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Functional dissection of the mouse *Hox-a5* gene

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The *Hox* genes are clustered in evolutionarily conserved complexes and encode DNA binding proteins that determine positional identity. Ubiquitous expression of fly or mammalian Hox proteins in *Drosophila* embryos provides an assay for gene function, since different *Hox* genes induce characteristic homeotic transformations. *Drosophila* Sex combs reduced (Scr) and its murine cognate Hox-a5 produce identical transformations in transgenic flies. To study the contributions of domains conserved between the two proteins, truncated versions of mouse Hox-a5 were assayed for their ability to activate transcription in cultured cells and to induce homeotic transformation and activate target gene expression in transgenic embryos. The homeodomain is essential for protein function and/or nuclear targeting; the N-terminal region contributes to transcription activity and transformation potential in the embryo, but plays no role in determining functional specificity. The YPWM motif is essential for biological specificity, although it does not contribute to transcriptional activation potential. It was recently shown that the Hox-a5 YPWM motif is necessary for *in vitro* interactions with the co-factor Pbx1. Our results suggest that this type of protein–protein interaction may be essential for the biological activities of Hox-a5 and Scr.

Keywords: functional specificity/Hox-a5/octapeptide motif/Sex combs reduced/YPWM motif

Introduction

Hox genes are a highly conserved group of homeobox-containing genes, first identified in *Drosophila*, that specify cell identity during embryonic development. The products of these genes are homeodomain-containing proteins which bind to DNA in a sequence-specific fashion and define segment identity in the embryo by regulating the transcription of downstream target genes (reviewed in Akam, 1989; McGinnis and Krumlauf, 1992). While each *Hox* gene has a unique and specific biological function, the homeodomain-containing proteins bind to very similar sequences *in vitro*. In addition, although this binding is sequence specific, the target recognition sequence is small, comprised primarily of an ATTA motif (Desplan *et al.*, 1988; Odenwald *et al.*, 1989), such that Hox proteins have the potential to bind to an unusually large number of sites in the genome. The differential biological activities of the *Hox* genes can be accounted for in part by their differential spatial and temporal expression patterns in the embryo. However, in *Drosophila*, ubiquitous, ectopic expression of a variety of *Hox* genes has revealed that distinct homeotic transformations are induced by different *Hox* genes (Schneuwly *et al.*, 1987; Gibson and Gehring, 1988; Gibson *et al.*, 1990; Malicki *et al.*, 1990, 1993; McGinnis *et al.*, 1990; Zhao *et al.*, 1993; reviewed in McGinnis and Kuziora, 1994). Thus, these proteins have intrinsic biological specificities that are not revealed by *in vitro* DNA binding studies. These observations suggest that the DNA binding and/or transcriptional activity of Hox proteins is modulated *in vivo*, possibly through protein–protein interactions with specific cofactors that limit the specificity of action of *Hox* gene products (reviewed in Hayashi and Scott, 1990).

In *Drosophila*, the *Hox* genes are clustered in the *Antennapedia* (ANT-C) and *bithorax* (BX-C) complexes that together comprise the *Drosophila* HOM-C (homeotic complex). The linear arrangement of *Hox* genes within HOM-C corresponds to the anterior–posterior activity of these genes along the *Drosophila* body axis such that genes at the 3′ end of the complex define anterior embryonic structures and genes at the 5′ end of the complex define posterior body structures (Lewis, 1978). *Hox* genes are highly conserved throughout the animal kingdom, as is the order of genes within the complex and the 5′ to 3′ collinearity of the genes with respect to their action along the embryonic anterior–posterior axis (McGinnis and Krumlauf, 1992). Mammals contain four independent copies of a putative ancestral Hox complex (HoxA, B, C and D); each cluster is found on a different chromosome (McGinnis and Krumlauf, 1992; Scott, 1992).

Sequence comparisons of *Hox* genes from different species revealed that homeobox sequences could be aligned across the complexes such that cognate genes were arranged in the same order in different clusters throughout the animal kingdom (Duboule and Dolle, 1989; Graham *et al.*, 1989). However, outside of the homeobox, little sequence conservation was found between cognate genes from different species (see below). Thus, biological tests were required to address the degree of functional conservation of cognate genes from different species. For example, Antp and its human and mouse cognate Hoxb6 differ by only 5/61 amino acids in the homeodomain, while Antp protein is less similar to other Hox products within *Drosophila* HOM-C (e.g. 11/61 differences between the homeodomains of Antp and Dfd). The fact that different *Drosophila Hox* genes caused unique homeotic transformations in flies after ubiquitous expression raised the possibility of using transgenic flies as an assay to test

directly the functional relationships between *Hox* genes from different species. Thus, it was shown that mouse Hox-b6 (formerly Hox-2.2) induced antenna to leg transformations similar to those induced by its *Drosophila* cognate Antp, when expressed ubiquitously in developing larvae (Malicki *et al.*, 1990). Similarly, Dfd and AbdB-like phenotypes were induced by their respective human and murine cognates (McGinnis *et al.*, 1990; Malicki *et al.*, 1993).

We showed previously that the mouse *Hox-a5* gene (formerly *Hox-1.3*) is functionally equivalent to *Drosophila Sex combs reduced* (*Scr*) in that it induced Scr-like transformations in both larvae and adult *Drosophila*. In addition, Hox-a5 activated the expression of *forkhead* (*fkh*), a downstream target gene of Scr, in the same manner as did Scr itself (Zhao *et al.*, 1993). Despite this surprising degree of functional conservation, mouse Hox-a5 and *Drosophila* Scr share only three regions of sequence homology. The longest of these is the 61 amino acid homeodomain which is 92% identical in the two proteins. In addition, two short regions are shared: the YPWM motif [because of extended conservation between cognate genes, this sequence has also been called the pentapeptide or hexapeptide (Mavilio *et al.*, 1986; Burglin, 1994)] just N-terminal to the homeodomain, and a short octapeptide near the N-terminus of the protein. These sequences are also present in other Hox proteins. However, the YPWM motif present in Scr and its cognates contains a three amino acid N-terminal extension that is not found in other Hox proteins. Similarly, the Scr N-terminal octapeptide is more similar to that of Hox-a5 than it is to *Drosophila* Antp or Dfd.

In order to test the contributions of these conserved domains to Hox-a5/Scr function, we have analyzed a series of truncated Hox-a5 proteins in transcriptional assays in cell culture and *in vivo* by expression in *Drosophila* embryos and larvae. We have found that deletion of the N-terminus of the protein, which contains a conserved octapeptide, decreased the protein's ability to activate transcription in mammalian cells and weakened its ability to generate homeotic transformations in *Drosophila*. In addition, this protein only weakly activated expression of *fkh*, which is readily activated by full-length Hox-a5 protein in embryos. Surprisingly, deletion of the YPWM motif had a drastic effect in *Drosophila*: no homeotic transformations were observed in larvae or adult flies, and *fkh* expression was not activated. Since this truncated protein activated transcription in cultured cells, we conclude that the transcriptional activation potential of this protein has not been affected. Rather, it appears that the YPWM motif is necessary for the biological activity of Hox-a5/Scr, likely mediating protein–protein interactions which are required for the *in vivo* function of Hox proteins.

