polyadenylation sites (Kuziora and McGinnis 1988). An enlarged homeo domain region is depicted below the vector, and the 66-amino-acid residues encoded by *Dfd* in this region are shown in one-letter format. The 22 positions in this region where the *Ubx* protein sequence differs from *Dfd* are also indicated. These 22 different amino acids were divided into four subregions according to their putative structural identities: the amino (N) end, helix I, helix II/turn, and the carboxyl (C) end. The four subregions were swapped between *Dfd* and *Ubx* using three restriction sites (*AccI*, *MluI*, and *BglIII*), some of which were created by site-directed mutagenesis (Materials and methods). This resulted in six *Dfd/Ubx* homeo box chimeras, designated as *hsS1*–*hsS6*, all in the context of *Dfd* cDNA sequence. The homeo domain composition of these sub-region chimeras are diagrammed with solid bars designating *Ubx*-like sequences and shaded bars showing *Dfd*-like sequences.

gineered (Materials and methods) into both *Ubx* and *Dfd* homeo box sequences, we could test four structural subregions of the homeo domain. As shown in Figure 1, these subregions are the amino end (N-end; residues 0–7), α -helix I (residues 11–24), α -helix II and turn region (residues 34–41), and the carboxyl end (C-end; residues 56–65). We constructed three pairs of reciprocal chimeric homeo boxes that were all introduced into a *Dfd* cDNA sequence under *hsp70* promoter control. The *hsp70* promoter/Swap 1 chimera (*hsS1*) encodes a normal *Dfd* protein sequence except in the amino end, helix I, and helix II/turn subregions, which encode *Ubx*-specific residues. The *hsS3* chimera encodes *Ubx*-specific residues only in the amino end and helix I subregions, and the *hsS5* construct encodes *Ubx*-specific residues only in the amino-end subregion (Fig. 1). In the reciprocal set of chimeras, *hsS2* encodes *Ubx*-specific residues only in the carboxy-end subregion, *hsS4* encodes *Ubx*-specific residues in both the helix II/turn and carboxy-end subregions, and *hsS6* encodes *Ubx*-specific residues in the helix I, helix II/turn, and carboxy-end subregions.

Multiple transgenic lines carrying genomic inserts of the *hsS1*–*hsS6* chimeric genes were generated using P-element transformation (Rubin and Spradling 1982; Spradling and Rubin 1982), and transformant functions were tested in fly strains carrying two chimeric gene copies. The expression of chimeric proteins was induced in *hsS1*–*hsS6* embryos with a double heat shock regimen. A 1-hr heat shock (37°C) was administered at the cellular blastoderm stage, followed by a 1-hr recovery at normal growth temperature (25°C); a second 37°C heat shock of 1 hr was then administered, after which embryos were returned to normal temperatures. To test the relative expression levels, stabilities, and cellular localizations of the chimeric proteins as compared with parental *hsDfd* and *hsDfd/Ubx-HD* proteins, all lines were stained with anti-*Dfd* antiserum at 1 and 3 hr after a double heat shock regimen (data not shown). The parental and chimeric proteins were expressed at approximately equal levels as judged by staining intensity, and all were localized in embryonic nuclei. In addition, all proteins were still detectable (barely) after 3 hr of recovery, so they appeared to have approximately the same levels of stability.

Homeotic transformations induced by *hsS1*–*hsS6* chimeras

To test the functional specificity of the chimeric proteins, we first evaluated the cuticular phenotypes induced by the chimeric proteins after heat shock-induced expression. The parental constructs *hsDfd* and *hsDfd/Ubx-HD* each induce homeotic transformations by indirect means. The ectopic and persistent autoactivated expression of *Dfd* protein that is induced by *hsDfd* triggers a transformation of parts of more posterior segments to maxillary identities, as evidenced by the development of maxillary cirri and (occasionally) mouth hooks in labial and thoracic segments (Fig. 2b; Kuziora and McGinnis 1988). In *Dfd* mutant backgrounds, the *hsDfd* construct

does not induce ectopic maxillary structures. The *hsDfd/Ubx-HD* construct ectopically activates the expression of *Antp* transcripts and protein, which triggers a transformation of parts of more anterior segments toward thoracic identities, as evidenced by the development of thoracic denticle belts in the head (Fig. 2c; Kuziora and McGinnis 1989). In *Antp* mutant backgrounds, the *hsDfd/Ubx-HD* construct does not induce ectopic thoracic structures. Thus, whether using single or double heat shock regimens, neither *hsDfd* nor *hsDfd/Ubx-HD* induces a strong phenotypic transformation that is directly caused by protein produced from the heat shock promoter construct, which may be due in part to the relative instability of ectopically expressed *Dfd* and *Dfd*–chimeric proteins when compared with ectopically expressed *Ubx* or *Antp* proteins (Gibson and Gehring 1988; Kuziora and McGinnis 1988; González-Reyes and Morata 1990; Mann and Hogness 1990).

The *hsS1*–*hsS6* subregion chimeras can be divided into two groups based on the cuticular phenotypes they induced. When treated with a double heat shock regimen, embryos carrying *hsS1*, *hsS3*, and *hsS5* chimeras had severe head involution defects and developed thoracic denticle belts in place of normal head structures. Almost all of the *hsS1*, *hsS3*, and *hsS5* embryos die before hatching when heat-shocked, although the different chimeric genes yield different levels of penetrance and expressivity for the head-to-thorax transformations. *hsS1* and *hsS3* give strong transformations, yielding phenotypes essentially identical to the parental *hsDfd/Ubx-HD* construct (~90% of unhatched first-instar larvae of both transformants had ectopic denticles in the head segments; Fig. 2d,e). The *hsS5* construct is a slightly weaker version of the *hsDfd/Ubx-HD* parent, as patches of ectopic thoracic denticles appeared in the head in ~70% of heat-shocked *hsS5* larvae (Fig. 2f). None of these three transformants yield *hsDfd*-like homeotic transformations after heat shock.

The heat-shocked *hsS2*, *hsS4*, and *hsS6* transformants did not have cuticular phenotypes that resembled either of the parental constructs. The expression of the *hsS2* construct appeared to be rather innocuous, as almost 90% of *hsS2* embryos survived the heat shock treatment with no obvious ill effect. The remaining unhatched *hsS2* larvae exhibited only defects in head involution (Fig. 2g). Heat shock-induced expression of *hsS4* and *hsS6* constructs in embryos had stronger effects on embryonic viability, as only 40% of embryos hatched. The *hsS4* and *hsS6* animals that died as unhatched first-instar larvae had no specific morphological changes except for abnormal head involution (Fig. 2h,i). One rare exception to this is that in small percentages of *hsS4* (3%) and *hsS6* (6%) cuticles, there were a few randomly positioned denticles in the head regions.

Transcriptional regulatory effects of the *hsS1*–*hsS6* chimeras

As mentioned previously, the parental constructs have distinct embryonic targeting specificities, with *hsDfd*

Figure 2. (See facing page for legend.)

activating ectopic *Dfd* transcription (Fig. 3c), and *hsDfd/Ubx-HD* activating ectopic *Antp* transcription (Fig. 3f). To test the targeting specificity of the *hsS1–hsS6* chimeras, digoxigenin-labeled *Dfd* and *Antp* cDNA probes were used in whole-mount in situ hybridizations on heat-shocked embryos. As shown in Figure 3, *hsS1*, *hsS3*, and *hsS5* activate ectopic *Antp* transcription. Qualitatively, *hsS1* and *hsS3* appear to have stronger regulatory effects, variably activating *Antp* transcription in the first thoracic, labial, and head epidermis (Fig. 3h,j). These patterns of ectopic *Antp* transcription closely resemble those induced by *hsDfd/Ubx-HD* (Fig. 3f). The *hsS5* construct also induces ectopic *Antp* transcripts, but appears to act in fewer cells overall (Fig. 3m). We have detected no significant effects on *Antp* expression levels within the normal *Antp* expression domain. However, the abundance and pattern of *Antp* transcripts evolves rapidly during development, so modest differences from the normal patterns would be difficult to detect.