Results

Hox-a5 protein activates transcription in cultured cells

It was shown previously that Hox-a5 protein binds to the promoter region of *Hox-a5* itself (Odenwald *et al.*, 1989). To test the ability of Hox-a5 protein to activate transcription via this 5′ flanking region, a transient expression

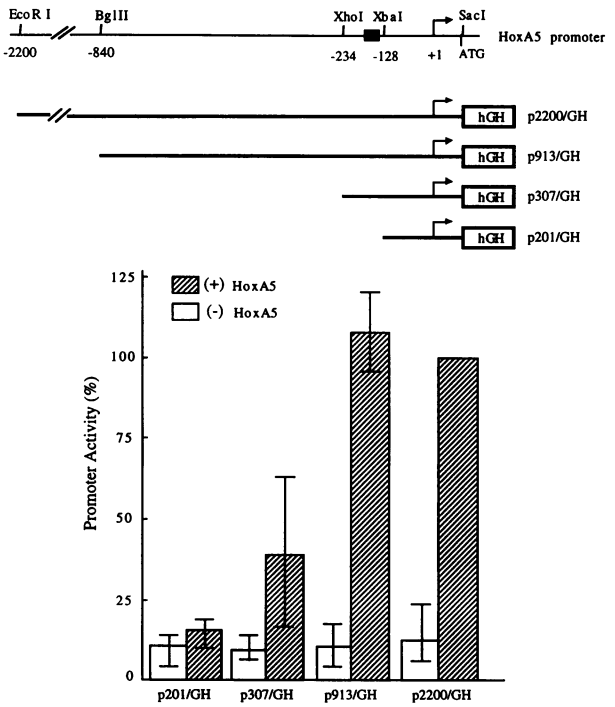

Fig. 1. Hox-a5 protein activates transcription via *Hox-a5* promoter sequences. Top: a schematic view of Hox-a5 promoter constructs used in co-transfection assays. The putative Hox-a5 responsive elements are located at −160 to −137 bp (shown as a shaded rectangle). Bottom: GH activities in the absence (−) or presence (+) of Hox-a5 protein are expressed relative to those seen with the 2.2 kb *Hox-a5* promoter construct in the presence of Hox-a5 protein.

assay in cultured COS7 cells was utilized. For these experiments, cells were co-transfected with an expression plasmid encoding the full-length Hox-a5 protein together with a plasmid containing the putative target *Hox-a5* promoter fused to the sensitively assayable human growth hormone (GH) gene (Selden *et al.*, 1986). As shown in Figure 1, a 2.2 kb *Hox-a5* promoter fragment (*Eco*RI–*Sac*I) mediated an ~8-fold increase in growth hormone levels when the COS7 cells were co-transfected with a plasmid producing the Hox-a5 protein. We conclude from these experiments that Hox-a5 protein has the potential to activate transcription. This cell culture system therefore provides an assay for monitoring the transcriptional activation potential of Hox-a5 proteins.

The 201 bp proximal promoter sequence which lacks any putative Hox-a5 binding sites did not stimulate expression above the basal level. The inclusion of one single Hox-a5 protein binding site, even in the context of its natural promoter, was not sufficient for the full activity of the *Hox-a5* promoter, since the 307 bp promoter construct mediated only a mild enhancement of expression. Interestingly, the strongest expression was observed with a 913 bp promoter construct. Sequence analysis of this region identified additional potential Hox-a5 binding consensus sequences (not shown). The presence of these elements indicates that they could serve as *cis*-acting sites for *Hox-a5* gene regulation by Hox-a5 and/or other Hox proteins.

Characterization of functional motifs of Hox-a5 protein in cultured cells

Since Hox-a5 protein functions as a transcriptional activator, the cell culture system was used to study the

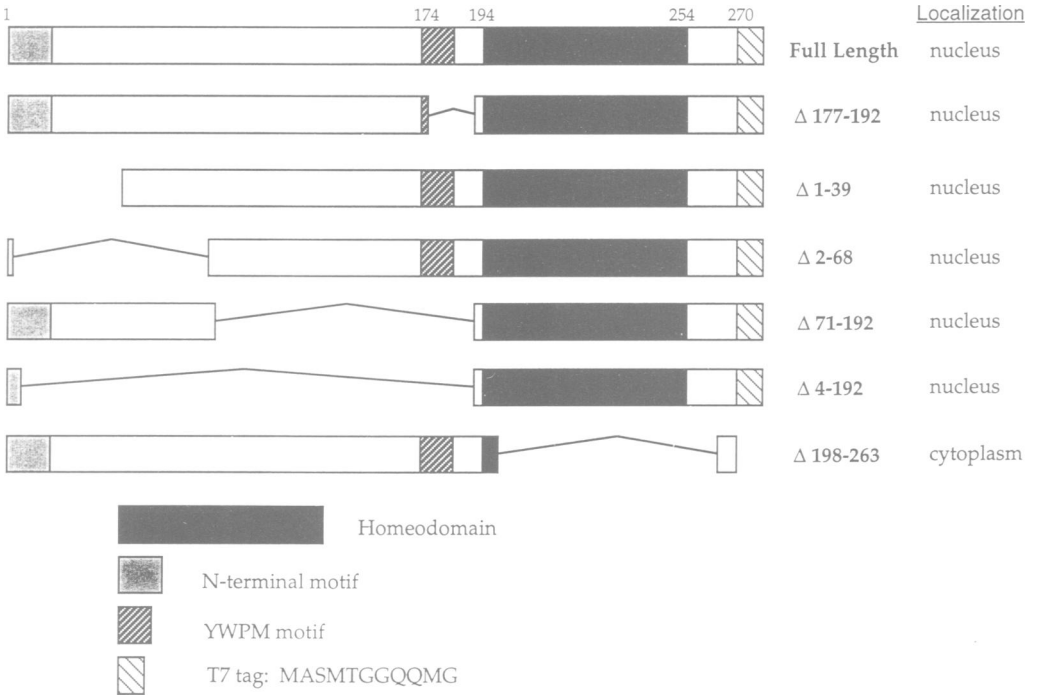

Fig. 2. Structures of the recombinant Hox-a5 protein and its truncated forms. Domains within Hox-a5 protein are indicated schematically. The N-terminal motif contains a conserved octapeptide, MSSYFVNS. The YPWM and homeodomain regions are indicated. The C-terminal 12 amino acid peptide derived from bacteriophage T7 gene 10 is indicated as 'T7 tag'. The subcellular localization of each protein product is summarized on the right.

contributions of different portions of the protein to its transcriptional activation ability. A series of DNA constructs which encode truncated proteins was made by digesting the DNA with restriction enzymes and using linkers for in-frame ligations (Figure 2). The full-length *Hox-a5* cDNA encodes a protein with a long N-terminal segment (194 residues), containing serine-rich and proline-rich regions, followed by the 61 residue homeodomain and a short (16 residue) C-terminal tail. Two short conserved peptide sequences, one at the N-terminus of the protein (octapeptide) and the other just upstream of the homeodomain (YPWM motif), are found in many *Drosophila* and vertebrate Hox proteins. To investigate whether these domains in Hox-a5 protein function as potential transactivating domains, five *Hox-a5* cDNA constructs which delete amino acids 1–39, 2–68, 69–192, 177–192 or 4–192 were analyzed. Gel-retardation analysis using proteins synthesized by *in vitro* transcription and translation indicated that the full-length Hox-a5 protein and three truncated forms (Δ2–68, Δ68–192 and Δ4–192), all of which retain the homeodomain, bind to Hox-a5 target sites *in vitro*, although reproducible differences in affinities were observed for the truncated proteins (data not shown).

The ability of the truncated proteins to transactivate was tested in co-transfection experiments, as summarized in Table I. The data indicate that the N-terminal 39 residues are important for transcription activation. This domain contains a conserved N-terminal MSSYFVNS octapeptide and a serine-rich region. An internal deletion of 16 amino acids, removing the highly conserved YPWM motif just upstream of the homeodomain, did not affect transactivation activity. The homeodomain and carboxyl tail alone are insufficient to activate transcription.

Table I. Transacting activites of HoxA5 truncated proteins

Expression construct	Relative activity (%)
HoxA5 (full length)	100.0
HoxA5Δ177–192	98 ± 3.0
HoxA5Δ1–39	20.0 ± 7.3
HoxA5Δ2–68	9.1 ± 1.4
HoxA5Δ71–192	22.8 ± 8.5
HoxA5Δ4–192	9.5 ± 3.3
Vector only	9.2 ± 2.0

To confirm that loss of transactivation activity was not due to the lack of nuclear targeting, the transfected culture cells were immunostained with an anti-TAG antibody which recognizes a 12 peptide bacterial sequence that had been inserted at the carboxyl end (see details in Materials and methods). Both the full-length Hox-a5 (Figure 3A and B) and all of the truncated proteins containing the homeodomain (Figure 3C–L) are targeted to the nucleus. Thus, the DNA binding domain and transactivating domains are independent and separable in the murine Hox-a5 protein.

Ectopic expression of truncated Hox-a5 proteins in Drosophila

We showed previously that ectopic expression of Hox-a5 protein generates homeotic transformations in both larvae and adult *Drosophila* equivalent to those produced by its fly cognate Scr (Zhao *et al.*, 1993). We have taken advantage of this biological assay to assess the *in vivo* function of truncated forms of Hox-a5. The truncated forms of *Hox-a5* cDNA—Δ1–39, Δ177–192, Δ4–192

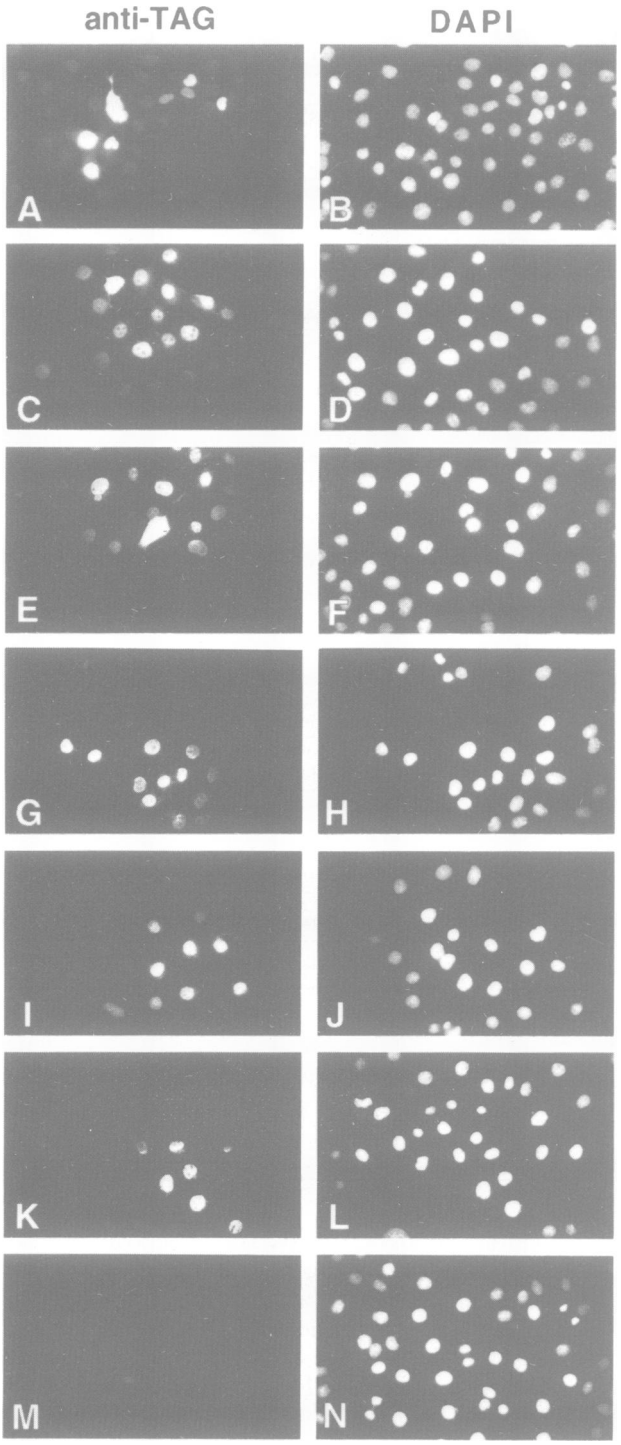

Fig. 3. Subcellular distribution of the full-length and truncated forms of Hox-a5 polypeptides. The anti-TAG antibody was used to detect Hox-a5 proteins in transfected COS7 cells (A, C, E, G, I, K and M). Nuclei stained with DAPI are shown in (B), (D), (F), (H), (J), (L) and (N). Cells expressed the following proteins: (**A**) and (**B**), full-length Hox-a5 protein; (**C**) and (**D**), Δ177–192 polypeptide; (**E**) and (**F**), Δ1–39 polypeptide; (**G**) and (**H**), Δ2–68 polypeptide; (**I**) and (**J**), Δ71–192 polypeptide; (**K**) and (**L**), Δ4–192 polypeptide; (**M**) and (**N**), untransfected COS cells. All proteins that retain the homeodomain are localized in the nucleus.

and Δ198–263—were each inserted into the pCaSpeR-hs vector (Thummel and Pirrota, 1992). Each construct was introduced into the germline of flies and multiple independent lines were generated for each, as was done for the full-length *Hox-a5* (Zhao *et al.*, 1993). All lines are viable under normal growth conditions.