None of the three constructs activate ectopic *Dfd* transcription in any epidermal cells in a manner similar to the *hsDfd* parent (Fig. 3c,g,i,k). However, the level of normal *Dfd* expression in the maxillary and mandibular segments is repressed in almost all the *hsS3* embryos after heat shock (Fig. 3i).

In heat-shocked *hsS2*, *hsS4*, and *hsS6* embryos, the distribution of *Antp* transcripts closely resembles the normal pattern: No ectopic expression is detected (Fig. 4b,d,f); nor are the levels or patterns of *Antp* transcription obviously affected within the normal *Antp* expression domain, though again, modest differences from the normal patterns would be difficult to detect.

The *hsS2* and *hsS4* constructs induce weak regulatory effects on *Dfd* transcription that do not resemble those induced by the parental *hsDfd* construct. About 45% of *hsS2* embryos accumulate *Dfd* transcripts in a few cells of the most anterior segment, the clypeolabrum (Fig. 4a), whereas the rest of the *hsS2* embryos display a normal *Dfd* expression pattern. Ectopic *Dfd* expression in the clypeolabrum has not been observed in *hsDfd* embryos (Fig. 3c). The only detectable effect of *hsS4* expression is a repression of *Dfd* transcript levels within the normal expression domain of the maxillary and mandibular segments (Fig. 4c). In 74% of *hsS6* embryos, ectopic *Dfd* transcripts are detected in a few cells of the labial segment, which weakly resembles the *hsDfd* regulatory effect (Fig. 4e). *hsS6* also induces *Dfd* transcription in a few cells of the clypeolabrum.

Discussion

The data from both the cuticular and transcriptional regulatory assays of *hsS1–hsS6* is summarized in Figure 5, in which each construct is compared with the parental *hsDfd* and *hsDfd/Ubx-HD*. The most important conclusion from these data is that only a few amino-end Ubx-specific homeo domain residues are required to confer targeting specificity for the regulation of *Antp* transcription. *hsS1*, *hsS3*, and *hsS5* all retain the specificity of the parental *hsDfd/Ubx-HD* construct, and the protein encoded by *hsS5* only differs from *Dfd* at 6 Ubx-specific residues at the amino end of the homeo domain (Fig. 6). The same region is also necessary for Ubx-like targeting, as *hsS6* is incapable of regulating *Antp* even though the *hsS6* homeo domain is entirely Ubx-like except at the amino end.

A second conclusion is that both amino- and carboxy-end *Dfd*-specific homeo domain residues are required for full ectopic activation of the endogenous *Dfd* transcription unit, although the amino end is sufficient for a weak *Dfd*-like regulatory effect. As can be seen in *hsS2* and *hsS5* (Fig. 5), changing either amino or carboxyl ends to Ubx-specific residues abolishes the ability to activate *Dfd* transcription. Only the *hsS6* chimera had a detectable, although weak, ability to activate *Dfd* transcription in a way similar to *hsDfd*, again emphasizing the importance of the homeo domain amino end. However, it is puzzling that the *hsS2* and *hsS4* proteins, with even more *Dfd*-like homeo domain residues than *hsS6*, were incapable of activating *Dfd* transcription.

Curiously, the *hsS3* and *hsS4* chimeras that are half-*Dfd*/half-Ubx (recombined in the helix I/II region) both appear to partially repress *Dfd* transcription. This result is puzzling because the *hsS3* and *hsS4* swaps are nonoverlapping and also because the regulatory effect on *Dfd* does not resemble either of the parental constructs. This seems to suggest that an intact helix I and II region (either Ubx- or *Dfd*-specific) is required to prevent the *Dfd* or Ubx homeo domains from mediating a repressive effect on *Dfd* transcription. None of the other chimeric proteins have a detectable repressive effect on *Dfd* transcription, so it seems possible that the generation of chimeric helix I/II regions in *hsS3* and *hsS4* supplies novel functions not present in either of the parental constructs.