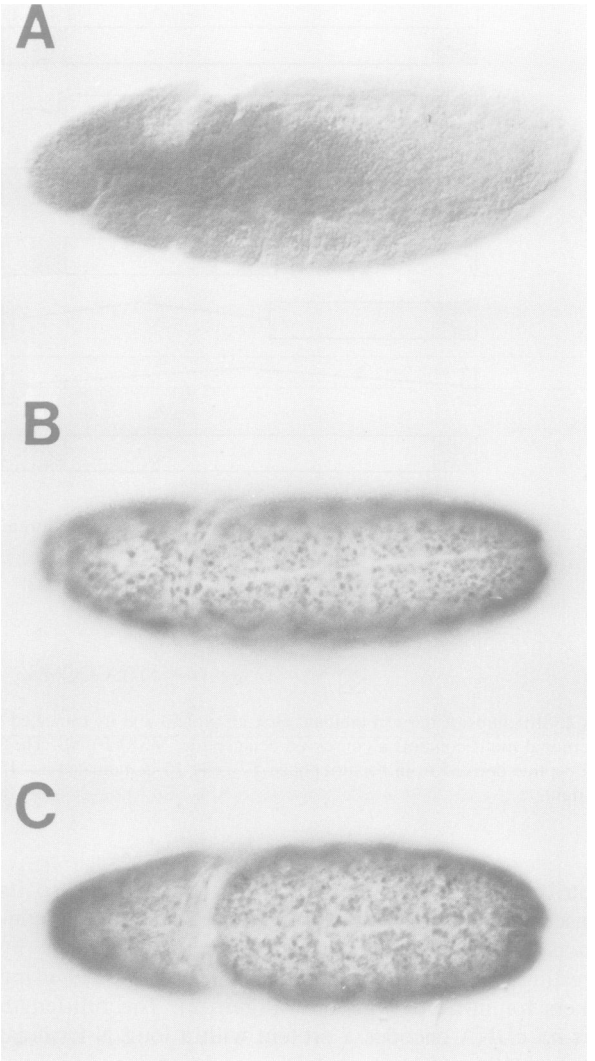

Fig. 4. Ectopic expression of different truncated forms of mouse Hox-a5 protein in transgenic *Drosophila* embryos. Expression of Hox-a5 proteins was monitored with anti-Hox-a5 antibody (Odenwald *et al.*, 1987) after a 1 h heat shock at 37°C at 6 h ± 30 min after egg laying (AEL), followed by 1 h of recovery at 25°C. Embryo staining was carried out in parallel for all panels. (**A**) Heat-induced expression of *hs-Hox-a5*Δ198–263; (**B**) heat-induced expression of *hs-Hox-a5*Δ1–39; (**C**) heat-induced expression of *hs-Hox-a5*Δ177–192. Hox-a5 lacking the homeodomain fails to accumulate in the nucleus, while other truncated proteins and full-length Hox-a5 protein (Zhao *et al.*, 1993) are localized in the nucleus.

As shown in Figure 4, ectopic expression of these truncated forms was detected after a 1 h heat pulse with an antibody directed against a Hox-a5 peptide (amino acids 53–72; Odenwald *et al.*, 1987). Only the Hox-a5Δ4–192 protein which is missing the epitope could not be detected. The protein products of *Hox-a5*Δ198–263, which lack the coding sequence for the homeodomain and part of the C-terminus, were detected in the cytoplasm of cells and failed to accumulate in the nucleus (Figure 4A). Thus, this portion of the protein presumably contains the Hox-a5 nuclear localization signal. Both Hox-a5Δ1–39 and Δ177–192 were detected as nuclear proteins in heat-shocked embryos (Figure 4B and C).

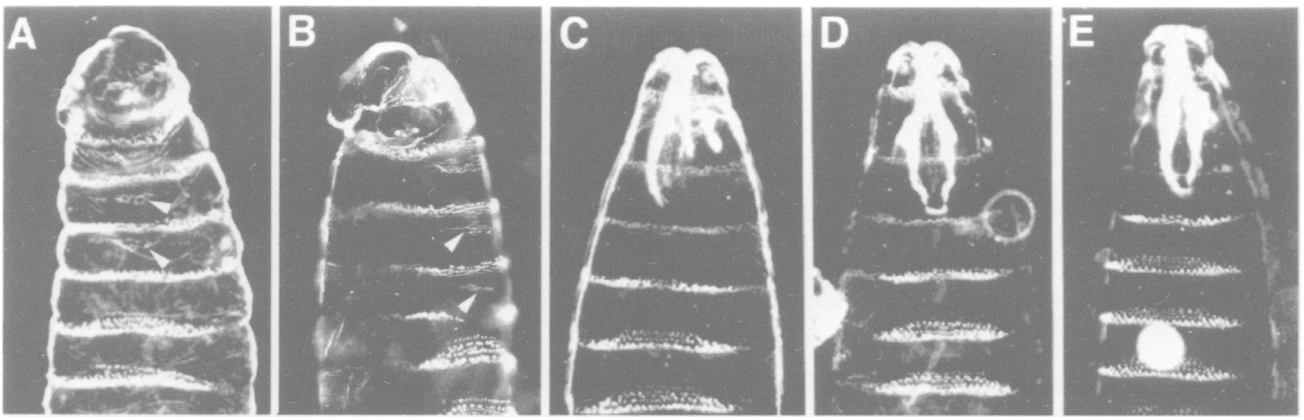

Fig. 5. The YWPM motif is necessary for Hox-a5-induced homeotic transformation of the larval cuticle. Transgenic embryos were subjected to a 60 min heat shock at 5.5–6.5 h AEL in a 37°C water bath and then allowed to develop into larvae. Larvae shown carried the following transgenes: (**A**) *hs-Hox-a5*; (**B**) *hs-Hox-a5*Δ1–39; (**C**) *hs-Hox-a5*Δ177–192; (**D**) *hs-Hox-a5*Δ4–192; (**E**) *hs-Hox-a5*Δ198–263. Transformation of T2 and T3 towards T1 is indicated by the appearance of ectopic ventral beards of denticles (see arrowheads). Such a transformation is observed with the full-length Hox-a5 protein (A) and also when the N-terminal octapeptide region is deleted (B). Additional defects in head involution are apparent for these panels. Neither defect is observed for other truncated proteins, the shortest of which lacks only the YPWM region (C).

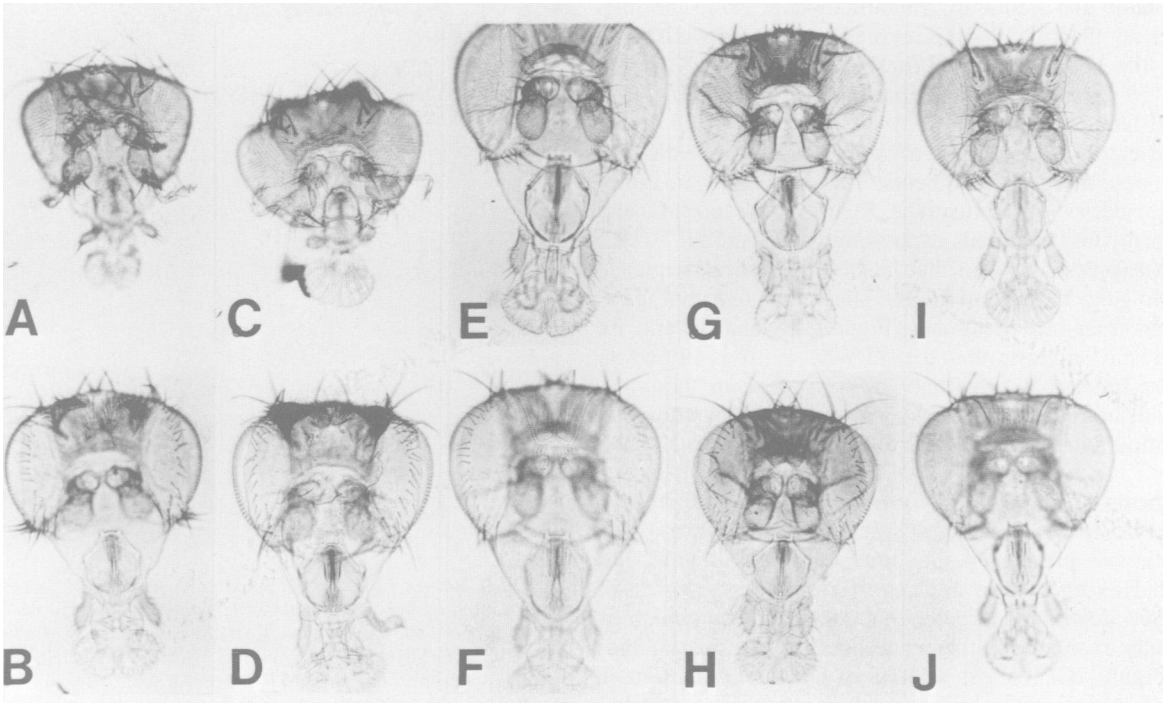

Fig. 6. Adult phenotypes in transgenic flies expressing truncated mouse Hox-a5 proteins. Larvae carrying the individual truncated transgenes were subjected to multiple heat shocks from 68 to 92 h AEL for 45 min at minimal 4 h intervals. The larvae expressing the full-length Hox-a5 protein, shown in (A) and (B), were heat shocked only once at 70 h AEL. Cephalic views of adult heads are shown in (A), (C), (E), (G) and (I); caudal views of adult heads are shown in (B), (D), (F), (H) and (J). Transgenic animals carry the following transgenes: (**A**) and (**B**), *hs-Hox-a5*; (**C**) and (**D**), *hs-Hox-a5*Δ1–39; (**E**) and (**F**), *hs-Hox-a5*Δ177–192; (**G**) and (**H**), *hs-Hox-a5*Δ4–192; (**I**) and (**J**), *hs-Hox-a5*Δ198–263. Note the transformation of antenna towards leg in (A) and (C). Transformations are less severe for the *hs-Hox-a5*Δ1–39 transgene. Deletion of the homeodomain (I), the N-terminal half of the protein (G) or the YPWM motif (E) renders the proteins inactive in this assay. These heads are all indistinguishable from wild type.

Effects of Hox-a5 motifs on induction of homeotic transformations in Drosophila

The expression of these truncated forms of Hox-a5 was induced in transgenic animals by heat shock, as described previously for full-length Hox-a5 (Zhao *et al.*, 1993). The results are shown in Figure 5 for larval cuticular patterns and in Figure 6 for phenotypes of adult heads. As expected, the transgenic flies which expressed a truncated protein lacking most of the homeodomain and 9 of 16 C-terminal amino acid residues (*hs-Hox-a5*Δ198–263) and which was targeted to the cytoplasm, were indistinguishable from wild-type flies after heat induction either in embryos or larvae (Figures 5E and 6I and J). Therefore, this construct actually serves two purposes: first, it demonstrates that the

observed transformations cannot be attributed to general effects of heat shock and ectopic protein expression; second, it provides direct evidence that a murine Hox homeodomain is necessary for its biological function.

In heat-shocked *hs-Hox-a5*Δ1–39 embryos, larval transformations closely resembled those of the *hs-Scr* and full-length *hs-Hox-a5*. As shown in Figure 5B, thoracic segments T2 and T3 were clearly transformed towards T1, as indicated by the appearance of ectopic T1 ventral beards. The ectopic expression of *hs-Hox-a5*Δ1–39 also blocked head involution and generated abnormalities in the head segments. The adult phenotype resulting from the ectopic expression of *hs-Hox-a5*Δ1–39 in larvae (Figure 6C and D) corresponded to homeotic transformations in the head induced by full-length Hox-a5 (Figure 6). However, closer comparisons with the full-length Hox-a5 protein indicate that the transforming activity of this truncated protein may be slightly different in different tissue types. Both of them can dramatically affect the size and shape of the eye, and induce the obvious *Cephalothorax*-like transformation in the hindhead. However, the antenna to leg transformation was observed more completely and frequently in animals expressing the full-length a5 than in animals expressing *hs-Hox-a5*Δ1–39. Thus, the homeotic transformations induced by *hs-Hox-a5*Δ1–39 appear to be weaker than those induced by Hox-a5 full-length protein.

The ectopic expression of *hs-Hox-a5*Δ4–192 did not cause any detectable homeotic transformation in either larval cuticles or adults (see Figures 5D and 6G and H). Surprisingly, animals expressing *hs-Hox-a5*Δ177–192, which express a protein that lacks only a short sequence including the YPWM motif, also showed no morphological changes after heat treatment (Figures 5C and 6E and F). This result suggests that the YPWM motif is important for the functional specificity of Hox-a5 even though it may not be responsible for the potency of transcriptional activation as assayed in cultured cells (see Table I).

Deletions affect the ability of Hox-a5 to activate fkh, a downstream target gene in Drosophila

We showed previously that the overexpression of full-length Hox-a5 protein induces the ectopic expression of one *Scr* downstream gene, *fkh* (Figure 7A), which is normally expressed in the primordia of the salivary glands (see Figure 7E). To test whether or not the truncations in Hox-a5 protein affect this regulatory function, the p[ry$^+$, HNK01] line, which carries a *fkh–lacZ* fusion gene, was crossed individually with either *hs-Hox-a5*Δ1–39, Δ4–192, Δ177–192 or Δ198–263 transgenic strains. After embryos were shifted to a 37°C water bath for 30 min, and allowed to recover at normal temperature (25°C) for 6 h, the expression of the *fkh–lacZ* fusion was examined in whole-mount embryos (Figure 7). Ectopic expression of the *fkh–lacZ* transgene was detected only in embryos carrying *hs-Hox-a5*Δ1–39, but not in others (Figure 7B versus C–E). The activation ability of Hox-a5Δ1–39 on *fkh–lacZ* was considerably lower than that of the full-length Hox-a5. The ectopic β-galactosidase was limited to the procephalon region and was not detected in more posterior segments (compare Figure 7A and B). This is consistent with the ability of this truncated protein to induce weak homeotic transformations in larvae and adult flies. Notably, Hox-a5Δ177–192, which lacks the YPWM motif and apparently retains full transactivating activity in cultured cells (see above), failed to activate expression of the *fkh–lacZ* reporter gene in embryos. This observation is consistent with the protein's inability to produce homeotic transformations in transgenic larvae or adults.