Finally, we note that there is a correlation between abundant ectopic activation of an endogenous target

Figure 2. Cuticular phenotypes induced by *hsS1–hsS6* proteins in embryos. Experimental (*hsS1–hsS6*) and control (*hsDfd*, *hsDfd/Ubx-HD*, and *ry⁵⁰⁶* or *w¹⁰⁰⁶* host strains) embryos were heat-shocked for 1 hr at cellular blastoderm stage, recovered for 1 hr, and heat shocked for 1 hr. The resulting first-instar larvae were scored for survival and for cuticular defects and segmental transformations (Van der Meer 1977). The first thoracic segment of each larvae is marked by T1. (a) *ry⁵⁰⁶* (P-element host strain) larva (note the normal, fully involuted head). (b) *hsDfd* larva: Ectopic maxillary cirri in labial and thoracic segments are shown by arrowheads. (c) *hsDfd/Ubx-HD* larva: Strong homeotic transformation of head to thorax is shown by T2-like denticle belts in the head (arrow). (d–f) *hsS1*, *hsS3*, and *hsS5* larvae, respectively. All had head-to-thoracic transformations, similar to those seen in *hsDfd/Ubx-HD* larvae as shown in c. Arrows indicate the ectopic thoracic-like denticles in the head region. (g–i) The *hsS2*, *hsS4*, and *hsS6* larval cuticles. No reproducible homeotic transformations were observed, although some larvae, as shown, had moderate head involution defects.

Figure 3. (See facing page for legend.)

gene (*Dfd* or *Antp*) and the induction of a cuticular homeotic transformation (Fig. 5), consistent with all of the cuticular phenotypes deriving from ectopic expression of endogenous homeotic genes. Head-to-thorax transformations were only detected in *hsS1*, *hsS3*, and *hsS5* strains, all cases in which endogenous *Antp* was ectopically expressed at abundant levels in the embryonic head. Unlike the *hsDfd* parental construct, none of the subregion chimeras induced abundant ectopic expression of the endogenous *Dfd* gene, and no ectopic maxillary structures were reproducibly seen in the *hsS1*–*hsS6* strains. This is in agreement with previous experiments on *hsDfd* and *hsDfd/Ubx-HD* constructs, indicating that their homeotic effects were reverted in *Dfd* and *Antp* mutant backgrounds, respectively (Kuziora and McGinnis 1988, 1989).

Previous experiments have shown that *Dfd* protein-binding sites are required for the function of an autoregulatory element module that supplies late expression in maxillary epidermal cells (Bergson and McGinnis 1990; Regulski et al. 1991). Thus, the action of *hsDfd* is likely to be accomplished by direct binding of *Dfd* protein to sites in the upstream autoregulatory element of the endogenous *Dfd* gene (Regulski et al. 1991). Our working model is that *Dfd/Ubx-HD* protein and the chimeric proteins that mimic its function (*hsS1*, *hsS3*, and *hsS5*) also act directly on *Antp* cis-regulatory sequences (Beachy et al. 1988), but no strong evidence exists at present to support this idea. Regulatory regions that mediate the effect of *Ubx* on *Antp* in embryos have not yet been defined precisely, although recent experiments suggest that sequences in the *Antp* P1 first exon are required for the repressive effects of *Ubx* as well as for the activating effects of *Dfd/Ubx-HD* proteins (Zink et al. 1991; L. Lin and Y. Engstrom, unpubl.).

At present, the biochemical interactions that explain how HOM-C homeo domain proteins sort out target regulatory elements are poorly understood. Some HOM-C homeo domain proteins show modest distinctions in their preference for different binding sites (Affolter et al. 1990; Ekker et al. 1991; Florence et al. 1991; Dessain et

al. 1992), but it is unclear whether these modest binding differences are important in distinguishing embryonic target elements. It seems likely, on the basis of analogy to yeast mating-type homeo domain proteins $\alpha 1$ and $\alpha 2$, and POU-class homeo domain proteins like Oct-1, Oct-2, and I-POU (Keleher et al. 1988; Jarvis et al. 1989; Stern et al. 1989; Ingraham et al. 1990; Treacy et al. 1991), that stable protein–protein interactions involving different homeo domain and as yet unidentified cofactors may make an important contribution to HOM-C targeting specificity (Hayashi and Scott 1990).