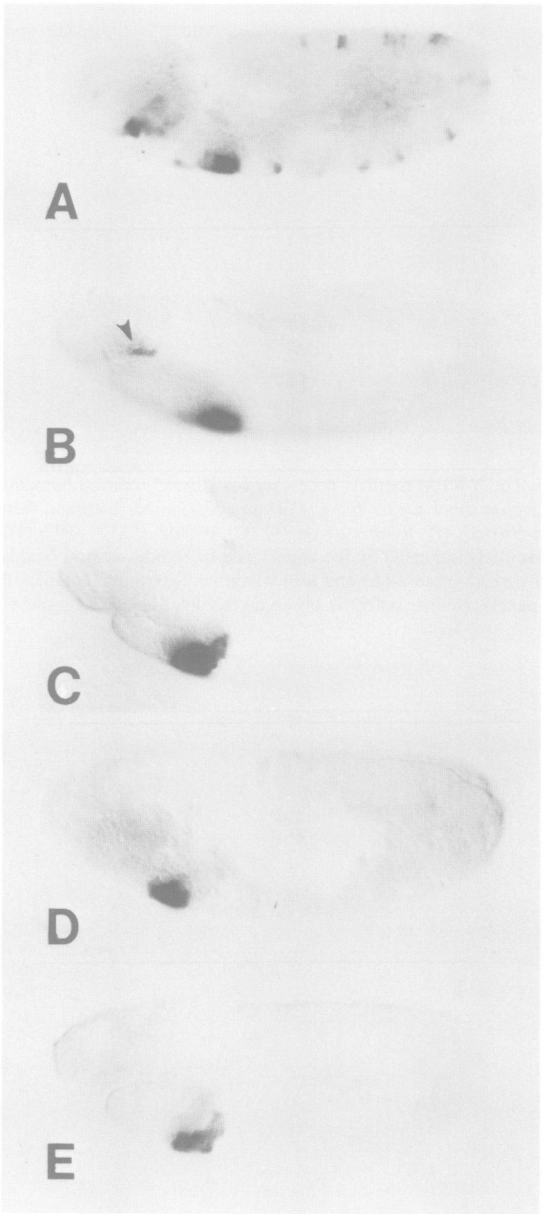

Fig. 7. Ectopic expression of *fkh* requires the YPWM motif and is enhanced by the octapeptide motif. Embryos carrying a *fkh–lacZ* fusion gene and (**A**) *hs-Hox-a5*, (**B**) *hs-Hox-a5*Δ1–39, (**C**) *hs-Hox-a5*Δ177–192, (**D**) *hs-Hox-a5*Δ4–192 or (**E**) *hs-Hox-a5*Δ198–263 were heat shocked for 30 min at 37°C at 1–4 h AEL. After 6 h of recovery at 25°C, *fkh–lacZ* expression was detected by immunostaining with an anti-β-galactosidase antibody. Hox-a5 protein lacking the homeodomain (E) has no effect on *fkh–lacZ* expression; this pattern of expression is indistinguishable from wild type. The ectopic expression of *fkh–lacZ* in response to *hs-Hox-a5*Δ1–39 is indicated by an arrow in (B). This Hox-a5 protein only stimulates *fkh* expression weakly; ectopic expression in posterior segments (A) is not detected. No ectopic expression is detected when protein lacking the N-terminal half of Hox-a5 (D) or the YPWM motif is expressed (E).

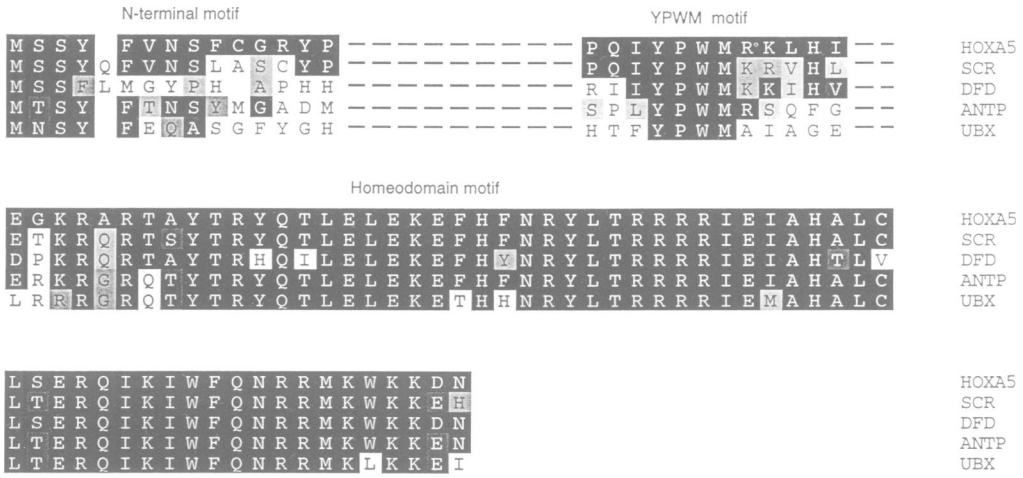

Fig. 8. Alignment of conserved motifs of mouse Hox-a5 and related *Drosophila* HOM-C proteins. Sequence comparison of murine Hox-a5 with *Drosophila* Scr, Dfd, Antp and Ubx proteins was carried out using DNASTAR Inc. software. Mouse Hox-a5 is more similar to *Drosophila* Scr than to the other proteins in the N-terminal region and YPWM motif. The octapeptides diverge at one position only. The YPWM motifs share extended homology that is not found in other Hox proteins. The homeodomains are highly conserved. Five out of six of the differences are conservative substitutions.

Discussion

The Hox-a5 homeodomain is necessary but not sufficient for biological activity

Our studies have distinguished three independent functions of the mouse Hox-a5 protein that are shared with its *Drosophila* cognate Scr. First, the homeodomain is most likely to harbor both the nuclear targeting signal and the protein's DNA binding activity. It is necessary but not sufficient for all observed Hox-a5 regulatory functions, since the homeodomain alone (see results with *Hox-a5*Δ4–192) is unable to function in cultured cells and fails to produce any visible homeotic transformation in transgenic animals, but its presence is required for both transcriptional activation and *in vivo* functions in transgenic embryos. Potential homeodomain-independent functions of Hox-a5/Scr, as have been found recently for *Drosophila* fushi tarazu (FTZ) protein (Fitzpatrick *et al.*, 1992; Hyduk and Percival-Smith, 1996), cannot be ruled out since Hox-a5 proteins lacking the homeodomain were not localized in the nucleus. Previous studies implicated the N-terminus of the homeodomain as being important for the functional specificity of Hox proteins. Chimeric *Drosophila* proteins containing portions of Dfd and Ubx (Kuziora and McGinnis, 1989; Lin and McGinnis, 1992), Antp and Scr (Furukubo-Tokunaga *et al.*, 1993; Zeng *et al.*, 1993) or Ubx and Antp (Chan and Mann, 1993) were analyzed using the types of ectopic expression assay described in this paper for specificity of cuticular homeotic transformations and for effects on downstream target gene expression. Swapping of the eighth amino acid from the N-terminus of the homeodomain from Ubx into the Dfd protein switched the specificity of Dfd to Ubx-like in that *Antp* was a target of Ubx and of the chimeric protein, but was not a target of Dfd itself (Lin and McGinnis, 1992). Similarly, this short N-terminal region of the homeodomain determined regulatory specificity in Antp/Scr chimeras (Furukubo-Tokunaga *et al.*, 1993; Zeng *et al.*, 1993). Similar chimeras have pointed also to the importance of the regions of Hox proteins C-terminal to the homeodomain (Chan and Mann, 1993).

Our experiments have not re-examined the function of these specific sequences within the homeodomain. However, it is interesting to note that both Scr and Dfd show equal similarity to Hox-a5 in the flexible N-terminal arm of the homeodomain (Figure 8). This sequence similarity to Dfd may account for the weak Dfd-like transformations of the maxillary palps noted in our previous studies with full-length Hox-a5 (Zhao *et al.*, 1993; Figure 5). However, the fact that ectopic expression of Hox-a5 induces Scr-like homeotic transformations that are not seen with Dfd in transgenic flies indicates that individual amino acid residue differences in the N-terminal arm of the homeodomain may not be sufficient to determine the functional specificities of these homeodomain proteins *in vivo*.

The N-terminus of Hox-a5 is involved in transcription activation in vivo

The second functional domain is the N-terminal 1–39 amino acid residues of Hox-a5 including the conserved octapeptide motif and a serine-rich region. Lacking this region, the transcriptional activity of Hox-a5 protein decreased ~80% in the transient expression assay system. Effects of this deletion on transacting activity were also observed in transgenic animals in that qualitatively similar but significantly weaker transformation was observed in both larvae and adult flies when this motif was deleted. Since the octapeptide is the only conserved portion of this domain, we suggest that the effects observed on transcription are due to deletion of these amino acids. Direct demonstration of this will require more specific mutagenesis within the N-terminal region.

These observations are similar to those of Gibson *et al.* (1990), who observed that the N-terminal half of Antp protein was important for 'potentiating' its biological activity in ectopic expression assays in *Drosophila*. Our experiments have narrowed down this 'potentiating' or

activating function to the 39 amino acids at the N-terminus of Hox-a5. This region contains a highly conserved octapeptide sequence that is found in many Hox proteins (see Figure 8; Mavilio *et al.*, 1986; Odenwald *et al.*, 1987; Schugart *et al.*, 1988; Taniguchi *et al.*, 1991). This sequence is more highly conserved and is extended between cognate genes of different species rather than between members of different cognate groups. For example, the Scr and Hox-a5 octapeptides share eight amino acids, differing only at a single amino acid insertion in Scr, while the octapeptides of Antp and Dfd differ at three and six positions, respectively (Figure 8). Although we have not observed any specific biological requirements for this highly conserved motif other than raising levels of transcriptional activation, we cannot exclude the possibility that it may be required in a different type of tissue or cell to modulate target specificity. Zappavigna *et al.* (1994) recently proposed that the N-terminal 46 amino acids of HoxD8 carry out an 'effector' function involved in inhibition of transcription of HoxD9 by HoxD8. This inhibition also requires the N-terminus of the homeodomain which was shown to mediate protein–protein interactions between these two Hox products. Thus, the effects of deletion of this domain seen on transcription activity in cells and in *Drosophila* may reflect interactions with specific transcription factors that modulate activity rather than interactions with general factors that are part of the basal transcription machinery.

The YPWM motif is essential for biological activity of Hox-a5

Finally, we have shown that the YPWM motif N-terminal to the homeodomain is crucial for biological activity of the Hox-a5 protein. While the deletion of this motif does not affect the transcription activity of Hox-a5 in cultured cells, it significantly affects the function in transgenic flies. Hox-a5Δ177–192 is no longer able to induce any homeotic transformation, even though it has been expressed abundantly in most nuclei of heat-treated embryos. These observations suggest that the YPWM motif may be involved in protein–protein interactions *in vivo* that define target specificity.

The gene *extradenticle* (*exd*) encodes a cofactor for Ubx and abd-A which interacts cooperatively with and increases the DNA binding affinity of these Hox proteins (Chan *et al.*, 1994; van Dijk and Murre, 1994). The *Drosophila exd* gene shares extensive sequence homology with the human proto-oncogene *pbx* and related genes which encode diverged homeodomain-containing proteins (Rauskolb *et al.*, 1993). The DNA binding ability of Pbx proteins suggests that they are themselves transcription factors that may modulate the specificity of mammalian Hox proteins. Interaction of the *Drosophila* EXD and UBX proteins in a yeast two-hybrid system was shown to require the UBX homeodomain and its YPWM motif (Johnson *et al.*, 1995). No interaction with EXD was observed when the UBX homeodomain was replaced with either the Antp or Scr homeodomains (Johnson *et al.*, 1995). However, it has been shown more recently that mammalian Hox proteins, including Hox-a5, interact directly with PBX1 (Lu *et al.*, 1995). The YPWM motif in Hox-a5 is necessary for cooperative interactions of these proteins (Knoepfler and Kamps, 1995). Similarly, interactions between human PBX1 and mammalian HoxB6 and B7 require an intact YPWM motif (Chang *et al.*, 1995). While the Antp and Scr homeodomains are more closely related than either is to Ubx, the YPWM box of Scr diverges from both sequences outside of the core YPWM (Figure 8; Burglin, 1994). These observations suggest the existence of other EXD-like cofactors that interact specifically with the YPWM/homeodomain combinations found in Antp and in Scr. Interestingly, we found a nine nucleotide motif (ACATATCAA) in the *Hox-a5* promoter, which matches the core motif of one previously identified EXD binding site. It is positioned at +53 to +61, 200 bp downstream of the Hox-a5 binding region (Odenwald *et al.*, 1987; van Dijk *et al.*, 1993). A putative Scr cofactor would be expected to interact with the regions conserved between Scr and Hox-a5, including the homeodomain and the extended YPWM motif (see Figure 8). Thus, using a small number of cofactors, each of which interacts with only a small number of Hox proteins, a combinatorial code could be sequentially built up such that each Hox product would only bind to and/or regulate transcription via discrete and distinct DNA target sites.

Materials and methods

Plasmid preparation

Plasmids for in vitro translation. Translation vectors were based on pT7βGΔ6Sal, a derivative of pGEM2 in which the β-globin sequence is inserted between the polylinker *Hin*dIII and *Pst*I sites. Transcripts synthesized from this plasmid by T7 RNA polymerase begin with the nucleotides GGAGACCGGAAGCUUGCUU, followed by the β-globin leader sequence. pT7β*Hox-a5* contains the 1 kb *Sac*I–*Sal*I *Hox-a5* cDNA fragment from pUC-Xho-rhomeo (obtained from W.F.Odenwald; originally constructed by E.Linney) inserted into *Nco*I–*Sal*I-cleaved pT7βGΔ6Sal. An adapter oligonucleotide was used to allow the in-frame ligation of *Hox-a5* cDNA to the β-globin ATG codon. pT7β*Hox-a5*Δ1–39: pUC-Xho-rhomeo was cut with *Sph*I and blunt ended with T4 DNA polymerase. A 0.88 kb DNA fragment, excised with *Sal*I, was inserted into pT7βGΔ6Sal that had been digested first with *Nco*I, blunt ended and digested with *Sal*I. pT7β*Hox-a5*Δ2–68: a 201 bp *Sac*I–*Bss*HII fragment was deleted from pT7b*Hox*. After the two incompatible ends were blunt ended with T4 DNA polymerase, the linearized plasmid was religated. pT7β*Hox-a5*Δ71–192: a 387 bp coding sequence was deleted out of pT7β*Hox-a5* with *Bss*HII and *Apa*I. An annealed synthetic oligonucleotide pair (CGCGCCGATATCCCAGAAGGCAAAAGGG-CC/CTTTTGCCTTCTGGGATATCGG) was inserted to give an in-frame ligation, reconstitute *Bss*HII and *Apa*I sites, and engineer an *Eco*RV site between them. pT7β*Hox-a5*Δ4–192: the *Sac*I–*Apa*I-cleaved pT7β*Hox-a5* was recircularized with an annealed linker (CTGATATCCCAGAA-GGCAAAAGGGCC/CTTTTGCCTTCTGGGATATCAGAGCT) which reconstituted the *Sac*I and *Apa*I sites, and introduced an *Eco*RV site. pT7β*Hox-a5*Δ198–263: the homeobox sequence was deleted from pT7β*Hox-a5* with restriction enzymes *Apa*I and *Sac*II. After blunt ending with T4 DNA polymerase, both sites were lost, and the plasmid was religated.