Our results indicate that the amino-terminal residues are very important for both *Ubx* and *Dfd* homeo domain specificity in embryos but give no hint as to mechanism. Nevertheless, it is interesting to consider our results on the importance of different homeo domain subregions in light of recent structural descriptions of homeo domain–DNA complexes (Kissinger et al. 1990; Otting et al. 1990; Wolberger et al. 1991). An intriguing feature of the homeo domain–DNA complexes is the interactions between residues in an amino-terminal “arm” and bases in the minor groove. In the engrailed homeo domain, Arg-3 and Arg-5 (Fig. 6) interact directly with A-T base pairs at the 5′ end of a TAAT core sequence. However, differences in positions 3 and 5 could not account for the amino-terminal targeting differences documented here as the *Dfd* and *Ubx* homeo domains have Arg at both positions.

It seems plausible that other amino acid differences in the amino-terminal arm might directly or indirectly control differences in DNA-binding affinity, which might explain the functional differences that distinguish the *hsS5* and *Dfd* proteins. However, we have not yet been able to detect such DNA-binding differences using *in vitro* assays. Using monomer-binding sites with high affinity for either *Ubx* or *Dfd* homeo domains (Dessain et al. 1992; Ekker et al. 1991; Regulski et al. 1991), we have compared *hsS1*–*hsS6* protein binding to the parental *Dfd* and *Dfd/Ubx-HD* proteins. The *in vitro* binding preferences of *hsS1*–*hsS6* proteins for high-affinity *Ubx* or *Dfd* monomer-binding sites show no correlation with the tar-

Figure 3. Regulatory effects on *Dfd* and *Antp* transcription in *hsS1*, *hsS3*, and *hsS5* embryos. Experimental (*hsS1*, *hsS3*, *hsS5*) or control (*hsDfd*, *hsDfd/Ubx-HD*) embryos were heat-shocked at cellular blastoderm stage for 1 hr at 37°C, allowed to recover for 1 hr at 25°C, and heat-shocked again for 1 hr. After another hour at 25°C, embryos were fixed and *Dfd* and *Antp* transcripts were detected by whole-mount *in situ* hybridization [Materials and methods]. The different strains tested are indicated at the bottom right corner of each panel. Embryos shown in the left column were hybridized with *Dfd* probe. The curved arrows indicate the border between maxillary and labial segment in each embryo. (a) A wild-type (wt) embryo showing the normal *Dfd* transcript expression pattern. (c) *hsDfd* embryos exhibit ectopic *Dfd* transcripts in labial, thoracic and abdominal segments. These *Dfd* transcripts derive from the endogenous *Dfd* locus, as the *Dfd* or *Dfd/Ubx* chimeric transcripts that arise from the heat shock promoter are very unstable, with a half-life on the order of a few minutes (Kuziora and McGinnis 1988; M. Kuziora, unpubl.). (e) *hsDfd/Ubx-HD* embryos *Dfd* expression was normal. (g,i,k) No *Dfd* transcription was ectopically activated in *hsS1*, *hsS3*, and *hsS5* embryos. However, in nearly all of the *hsS3* embryos (i), *Dfd* expression was repressed in the maxillary and mandibular regions. Embryos shown in the right column were hybridized with *Antp* probe. Arrows indicate the parasegment 3/4 boundary in each embryo, and arrowheads indicate sites of ectopic *Antp* transcription. (b) A wild-type (wt) embryo showing the normal *Antp* transcript expression pattern. (d) *hsDfd* embryos have normal *Antp* transcription patterns. (f) *hsDfd/Ubx-HD* embryos have ectopic *Antp* transcripts in thoracic, labial, and head segments. (h,j,m) In *hsS1*, *hsS3*, and *hsS5* embryos, ectopic *Antp* transcripts were expressed in thoracic, labial, and head segments, similar to the pattern in *hsDfd/Ubx-HD* embryos. *hsS5* embryos (m) generally have fewer cells expressing ectopic *Antp* transcripts. All transformant strains in this figure and in Fig. 4 were heat shocked in parallel with control strains, and hybridized and stained for the same intervals, with aliquots of the same probes and solutions.