Plasmids for the transient expression assay. The *Hox-a5* promoter sequence was derived from a plasmid, pJG4 (a gift from J.Garbern, University of Pennsylvania), carrying a 3.8 kb *Eco*RI genomic fragment which starts in the *Hox-a6* homeobox and extends to the *Hox-a5* homeobox. From this plasmid, a 2.2 kb *Sac*I–*Eco*RI fragment containing the whole 5′ untranslated sequence of *Hox-a5* (see the diagram in Figure 1) was subcloned into pUC19 to generate pJZH1. Since this fragment retains the initial codon ATG, a primer, GGGAGCTCGGATCCTTGTTT-TTTGATATGTGTGCTTGA, which matches the sequence from +82 to +51 bp, was designed to delete the ATG in a polymerase chain reaction (PCR). This introduces a novel *Bam*HI site at this position. The second primer used in this PCR was GGCTCGAGTCCGACTGAACGGCGGC-AACGGGT, derived from upstream sequence at bases −235 to −205. The 320 bp PCR product was cut with *Bam*HI and *Xba*I, and the 201 bp fragment was inserted into pϕGH (Selden *et al.*, 1986), to generate p201/

GH. p2200/GH: a 2.0 kb *Hin*dIII–*Xba*I fragment from pJZH1 was inserted into p201/GH linearized with *Hin*dIII and *Xba*I. p913/GH: a 913 bp *Bgl*II–*Bam*HI fragment from p2200/GH was inserted into the *Bam*HI site of pΦGH. p307/GH: a 1.9 kb *Hin*dIII–*Xho*I fragment was deleted from p2200/GH. The linearized plasmid was blunt ended with Klenow enzyme and religated.

Constructions for the full-length and truncated *Hox-a5* cDNA expression plasmids (p*JZHox* series) were made by inserting *Hin*dIII–*Bam*HI fragments from constructs prepared for *in vitro* translation (described above) into the expression vector pCMV5 (Anderson *et al.*, 1989). The heterologous 12 amino acid 'tag' was introduced into these expression plasmids, except p*JZHox*Δ198–263 which had no *Sac*II site, by placing the synthetic sequence (upper strand: GGCAGGCGGCGCTTTCCG-CCCCATGGCTAGCATGACCGGCGGCCAGCAGATGGGCCGCTG-ATAG/lower strand: GATCCTATCAGCGCCGCATCTGCTGGCCG-CCGGTCATGCTAGCCATGGGGCGGAAAGCGCCGCCTGCCGC) between *Sac*II and *Bam*HI sites.

Plasmids for transgenic flies. p*hs-Hox-a5*Δ4–192: p*hs-Hox-a5* (formerly p*hs-Hox-1.3*; see Zhao *et al.*, 1993) was digested with *Bgl*II and *Xba*I, the ends were filled in with Klenow enzyme and the two blunt ends were ligated. p*hs-Hox-a5*Δ1–39: the vector pCaSpeR-hs was cut with *Hpa*I and *Bgl*II, and a *Hin*dIII (blunt ended)–*Bam*HI fragment from p*JZHox*Δ1–39 was inserted. p*hs-Hox-a5*Δ177–192: a *Hin*dIII (blunt ended)–*Bam*HI fragment from p*JZHox-a5*Δ177–192 was inserted between the *Hpa*I and *Bgl*II sites of pCaSpeR-hs. p*hs-Hox-a5*Δ4–192: the 330 bp *Hin*dIII (blunted)–*Bam*HI fragment from p*JZHox-a5*Δ4–192 was inserted into the *Hpa*I–*Bgl*II sites of pCaSpeR-hs.

Co-transfection and transient expression assays

Monkey kidney COS7 cells were grown on 100 mm tissue culture dishes or T75 flasks in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Calcium phosphate transfection was performed as described by Ausubel *et al.* (1987). Equimolar amounts of test plasmid (10–20 μg) plus 5 μg of pSV2CAT (Gorman *et al.*, 1982) were transfected per 100 mm dish. Cells were transfected for 6 h, followed by a 2 min 10% glycerol shock. For GH transient expression assays, 0.5 ml of culture media was collected at 12 h intervals from 36 to 72 h post-transfection. The level of growth hormone secreted in the culture medium was determined by radioimmunoassay as suggested by the manufacturer (Nichols Diagnostics, San Jaun, Capastrano, CA). For CAT transient expression assays, cells were harvested at 72 h post-transfection. CAT enzymatic activity was monitored by a standard liquid scintillation counting (LSC) assay (Promega).

Immunohistochemistry

Transfected cells were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4) for 30 min. After rinsing with PBS, the cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min. Following rinsing with PBS, the cells were blocked with 20% normal goat serum, 0.02% biotin and 0.1% Triton X-100 in PGBA [0.1% gelatin, 1% bovine serum albumin (BSA) and 0.05% sodium azide in PBS] for 30 min. The rabbit anti-TAG antiserum (a gift from Dr J.Keene, Duke University) was diluted in block solution (1:200). The primary antibody incubation was performed overnight at 4°C. Cells were then washed three times for 20 min with three changes each of 0.1% Triton X-100 in PBS. The donkey biotinylated anti-rabbit Ig antiserum (Amersham) was diluted 1:200 with 0.1% Triton X-100 in PGBA. After incubation with the secondary antibody for 1 h, cells were washed as described above. The secondary antibodies were labeled with streptavidin–rhodamine B (Molecular Probes, 12 ng/ml in 0.1% Triton X-100, PGBA) for 1 h. After three 10 min rinses in buffer containing 0.1% Triton X-100 and PBS, DAPI was used to stain the cellular DNA. Cells were stained with 1 μg/ml of DAPI in 0.1% Triton X-100 and PGBA for 40 min, followed by three 10 min of destaining in 0.1% Triton X-100 and PBS. Protein expression in transgenic embryos was monitored with specific antibodies as described previously (Gutjahr *et al.*, 1993; Zhao *et al.*, 1993).

Transgenic flies and heat shocks

All procedures used were identical to those described by us previously (Zhao *et al.*, 1993). The presence of appropriate transgenes in all independent transformant lines was analyzed by PCR using specific primers. At least three independent transformant lines were established and analyzed for each construct.

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