Figure 4. Regulatory effects on *Dfd* and *Antp* transcription in *hsS2*, *hsS4*, and *hsS6* embryos. Experimental (*hsS2*, *hsS4*, *hsS6*) or control (*hsDfd*, *hsDfd/Ubx-HD*) embryos (shown in Fig. 3c–f) were heat-shocked at cellular blastoderm stage for 1 hr at 37°C, allowed to recover for 1 hr at 25°C, and heat shocked again for 1 hr. After another hr at 25°C, embryos were fixed, and *Dfd* and *Antp* transcripts were detected by whole-mount in situ hybridization [Materials and methods]. The different strains tested are indicated at bottom right corner of each panel. Embryos shown in the left column were hybridized with *Dfd* probe. The curved arrows indicate the border between maxillary and labial segment in each embryo; arrowheads indicate sites of ectopic *Dfd* transcription. No significant *Dfd* ectopic expression was detected in *hsS2* (a) and *hsS6* (e) embryos, as compared with *hsDfd* (Fig. 3c), except for a few cells of the clypeolabrum in some *hsS2* and *hsS6* embryos, and a few cells of the labial segment in *hsS6* embryos. In *hsS4* embryos (c), *Dfd* transcript levels in mandibular and maxillary segments were repressed. Embryos shown in the right column were hybridized with *Antp* probe. Arrows indicate the parasegment 3/4 boundary in each embryo. *Antp* transcript patterns were normal in *hsS2* (b), *hsS4* (d), and *hsS6* (f) embryos compared with wild-type embryos at comparable stages.

getting specificities that they exhibit in embryos (Fig. 5). For example, the *hsS5* protein does not preferentially bind to monomer Ubx-binding sites in a way that closely resembles the parental *Dfd/Ubx-HD* (C. Gross, unpubl.).

Perhaps the different side chains in the amino-terminal region, many of which are directed away from the DNA in the homeo domain–DNA structures of Kissinger et al. (1990) and Wolberger et al. (1991), control required interactions with homeo domain cofactors. Specific amino-terminal residues of the homeo domain region of the *Drosophila* I-POU protein have been shown to be important in conferring its ability to form heteromeric complexes with the Cfl-1 protein (another protein of the POU–homeo domain class; Treacy et al. 1991). These observations suggest that the mechanistic explanation for the ability of the *S5* protein to specifically regulate

Antp may reside in its ability to interact productively with normal Ubx cofactors.

Materials and methods

Site-directed mutagenesis and construction of chimeric genes

Three natural or engineered restriction sites were used to swap the four subregions between the *Ubx* and *Dfd* homeo boxes (the positions of the restriction sites are shown in Fig. 1). *Bgl*II sites are present in both wild-type homeo box sequences, whereas an *Mlu*I site is present only in the *Dfd* homeo box. Using site-specific mutagenesis (Kunkel 1985), we introduced *Acc*I sites into both homeo boxes and an *Mlu*I site into the *Ubx* homeo box without changing the amino acid code. Both *Mlu*I and *Acc*I sites were engineered in the *Bam*HI–*Eco*RV fragment of the *Dfd/Ubx* chimeric gene (Kuziora and McGinnis 1989) in MP18

Figure 5. Summary of phenotypic and gene regulatory effects of *hsS1–hsS6*. (Left) The amino acid sequence compositions of the Dfd/Ubx homeo domain chimeras *hsS1–hsS6* and their parental constructs *hsDfd* (*hsD*) and *hsDfd/Ubx-HD* (*hsD/U*). The first open bar represents full-length Dfd protein, with two dotted lines delimiting the homeo domain region from amino acids 0–65. In the remaining constructs, Ubx-like amino acids in the homeo domain are represented as vertical lines in Dfd protein context. The next two columns summarize the two levels of regulatory effects induced by overexpressing the *hsS1–hsS6* constructs in embryos. The *hsS1*, *hsS3*, and *hsS5* proteins induce head-to-thorax transformations, whereas *hsS2*, *hsS4*, and *hsS6* induce no significant phenotypic changes. *hsS1*, *hsS3*, and *hsS5* are able to activate ectopic *Antp* transcription similar to Dfd/Ubx-HD protein [(+) activation; (–) no activation]. None of the chimeric proteins ectopically activate *Dfd* transcription in a manner similar to Dfd protein, although a significant percentage of *hsS6* embryos have weak ectopic *Dfd* expression in certain cells of the clypeolabrum and labial segment (–/+).

PhsS3 and PhsS4 Three-fragment ligations were carried out with the following fragments: For hsS3, these included the *Bam*HI-*Eco*RV fragment from phsDfd, the *Bam*HI-*Mlu*I fragment from MP18DU-M, and the *Mlu*I-*Eco*RV fragment from MP18D; for hsS4, these included the *Bam*HI-*Eco*RV fragment from phsDfd, the *Bam*HI-*Mlu*I fragment from MP18D, and the

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MluI-EcoRV fragment from MP18DU-M. The *NotI-SalI* fragments of the two resulting recombinant plasmids from the three-fragment ligations were subcloned into the *PstI* site of the CaSpeR injection vector (Piotto 1988) using oligonucleotide linkers.

PhsS5 and PhsS6 Three-fragment ligations were performed with the following fragments: For *hsS5*, these included the *BamHI-EcoRV* fragment from *phsDfd*, the *BamHI-AccI* fragment from MP18DU-A, and the *AccI-EcoRV* fragment from MP18D-A; for *hsS6*, these included the *BamHI-EcoRV* fragment from *phsDfd*, the *BamHI-AccI* fragment from MP18D-A, and the *AccI-EcoRV* fragment from MP18DU-A. *NotI-SalI* fragments of the two resulting recombinant plasmids were subcloned into the CaSpeR P-transformation vector.

Transformation and functional tests of chimeric genes

The injection procedure has been described previously (Rubin and Spradling 1982; Spradling and Rubin 1982). *PhsS1* and *PhsS2* constructs were coinjected with helper plasmid $\pi\pi 25.7wc$ (Karess and Rubin 1984) into *ry⁵⁰⁶*, whereas *PhsS3*, *PhsS4*, *PhsS5*, and *PhsS6* were coinjected with $\pi\pi 25.7wc$ into *w¹⁰⁰⁶* embryos. All transformants were made homozygous, and at least two independent lines for each inserted construct were tested in our experimental assays to avoid potential problems with position effects.

For analysis of cuticular phenotypes, embryos were collected and staged to 2.5–3 hr after egg lay (approximately cellular blastoderm/early gastrulation stages; Campos-Ortega and Hartenstein 1985), heat-shocked at 37°C for 1 hr, recovered for 1 hr at 25°C, and heat-shocked again at 37°C for 1 hr. The embryos were allowed to develop at 25°C for ~36 hr. Cleared cuticular preparations were done following the procedure of Van der Meer (1977). Cuticular defects were examined and scored under a phase-contrast microscope.

For anti-Dfd protein antibody staining and whole-mount in situ hybridization, embryos at cellular blastoderm stage were heat shocked for 1 hr at 37°C, returned to 25°C for 1 hr, and heat shocked again for 1 hr. The embryos were then allowed to develop for 1 (or 3) more hr at 25°C before fixing and further processing. Staining with rabbit anti-Dfd protein antiserum was performed as described previously (Jack et al. 1988). Digoxigenin-labeled DNA probes for detection of *Dfd* and *Antp* transcripts were prepared from *pBstAntp* digested with *BamHI-EcoRI* and from *pDfd41* digested with *HindIII-EcoRV* (Regulski et al. 1987; McGinnis et al. 1990) using the Genius labeling kit from Boehringer Mannheim. The procedure of whole-mount in situ hybridization was based on the method of Tautz and Pfeifle (1989).

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